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Introducing the New Editor of *Arthritis & Rheumatism*, Michael D. Lockshin, MD

Michael D. Lockshin, MD assumed the editorship of *Arthritis & Rheumatism* on July 1, 2005. He succeeds Dr. David S. Pisetsky, for a five-year term.

Dr. Lockshin is the Director of the Barbara Volcker Center for Women and Rheumatic Disease and Co-Director of the Mary Kirkland Center for Lupus Research at the Hospital for Special Surgery, and Professor of Medicine and Obstetrics-Gynecology at Joan and Sanford I. Weill Medical College of Cornell University in New York. He received his AB and MD degrees from Harvard University and his clinical training at the Second (Cornell) Medical Division Bellevue Hospital and Memorial Sloan-Kettering Hospital in New York, followed by a rheumatology fellowship at Columbia-Presbyterian Medical Center. As an Epidemic Intelligence Service officer for the Communicable Disease Center of the US Public Health Service, Dr. Lockshin was Assistant Professor of Epidemiology at the University of Pittsburgh School of Public Health, working on the health problems of coal miners. In 1970, he joined the Hospital for Special Surgery and Cornell University Medical College, where he became Professor of Medicine and Attending Physician. He moved to the National Institutes of Health in 1989, serving there as Extramural Director and then Acting Director of the National Institute of Arthritis and Musculoskeletal and Skin Diseases. He then was senior advisor to the Director of the Clinical Center, NIH, before returning to the Hospital for Special Surgery in 1997.

A renowned investigator in the field of rheumatology, Dr. Lockshin’s research interests have focused on systemic lupus erythematosus, the antiphospholipid antibody syndrome, and vasculitis. He coauthored the first reports on hepatitis B–associated polyarteritis nodosa, early reports on twins with lupus, studies on neurologic lupus including its treatment and the development of cognitive dysfunction, pregnancy and lupus, atherosclerosis and lupus, and many reports on the antiphospholipid antibody syndrome. His most recent interests have been on the sex distribution of disease.

Dr. Lockshin chaired the American Board of Internal Medicine Committee on Rheumatology and has served on many committees of the Arthritis Foundation and the American College of Rheumatology. He chaired the Arthritis Foundation Professional Education Committee and the ACR Audiovisual Aids Committee that produced the first Clinical Slide Collection. He was the first chairman of the ACR Committee on Rheumatologic Practice and was Second Vice President of the ACR in 1984–5. Dr. Lockshin was named a Master of the ACR in 2003. He has served on editorial boards of *Arthritis & Rheumatism*, *Journal of Rheumatology*, *Lupus*, *American Journal of Reproductive Immunology*, and other journals. He convened the first International Conference on Pregnancy and Rheumatic Disease and the first Conference on Gender, Biology, and Human Disease. He is the author of more than 230 scientific papers and textbook chapters and a book on health policy, *Guarded Prognosis*. He is a member of the Institute of Medicine Committee on Understanding the Biology of Sex and Gender Differences, its Committee to Review the CDC Anthrax Vaccine Safety and Efficacy Research Program, its Health Sciences Policy Board, and its Committee on (NIH) Centers of Excellence Programs.

Some of Dr. Lockshin’s stated goals for his editorship of *Arthritis & Rheumatism* include instituting means by which the journal can better accommodate the needs of both clinicians and researchers, increasing the visibility of the field of rheumatology to the public, and guiding the journal’s integration into the electronic world. Along with his impressive team of Associate Editors, he has assembled a group of distinguished Co-Editors—Drs. Steven Abramson, Jill Buyon, Daniel Clauw, Mary Goldring, Joshua Jacobs, Alisa Koch, Nancy Lane, James O’Dell, Stephen Paget, Richard Pope, and Jane Salmon—to work with him to accomplish these goals. Under Dr. Lockshin’s leadership, the American College of Rheumatology can look forward to our journal’s maintaining and improving upon its already high standard of excellence.

Elizabeth A. Tindall, MD
President, American College of Rheumatology
EDITORIAL

PTPN22 and Rheumatoid Arthritis: Gratifying Replication

Peter K. Gregersen and Franak Batliwalla

Two articles in this issue of *Arthritis & Rheumatism* (1,2) provide critical confirmation of the association between rheumatoid arthritis (RA) and a functional polymorphism located in the coding region of *PTPN22*, the gene that encodes the intracellular protein tyrosine phosphatase nonreceptor 22 (*PTPN22*; also known as Lyp, a lymphoid-specific phosphatase). This observation now stands as the most robust and reproducible genetic association with RA outside of the HLA region. It is especially satisfying that the *PTPN22* variant also predisposes to a variety of other autoimmune disorders in addition to RA, lending strong support to the opinion that common mechanisms and common molecular pathways underlie these disorders. What is most important is that this discovery is clearly useful in the sense that it raises a host of intriguing questions. We know just enough about the function of *PTPN22* to proceed immediately with a rich variety of new experimental approaches that will encompass biochemistry, cell biology, animal disease models, population genetics, and epidemiology.

The year 2004 was clearly a landmark in terms of *PTPN22* and human autoimmune disease. In March 2004, using a candidate gene approach, Bottini et al (3) reported that the minor allele (T) at nucleotide 1858 of *PTPN22* confers a predisposition to type 1 diabetes in US and Italian populations. This polymorphism results in a substitution of tryptophan (W) for arginine (R) at codon 620 of the PTPN22 protein. Working independently and combining a broad screen of functional single-nucleotide polymorphisms guided by previously published linkage studies, Begovich et al (4) reported a similar association with RA, in the summer of 2004. These studies were followed by several confirmations of the *PTPN22* association with type 1 diabetes (5–7) as well as convincing associations with systemic lupus erythematosus (8), Graves’ disease, and Hashimoto thyroiditis (9,10). More recently, several confirmations of the RA association have been reported (11,12).

The 2 independent studies in this issue of *Arthritis & Rheumatism* add important new observations concerning the association of the *PTPN22* 620W allele with RA in several Canadian (1) and New Zealand (2) populations. Both studies confirm that the 620W allele confers a risk for RA of ~1.5–2.0. In addition, these studies demonstrated that with the exception of one of the Canadian populations, homozygosity for the 620W variant more than doubles this risk, which is consistent with previous reports (4,11,12). Thus, overall, there is convincing evidence of a dose effect in disease susceptibility.

In both the study by van Oene et al and that by Simkins et al, the association with *PTPN22* extends to both rheumatoid factor–positive and rheumatoid factor–negative patients. This result is of interest because it contrasts with previous reports suggesting that the association is primarily with seropositive disease (4,12). This discrepancy may reflect heterogeneity in the clinical populations or differences in other background genes in these populations. In general, the presence of autoantibodies is a prominent feature of the autoimmune diseases that have been associated with *PTPN22*. It will therefore be of great interest to determine whether the *PTPN22* 620W allele is associated with the presence of anti–citrullinated peptide antibodies in the setting of RA, which is a topic that has not yet been thoroughly addressed.

Importantly, despite the association of *PTPN22* with the multiple different autoimmune disorders discussed above, there are some autoimmune diseases in which *PTPN22* does not appear to play a role in susceptibility. One of these diseases is multiple sclerosis (13). The study by van Oene et al now extends these negative...
data to Crohn’s disease. Autoantibodies are not a prominent feature of either of these disorders. In addition, results of studies in familial clustering of autoimmune disease suggest that the \textit{PTPN22}-associated disorders (i.e., RA, type 1 diabetes, autoimmune thyroid disease, and lupus) may form a related group (10). In contrast, support is much more limited for clustering of multiple sclerosis and Crohn’s disease with this group of disorders, although admittedly, the epidemiologic data are rather sparse. Given the fact that \textit{PTPN22} acts in part to regulate thresholds for T cell signaling (see below), these observations may lead to new insights into the different roles that T cells may play in these various disorders.

As noted above, \textit{PTPN22} belongs to a family of intracellular tyrosine phosphatases (14). It has been known for more than a decade that tyrosine phosphatase activity is associated with a negative regulatory effect on T cell function. Thus, early experiments showed that generalized phosphatase inhibition results in persistent proliferation of polyclonally activated T cells (15) or can induce spontaneous activation and cytokine release by resting T cells (16). A specific role of \textit{PTPN22} in T cell regulation has been confirmed by the results of knocking out the murine homolog of \textit{PTPN22} (PEST domain–enriched tyrosine phosphatase [PEP]), resulting in lowered thresholds for T cell receptor signaling in these animals (17). PEP-knockout mice on a nonautoimmune background (C57BL/6) exhibit a variety of phenotypes consistent with T cell hyperresponsiveness, including enlargement of the spleen and lymph nodes due to T cell proliferation. This T cell proliferation becomes more prominent in older mice, with the spontaneous development of germinal centers that appear to be largely dependent on the enhanced T cell function present in the PEP\textsuperscript{−/−} animals. Increased T cell proliferative capacity is primarily found within the effector/memory cell compartment in both CD4 and CD8 subsets and is accompanied by enhanced phosphorylation of activating tyrosine residues in both Lck and ZAP-70. Although there were increases in the levels of certain immunoglobulin isotypes in these knockout animals, autoantibodies did not develop, nor were there signs of overt

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**Figure 1.** Regulation of the Lck activation state. Lck (green shading) is an Src family kinase involved in early T cell signaling events and is maintained in an inactive state in resting T cells by phosphorylation (P) of a C-terminal tyrosine 505 (Y505), as shown on the left side of the figure. The literature suggests that dephosphorylation of Y505 (possibly mediated by CD45) causes a conformational change in Lck, resulting in phosphorylation of an activating tyrosine residue Y394, leading to Lck activation, as shown at the right side of the figure. \textit{PTPN22} binds the tyrosine kinase Csk via a proline-rich SH3 binding site (P1). This binding is thought to enable colocalization of \textit{PTPN22} to Lck, dephosphorylation of Lck Y394, and return of Lck to an inactive state, with concomitant rephosphorylation of Y505 by Csk. TCR = T cell receptor; PAG = phosphoprotein associated with glycosphingolipid-enriched microdomains.
autoimmune disease. Thus, PEP deficiency alone does not lead to clinical autoimmunity.

*PTPN22* has been shown to bind to an intracellular tyrosine kinase, Csk. This binding occurs by virtue of a proline-rich SH3 binding site on *PTPN22*, interacting with the SH3 domain of Csk. As shown in Figure 1, these molecules act in concert to inactivate Lck, an Src family kinase that is involved in early T cell signaling events. Csk acts to phosphorylate tyrosine 505 (an inhibitory phosphate for Lck), while *PTPN22* acts to remove the activating phosphate at tyrosine 394. The combined effect of these activities is to convert Lck to an inactive configuration (Figure 1).

The *PTPN22* R620W polymorphism is located within the SH3 binding site of *PTPN22*. A tryptophan (W) substitution at this position has been shown to disrupt the binding of *PTPN22* to Csk (3,4). Thus, the disease-associated 620W allele is likely to cause changes in the regulation of Lck and result in loss of negative regulation of T cell receptor signaling. Clearly, this polymorphism does not completely eliminate the functions of *PTPN22*, because even homozygous carriers of *PTPN22* 620W do not exhibit a phenotype such as that of the knockout mouse. It is more likely that the 620W polymorphism results in a change in the level of effective *PTPN22* activity in particular cell compartments. This view is supported by the dose effect that has been observed for the disease associations. Although reduction of *PTPN22* has been shown to change thresholds for T cell receptor signaling in human cells (4), the functional effect of the *PTPN22* 620W allele on T cell function in humans has not yet been demonstrated. It is likely that sensitive assays will need to be developed to detect such threshold changes in signaling in primary human cells.

Although the currently available data suggest that *PTPN22* acts primarily in T cells, it is now clear that this molecule is also expressed in other cell types, including B cells, monocytes, natural killer cells, and neutrophils (4,18). In addition, although *PTPN22* binds to the intracellular kinase Csk, there is also evidence that *PTPN22* can bind to other proteins such as c-Cbl and Grb2 (18,19). Baseline tyrosine phosphorylation is reduced in COS cells overexpressing Lyp/PEP, indicating that PEP may regulate the function of Cbl-associated proteins, such as ZAP-70 (20). Lyp also binds Grb2, a signaling adaptor molecule that is involved in CD28-mediated costimulation and T cell activation (19). Clearly, the full range of functions of *PTPN22* remain to be defined, in terms of both signaling pathways and the cell types in which they act. Indeed, there is now an explosion of interest in phosphatases as regulators of a wide variety of cellular functions (21). More than 100 different tyrosine phosphatases have been defined; this exceeds the number of tyrosine kinases (14). Although all of these molecules are likely to have interesting biologic effects (21), *PTPN22* is now going to receive a high level of scrutiny, given its clear involvement in RA and other forms of autoimmunity.

Finally, as alluded to in the beginning of this editorial, the association of *PTPN22* with autoimmunity was discovered by 2 separate experimental approaches: a candidate gene approach on the part of Bottini and colleagues, and a broader “discovery-driven” approach taken by Begovich et al. In general, candidate gene approaches can be frustrating because of the frequent lack of replication of initial positive results (22), related in part to publication bias as well as the tendency of investigators to perform preliminary studies that are statistically underpowered. Fortunately, this was clearly not the case for *PTPN22*. In contrast, discovery-driven approaches, based on genome-wide linkage or association, have the general problem of too many positive results, which need to be corrected for the simultaneous testing of multiple markers and then replicated (23). However, the confirmation of *PTPN22* as a risk gene for RA is an important validation of the discovery approach to gene identification used by Begovich et al, based on combining both genome-wide linkage and association.

Other discovery platforms, such as gene expression by microarray, are also beginning to yield valuable information for understanding autoimmune diseases, best exemplified by the identification of an interferon “signature” in the peripheral blood of patients with systemic lupus (24–26). Similar studies of RA have yielded evidence of monocyte activation (27,28) as well as other changes (29). It is currently unclear to what extent, if any, *PTPN22* signaling pathways are reflected in these findings. Morley and colleagues (30) have elegantly demonstrated that, by combining genetic analysis with these various discovery platforms, one can gain new insights into the relationship between genes and gene expression patterns and, ultimately, phenotype. The identification of *PTPN22* as an important risk gene for autoimmunity now provides for a more directed approach to using these powerful discovery-based technologies to understand the biology underlying complex autoimmune disorders.

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Disease Modification in Osteoarthritis: Are Drugs the Answer?

Paul Dieppe

Osteoarthritis (OA) is a difficult condition to manage (1,2). It is a common cause of pain and disability for which we have no simple, effective interventions. Drugs are widely used for symptom relief, but they do not work very well, and, as we have just been reminded by the cyclooxygenase 2 (COX-2) selective inhibitors debacle, they can have serious side effects. Physical, educational, and behavioral interventions are both safe and beneficial, but they have small effect sizes and may not be cost-effective. Major surgical interventions, such as total joint replacement, are the only effective options for people with severe disease, but such interventions are last resorts that are not without their own inherent problems.

For many years, we have been searching for ways to intervene in the disease process so that we can retard or prevent the progression of joint damage and thus theoretically reduce the symptom burden of the disorder and the number of patients who will need joint replacements. Most of these efforts have concentrated on trying to understand the mechanisms behind cartilage destruction and looking for pharmacologic ways of affecting them in a beneficial manner. Until recently, these endeavors have been more notable for their failures than for their successes, illustrated, for example, by the withdrawal of a number of proteinase inhibitor drugs that different pharmaceutical companies were hoping to launch as “disease-modifying OA drugs.”

In this issue of Arthritis & Rheumatism, Brandt and colleagues report results of a trial that shows that treatment with oral doxycycline can slow the rate of radiographic progression of OA of the knee joint in some patients (3). This is an exciting finding. Brandt and his colleagues are to be congratulated on the way they have developed the story of doxycycline therapy for OA. However, many concerns remain about the meaning, significance, and implications of this work.

This story begins, as with so many good scientific discoveries, in a serendipitous way. Brandt became aware of the work of Golub and colleagues on doxycycline and periodontal disease (4). He realized that its positive effects in rat models of periodontal connective tissue damage could have relevance to OA. He and his group therefore explored the mode of action of doxycycline in vitro, demonstrating an effect on the activity of both procollagenase and collagenase (5). They also showed that the drug had positive effects in an animal model of OA (6). Brandt and his group then moved on to humans, first doing some “proof-of-concept” experiments by looking at collagenase and gelatinase activity in excised OA femoral heads after preoperative treatment with doxycycline (7). This body of work represents a superb, careful, logical, and rational development of the science.

Does doxycycline improve the natural history of OA?

But does doxycycline really work in human OA? The study results reported in this issue of Arthritis & Rheumatism are all we have to go on so far. In order to increase the chances of finding an effect of doxycycline on the structural progression of OA, the authors chose to study obese women with unilateral knee OA, recruited from the community. This decision was based on previous work suggesting that relatively rapid progression of radiographic changes occurs in this patient group (8). Brandt et al describe a small but significant slowing of the rate of progression of joint space narrowing (JSN) (a surrogate for cartilage loss) in the index knees of these subjects over a 30-month period. There were no significant effects on pain, but there was a small group difference in other clinical outcomes which favored those treated with doxycycline over those treated with placebo.
These data raise 2 obvious questions about the efficacy of doxycycline in OA: 1) is this small, statistically significant effect of any clinical significance? 2) are the findings likely to be generalizable? The patients entered into the trial by Brandt et al had relatively mild knee OA, and they were not receiving extensive treatment for their condition. This situation reduces the scope for demonstrating improvement, but it also raises the question of whether a small change in someone with early disease has much meaning in the life of that individual. Other studies have suggested that radiographic and symptom progression may not go together (9), and we know that a variety of factors other than radiographic change (such as psychosocial problems) contribute greatly to the symptoms in knee OA (10). As such, these findings may not mean much to the clinical outcome of most people with knee OA. The group studied by Brandt et al consisted of obese, middle-age, predominantly white women with early knee OA. It is conceivable that OA of the knee is rather different in men than in women, that early and late disease are different entities, and that the natural history of knee OA is dependent on factors different from those of OA at different sites, such as the hip. Therefore, even if these findings turn out to have clinical as well as statistical significance, they may not be generalizable to other people with OA.

If doxycycline is effective in OA, how does it work?

Assuming that doxycycline has beneficial effects in OA, another question that needs to be asked is, how does it work? As noted above, Brandt et al, along with others, have shown that doxycycline can inhibit connective tissue loss through its effects on metalloproteinases. Doxycycline is also known to affect other pathways that might be important to cartilage integrity, such as nitric oxide production. Given the current emphasis on cartilage damage in OA, it is easy to conclude that the mode of action of the drug is via inhibition of cartilage damage by metalloproteinases and perhaps other cartilage-related mechanisms. But doxycycline does many other things (11,12). It affects bone and other tissues and interferes with many enzyme systems. Some investigators, including those in my own group, have suggested that bone is the crucial tissue involved in the progression of OA (13). I, for one, believe it is possible that any beneficial effect of doxycycline in OA has as much (or more) to do with the effect of the drug on bone as it does with its effect on cartilage. Furthermore, the fact that doxycycline has effects on several different biologic systems must raise concerns about its long-term use in older people with chronic disease, most of whom will have comorbidities.

What other interventions can modify structural changes in OA?

Several other pharmacologic agents might have structural benefits in OA. Perhaps the best established is diacerein. In a well-conducted, relatively large placebo-controlled trial reported in *Arthritis & Rheumatism* in 2001 (14), Dougados and colleagues found a positive effect of this agent on JSN in hip OA. Interestingly, in this trial, the beneficial effect on radiographic progression was not accompanied by any statistically significant improvement in symptoms, leading to the idea that disease modification in OA might be achieved without any symptomatic benefits (15). Claims have also been made for glucosamine (16) and for some hyaluronic acid products, but possible problems of methodology and concerns about various forms of study bias have been raised about the trials that purport to show their efficacy (17,18).

But OA is a disease that is mechanically driven as well as biochemically mediated, so biomechanical as well as pharmacologic interventions might have positive effects (1). Osteotomy and joint distraction are examples. Clear evidence of improvements in joint space width (JSW) and cartilage repair (although with fibrocartilage rather than hyaline cartilage) has been reported following osteotomies to correct mechanical abnormalities in OA of the hip or knee (19), and the beneficial effects of joint distraction on ankle joint OA appear to be accompanied by widening of the joint space due to cartilage repair (20). Therefore, perhaps other, simpler mechanical interventions, such as shoe wedging (21), will have the same effect.

Cartilage transplantation and cell-based therapies that might induce tissue regeneration dominate this research field at present (22). However, these approaches have not yet been shown to provide any lasting clinical benefits to people with OA. If they are to be effective, it seems likely that they will have to be combined with other interventions, including mechanical ones, that help to normalize weight bearing on the affected joint (perhaps it will prove to be easier just to do the mechanical intervention).
So where do we stand with OA disease modification today?

I conclude that we know that cartilage “repair” can occur following interventions that alter the loading of a joint (such as joint distraction or osteotomy), and that we now know that some pharmacologic interventions may be able to reduce the rate of cartilage loss in OA. In my view, the contribution made by the work of Brandt et al is proof of concept of this last point.

But that also worries me. Trials of drugs for OA (including doxycycline) are undertaken in relatively young, relatively fit people who are not representative of those with OA in the community (23). The reality of OA is that it is a disease of older people, most of whom have other health problems in addition to their joint failure. We have recently relearned how susceptible such people can be to drugs that are supposed to have a relatively simple and specific target, such as the COX-2 enzyme. What will happen if we start treating these people with drugs that interfere with connective tissue turnover? Furthermore, in spite of the promising results with doxycycline reported in this issue of *Arthritis & Rheumatism*, I still believe that the link between slowing of structural change and symptom improvement has yet to be established, and I myself would not want to take a drug that might improve my JSW if it was not going to help my pain or function.

In my view, we should not be chasing drugs as a means of modifying the outcome of OA. Rather, we should be looking at simple ways to achieve the benefits that accompany mechanical interventions such as joint distraction and osteotomy. Part of the problem appears to be financial, as well as the bias toward interventions that may be advanced more for the profits they provide for the pharmaceutical companies than for the benefits that accrue to society (24). While I applaud the superb work of Brandt and his colleagues, I worry that it might, paradoxically, increase the trend toward too much “medicalization” of people with OA as well as the rush to prescribe drugs for them that might affect radiographic findings but with unknown and potentially serious toxicity. If this occurs, our OA patients will not be among the “last well people” (25).

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EDITORIAL

The Many Worlds of Reducing Interleukin-1

Charles A. Dinarello

Which diseases respond best to blocking of a specific biologic pathway is not always apparent. In the case of reducing interleukin-1 (IL-1) activities, there is still much that is open for exploration. Although the IL-1 Trap (1), antibodies to IL-1β, antibodies to the IL-1 receptor type I (IL-1RI), and oral inhibitors of caspase 1 are undergoing clinical trials, anakinra, the recombinant form of the naturally occurring IL-1R antagonist (IL-1Ra), is the only agent presently approved for reducing IL-1 activities. Anakinra is approved in the US and Europe for treating the signs, symptoms, and structural damage in patients with moderate-to-severe rheumatoid arthritis. More than 100,000 patients have been treated with anakinra, some for as long as 5 years, and many continue to have benefit. But compared with agents that neutralize tumor necrosis factor (TNF), clinical responses to anakinra may require several weeks or even months of daily treatment before they are apparent. Anakinra binds to the IL-1RI as a pure receptor antagonist, preventing bona fide IL-1 from binding to and activating a cell. Therefore, following subcutaneous injection of anakinra, blocking of IL-1 receptors in the inflamed synovial space is the therapeutic objective in rheumatoid arthritis, and with a receptor antagonist, sustaining sufficient receptor blockade is concentration and time dependent. In contrast, direct intraarticular injection of anakinra into osteoarthritic joints provides some patients with pain relief lasting several weeks (ref. 2 and Weiss J: personal communication). Compared with TNF blockers in patients with rheumatoid arthritis, anakinra has a remarkable safety record.

There are, however, several systemic multisystem syndromes which respond to anakinra within hours or days, revealing a fundamental role for IL-1 in inflammation. These syndromes are characterized by recurrent fevers, neutrophilic leukocytosis, thrombocytosis, elevated levels of serum amyloid A and C-reactive protein, associated with rashes, and diffuse and/or frank deforming arthritis as well as hearing loss, developmental delay, and low grade aseptic meningitis in children. The symptoms are triggered by mild stresses, such as exposure to cold or routine viral infections of the upper respiratory tract. Anakinra rapidly and dramatically arrests each of the multisystem manifestations of these syndromes, commonly within hours or a few days. Upon cessation of anakinra therapy, clinical signs and symptoms, as well as biochemical and hematologic abnormalities, rebound within days.

These syndromes often occur in patients with single point mutations in a gene called cold-induced autoinflammatory syndrome 1 (CIAS1) (now termed NALP3) where the particular protein affected by the mutation is located. The mutations result in single amino acid changes in one of the proteins controlling the activity of the intracellular proteolytic enzyme called caspase 1 (formerly the IL-1β–converting enzyme). This enzyme converts the inactive IL-1β precursor molecule into active IL-1β. Active IL-1β is then released from the cell by a tightly controlled secretory process (3). Indeed, monocytes from patients with a mutation release greater amounts of IL-1β than monocytes from subjects without a mutation (4). However, there are patients with near identical syndromes who lack this particular mutation but experience the same dramatic resolution of disease activity within 24 hours of the first injection of anakinra (5–7).

The mutation is also absent in patients with refractory adult-onset Still’s disease, where a rapid resolution of the disease activity is observed within hours or days of treatment with anakinra (8–11). Anakinra is now the treatment of choice in patients with steroid-refractory adult-onset Still’s disease, mutations in NALP3, and Schnitzler’s syndrome (van der Meer J: personal communication). Systemic juvenile rheumatoid

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Fever, neutrophilia, and high levels of acute-phase proteins often characterize systemic inflammation, and skin rashes are indicative of endothelial cell activation. This is certainly the case in patients with NALP3 gene mutations and also in patients with adult-onset Still’s disease. Peak plasma levels of anakinra are between 1 and 1.5 μg/ml 3–4 hours after a subcutaneous injection, and upon entering the intravascular space, endothelial receptors for IL-1 have become saturated. However, the effect is short-lived, and plasma levels of anakinra return to baseline levels by 24 hours. Occupancy of the endothelial IL-1 receptors by anakinra is a global effect, resulting in blocking of all IL-1–mediated systemic inflammation. This action could explain the paradox of responses to anakinra; in systemic disease, endothelial IL-1 receptors are blocked rapidly and inflammation is arrested, whereas in joint disease, saturation of IL-1 receptors is dependent on synovial penetration, with clinical improvement and slowing of structural damage being observed only after prolonged use. Achieving pharmacologic occupancy of any receptor by a specific antagonist is no easy task, particularly when the receptor is not restricted to a particular tissue. In humans with rheumatoid arthritis, blocking of the type I receptor with anakinra is further complicated by its rapid renal clearance.

Clearly, the most marked responses to anakinra have been observed in patients with adult-onset Still’s disease (9,10), macrophage activation syndrome, familial Mediterranean fever, or mutations in the NALP3 gene. These mutations can cause Muckle-Wells syndrome (15), neonatal-onset multisystem inflammatory disease (7), and familial cold autoinflammatory syndrome (16). Although it is often difficult to demonstrate elevated circulating levels of IL-1β in these patients, the rapid reduction in fever, neutrophilia, and acute-phase reactants by anakinra demonstrates that these are IL-1–mediated diseases, since IL-1Ra blocks only the IL-1 receptor. IL-1 is the most pyrogenic of the fever-inducing cytokines (17); moreover, IL-1 is a bone marrow stimulant, particularly of neutrophilic responses (18). In contrast, TNFα suppresses bone marrow functions. IL-1 induction of endothelial IL-6 likely accounts for the rise in hepatic acute-phase proteins and thrombocytosis (16).

As shown in Figure 1, the endothelium is a primary target for the efficacy of anakinra in these systemic inflammatory diseases. The lesson learned from treating these rare diseases with specific blockade of IL-1 is that the clinical, hematologic, and biochemical manifestations are hardly rare; in fact, they are the hallmarks of systemic inflammation. These clinical findings also place IL-1 in a unique position in the cascade of cytokines during inflammation. They raise the question as to whether there are “unique IL-1 diseases” or whether IL-1 mediates the inflammation induced by more distal cytokines, such as TNF or IL-18. It should be noted that many patients with or without the NALP3 mutation, as well as patients with adult-onset Still’s disease, were initially treated with infliximab or etanercept with partial responses, suggesting that neutralization of TNF results in
decreased IL-1 activity in those patients. This author supports the concept that IL-1 contributes to the inflammatory component of most diseases and that efficacy of antibodies to TNF is due, in part, to a reduction in IL-1 activities. However, uniquely IL-1-mediated diseases do exist due to dysfunction in IL-1 gene expression, processing, and release, as well as receptor expression.

How best to reduce IL-1 activities: receptor antagonism and decoy receptors

It would seem that there is no need to have any other agent but anakinra to treat IL-1-mediated inflammation. However, there are several mechanisms by which nature limits IL-1 activity and each can be exploited for novel therapeutic targets. For treatment of any disease, blocking of the most proximal defect in the pathologic process reduces collateral damage. In the case of anticytokine therapies, reducing collateral damage means sparing impairment of host defenses. In rheumatoid arthritis, sparing host defenses becomes a particularly important consideration for long-term therapy since the disease itself exhibits a markedly reduced T cell repertoire even in young patients (19). T cell function is also reduced by the concurrent use of disease-modifying antirheumatic drugs, and the aging process itself is a progressive state of T lymphocyte senescence.

In a report in this issue of Arthritis & Rheumatism, Smeets et al (20) show that there is a selective effect on lymphocyte activation when 2 different methods for blocking IL-1 are compared in the mouse model of collagen-induced arthritis (CIA). They compared overexpression of IL-1Ra with overexpression of the soluble form of the IL-1 coreceptor, termed the IL-1 receptor accessory protein (sIL-1RAcP). The results were unexpected. Although overexpression of either IL-1Ra or sIL-1RAcP ameliorated joint and systemic manifestations of CIA in mice, lymphocyte populations affected by IL-1 blockade were not similar. The differential effects of the 2 IL-1 blockers occurred between B and T lymphocytes. With the report that antibodies to CD20 on B cells reduce disease severity in patients with rheumatoid arthritis, targeting B lymphocyte function in models of rheumatoid arthritis takes on increasing importance.

In the CIA model, both IL-1 blockers suppressed the levels of anticollagen IgG2a, as well as the production of IL-6. But, whereas overexpression of IL-1Ra blocked NF-κB signaling in both T and B lymphocytes, overexpression of sIL-1RAcP reduced activation only in B lymphocytes, sparing T cell activation. The findings have implications regarding the long-term safety of treating rheumatoid arthritis with anticytokine therapies. Sparing T lymphocyte function is the lesson from the human immunodeficiency virus 1 epidemic. When comparing TNF blocking therapies to anakinra treatment, there is a remarkable difference in the number of opportunistic infections between reducing TNFα and IL-1 activities (21). In marked contrast, there are hardly any voluntary reports, or findings in controlled trials, of opportunistic infections in rheumatoid arthritis patients treated with anakinra (22), including populations at high risk for reactivation of Mycobacterium tuberculosis infections (23). These clinical realities support the concept that preservation of T lymphocyte function using anticytokine therapies is a worthy objective for long-term safety. For that reason, the results of the study by Smeets et al need closer examination.

What accounts for the differences between overexpression of IL-1Ra and sIL-1RAcP?

The findings of Smeets and coworkers are novel with regard to the pathogenesis and treatment of rheumatoid arthritis, but not entirely surprising regarding the biology of IL-1 and its receptors. The difference between IL-1Ra and sIL-1RAcP in reducing IL-1 is best understood in terms of preventing IL-1 activity at the level of the cell surface compared with the extracellular space. IL-1Ra blocks IL-1 surface receptors, which are present on all nucleated cells, primarily by occupancy of the ligand-binding IL-1RI; in fact, IL-1Ra binds to this receptor with a greater affinity than IL-1β. Occupancy of IL-1RI by IL-1Ra prevents the recruitment of the IL-1RAcP coreceptor to form the heterodimer that initiates signal transduction (Figure 2A). However, there is another cell surface receptor for IL-1, termed IL-1RII. This receptor, which lacks a cytoplasmic domain and cannot participate in signal transduction, functions as a decoy receptor by competitive binding to IL-1β (24) (Figure 2B). The type II receptor can also form an inactive complex with the IL-1RAcP (Figure 2B), preventing the cell from participating in signal transduction (25,26).

Shedding of IL-1 cell surface receptors by proteolytic cleavage results in circulating levels of only the extracellular domains, termed soluble receptors. As shown in Figure 2C, sIL-1RI can bind IL-1Ra, IL-1α, or IL-1β. However, the soluble type I receptor has a higher affinity for IL-1Ra than for IL-1β, and is more likely to bind the antagonist than IL-1α or IL-1β. There is no evidence that sIL-1RAcP alone can neutralize IL-1
Figure 2. Natural mechanisms for reducing interleukin-1 (IL-1) activities. A, Most cells express both components of the IL-1 receptor complex. These receptors comprise 3 immunoglobulin-like domains and an intracellular domain containing the Toll-like regions (stippled area). IL-1 receptor type I (IL-1RI) binds IL-1β, which then recruits the IL-1 receptor accessory protein (IL-1RAcP). This forms the high-affinity heterodimeric signaling complex. X-ray diffraction studies of co-crystals of the complex revealed that IL-1β binds to the third domain of IL-1RI and that the first domain undergoes a structural change in shape. Proximity to the intracellular Toll–IL-1 receptor (TIR) domains of each receptor chain (2-headed arrow) initiates a signal. When the receptor antagonist (IL-1Ra) occupies the IL-1RI, the IL-1RAcP is not recruited, and there is no heterodimer and no signal. The affinity of IL-1Ra for the IL-1RI is greater than that for IL-1β. Endogenous IL-1Ra functions to limit the activities of IL-1 since mice deficient in IL-1Ra spontaneously develop inflammatory diseases including arteritis (39) and a destructive rheumatoid arthritis–like joint disease (40). There is no evidence that either IL-1β or IL-1Ra can bind to the IL-1RAcP alone, and hence this receptor chain is classified as an essential coreceptor. B, In neutrophils, monocyte/macrophages, B lymphocytes, and chondrocytes, another IL-1 receptor chain is expressed, termed the IL-1 receptor type II (IL-1RII). This receptor chain is also called the “decoy” receptor since it has a greater binding affinity to IL-1β than the type I receptor and hence serves as a “sink” for the ligand. This receptor lacks a significant intracellular segment, and there is no TIR domain. The IL-1RII binds IL-1β but does not signal (24). IL-1β bound to the IL-1RII can also form a high-affinity complex with the IL-1RAcP (25,26). Cells also expressing this receptor benefit from blockade of the type I receptor by IL-1Ra as well as the decoy effect of the type II receptor. C, The extracellular (also termed soluble) domains of each of the IL-1 receptor chains exist in the plasma as well as in interstitial fluids. In general, their concentrations in body fluids increase 2–4-fold in disease states. The soluble IL-1RI (sIL-1RI) binds IL-1Ra with a greater affinity than that for IL-1α or IL-1β. The soluble type I receptor may act as a sink for IL-1Ra at natural as well as pharmacologic levels. Not surprisingly, administration of sIL-1RI was not effective in reducing IL-1 activities in patients with rheumatoid arthritis (41) or in human volunteers injected with endotoxin (42). The sIL-1RII binds IL-1β but neutralizes its activities. The sIL-1RAcP does not bind IL-1β but rather forms a high-affinity complex with the sIL-1RII and neutralizes IL-1β activities (27). This cell is exposed to high levels of the soluble form of the IL-1RaCp. Although the source of sIL-1RaCp can be proteolytic release of the cell-bound IL-1RaCp, constitutive secretion of sIL-1RaCp from hepatocytes of an mRNA splice variant (29) likely contributes to high levels in body fluids. Soluble IL-1RaCp may form a complex with either IL-1RI or IL-1RII and IL-1β in vivo but without initiating a signal (29). Another splice variant of constitutively secreted sIL-1RaCp exists (30). During cell activation, there appears to be a down-regulation of the membrane form of IL-1RaCp and an increase in the translation of the constitutively secreted sIL-1RaCp (30). In cells primarily expressing the type II IL-1 receptor, the formation of the inactive complex of sIL-1RaCp with the cell-bound type II receptor provides for enhanced inhibition of IL-1 activities. In vivo, the combination of the sIL-1RII and sIL-1RaCp results in a greater inhibition of IL-1 activity than does sIL-1RII alone (27).
activities. There is also a paucity of in vitro data that the natural soluble type I receptor reacts with the soluble IL-1RAcP to form a complex with either IL-1α or IL-1β, but these complexes may form in vivo and may explain the data obtained by Smeets et al. Alternatively, it is possible to construct a high-affinity “trap” for IL-1β by combining the extracellular domains of both the IL-1RI and IL-1RAcP as a neutralization strategy. In fact, a recombinant bivalent chimeric containing the extracellular domains of both IL-1RI and IL-1RAcP linked toFc, termed the IL-1 Trap, has been engineered (1). Of clinical significance, the IL-1 Trap preferentially binds IL-1β and has been effective in clinical trials for the treatment of rheumatoid arthritis. The IL-1 Trap is also being studied in other inflammatory diseases.

Unlike the soluble type I receptor, the soluble type II receptor preferentially binds IL-1β and not IL-1Ra (Figure 2C). Furthermore, once IL-1β binds to the soluble type II receptor, sIL-1RAcP is recruited to form a complex with an affinity for IL-1β 100 times greater than that of sIL-1RII alone (27). This latter complex (Figure 2C) may be the dominant mechanism for the natural neutralization of IL-1β by endogenous soluble receptors. In monkeys, neutralization of IL-1β by sIL-1RII is greatly enhanced by sIL-1RAcP (27).

Several studies have demonstrated low (picogram and subpicogram/milliliter) levels of circulating IL-1β in human disease; however, levels of soluble type II receptor circulate in the nanogram/milliliter range (4–6 ng/ml). We now know that sIL-1RAcP circulates at an even greater concentration (median level 300 ng/ml) in healthy humans (27). Although most soluble receptors are generated by proteolytic cleavage from the cell surface receptors, this is apparently not the mechanism for high levels of sIL-1RAcP (27). It is likely that constitutive secretion of sIL-1RAcP explains the existence of these high levels. Supporting this concept is the existence of a splice variant of the IL-1RAcP, which lacks a transmembrane anchor (28–30). This sIL-1RAcP is synthesized and released by the liver in somewhat the same way IL-1Ra is released by the liver; as an acute-phase protein (31). In the report by Smeets and colleagues, overexpression of sIL-1RAcP likely formed a complex of IL-1β with sIL-1RII in the extracellular space (Figure 2C), with the cell surface IL-1RII (Figure 2D), or possibly with IL-1RI (Figure 2D). Thus, a large molar excess of sIL-1RAcP provides at least 3 mechanisms to entrap secreted IL-1β and reduce not only the arthritis, but also IL-6 production and antibodies to the collagen. In contrast, the sole mechanism for IL-1Ra is binding to the type I IL-1 surface receptor.

**Cell selectivity of decoy IL-1R**

The study by Smeets and coworkers revealed that sIL-1RAcP selectively reduces IL-1 activity on cells that express surface type II decoy receptors (B lymphocytes and chondrocytes). For example, B lymphocytes express 20-fold more type II receptors than type I receptors, but in T lymphocytes, this increase is only 5-fold. Relevant to IL-1–mediated cartilage breakdown and inhibition of proteoglycan synthesis, chondrocytes express an excess of type II receptors. As shown in Figure 2D, overexpression of sIL-1RAcP likely formed complexes on the cell surface of IL-1β bound to type II receptors. This complex as the type II receptor decoy mechanism was first proposed by Malinowsky et al (25) and Lang et al (26), and accounts for the ability of sIL-1RAcP to reduce B lymphocyte activation. It is thus likely that low levels of type II receptors on T lymphocytes prevent suppression of T lymphocytes in vivo and in vitro by sIL-1RAcP.

Since IL-1Ra preferentially binds to the type I receptor, and since the type I receptor is present on all nucleated cells, IL-1Ra inhibited both B and T lymphocyte responses.

**IL-1 effects on T and B lymphocyte functions**

Several studies have established the adjuvant properties of IL-1, particularly IL-1β. The adjuvant activity is likely due to the induction of B lymphocyte growth factors such as IL-6. In studies using mice deficient in both IL-1α and IL-1β, the primary B cell functions of antibody production to T cell–independent antigens were normal (32). In addition, antibodies to other antigens such as lipopolysaccharide, and proliferative responses to mitogens, were unaffected in these mice. However, both primary and secondary antibody production against the T lymphocyte–dependent sheep red blood cell antigen was significantly reduced in mice deficient in both IL-1α and IL-1β (32). Furthermore, antibodies to sheep red blood cells are normal in IL-1α-deficient mice, suggesting a specific role for IL-1β, since antibodies to common antigens require T helper lymphocyte interactions with antigen-presenting cells. On the other hand, the presence of IL-1α, but not IL-1β, was required during skin sensitization to a chemical antigen (33). In this case, transfer of antigen-conjugated IL-1α-deficient epidermal cells is unable to prime T lymphocytes for sensitization. This result is not unexpected, since IL-1α but not IL-1β is constitutively expressed in epidermal cells.
Why 2 IL-1s?

Interpretation of the results of the study by Smeets and coworkers focuses on the interaction of sIL-1RAcP with IL-1RII, the decoy receptor binding primarily IL-1β. But there are 2 IL-1s, and there is no dearth of data that IL-1α has its place in causing IL-1–mediated disease. IL-1β is the secreted form of IL-1 (Figure 1) and, although circulating levels of IL-1β are measurable, these levels are usually in the low picogram/milliliter range, even in severe diseases such as sepsis. In contrast, the IL-1 precursor is not cleaved by caspase 1, IL-1α is not secreted from cells, and only in severe disease can one detect serum IL-1α, which may result from its release from dying cells. IL-1α remains intracellular, where it can function as a DNA binding transcription factor, and perhaps as an oncogene (34–36). As shown in Figure 3, the IL-1α precursor is biologically active when inserted into the cell’s membrane, oriented in such a manner that it can bind to the IL-1RI surface receptor and recruit the IL-1RAcP. As with secreted IL-1β, the Toll domains (stippled areas) of both receptor chains trigger signal transduction. On the right, IL-1Ra occupancy of the type I receptor prevents the binding of membrane IL-1α, and no signal is transduced. See Figure 2 for definitions.

Juxacrine stimulation occurs when a cytokine expressed on the surface of one cell triggers the specific cytokine receptor on an adjacent cell (cell–cell contact), since the two cells are in juxtaposition to each other. Membrane IL-1α employs this mechanism of activity. IL-1α that is processed and released from the cell has a higher affinity for the type I receptor than does IL-1β. Membrane IL-1α also triggers the type I receptor. Therefore, the response to anakinra in systemic inflammation likely includes blocking of the juxacrine activity of IL-1α (Figure 3) as well as blocking of IL-1β secreted from the cell.

Exploiting nature’s mechanisms for limiting IL-1 activities

Are there better ways to achieve a reduction in IL-1 activities, particularly in localized disease? It is a general concept that IL-1–associated disease severity is regulated at the level of ligand production and activity, not the receptor level. For example, the IL-1 type I receptors are expressed on all cells in healthy subjects and increases of 2–3-fold occur in models of disease. On the other hand, in circulating monocytes and bone marrow macrophages, gene expression for IL-1β is absent under normal conditions but increases at least 100-fold with stimulation. Moreover, most of the messenger RNA coding for IL-1β is not translated into the IL-1β precursor, but instead degrades rapidly. The conversion of the precursor by caspase 1 to an active cytokine is tightly controlled. In fact, most of the IL-1β precursor that is synthesized is never cleaved despite the presence of constitutive caspase 1 in the same cell.

An elaborate complex of proteins termed the “IL-1β inflammasome” limits caspase 1 activity. The great lesson learned from the study of patients with a single point mutation in the NALP3 gene is that the “IL-1β inflammasome” has lost this tight control and that relatively minor stresses result in dramatic systemic and local disease. Thus, neutralization of IL-1β through sIL-1RAcP, IL-1 Trap, or inhibition of caspase 1 offers possible treatment options in these patients. However, there are patients without a mutation in this gene who also experience similar systemic IL-1–mediated disease, leaving the potential for novel therapies to reduce IL-1 activities. The implications of the study by Smeets and coworkers are that at pharmacologic levels, sIL-1RAcP may be an effective treatment option and may spare T lymphocyte activation by IL-1. Although the level of sIL-1RAcP that provides effective reduction in IL-1 activities is presently unknown, sIL-1RAcP has the
advantage of functioning as a neutralizing mechanism (Figure 2C) as well as participating in the formation of decoy complexes on the cell surface (Figure 2D). The IL-1 Trap (1) already takes advantage of the high-affinity complex of IL-1β (or IL-1α) with the type I receptor and the sIL-1RaCp for neutralization of IL-1. Future studies will further elucidate these interactions and define more clearly the role of IL-1 in inflammatory diseases and clarify optimal strategies for treatment.

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Selective Cyclooxygenase 2 Inhibitors and Cardiovascular Events

Daniel H. Solomon

Introduction

Just when rheumatologists thought we had more specific and less toxic drugs for our patients, we learned that selective cyclooxygenase 2 (COX-2) inhibitors (coxibs) may not provide a true overall safety advantage compared with traditional nonselective nonsteroidal antiinflammatory drugs (NSAIDs). A report in the lay press from the National Institutes of Health (1) even called into question the cardiovascular safety of naproxen, one of the “workhorses” in a rheumatologist’s armamentarium. Naproxen has been considered safe enough to be sold in the US without a prescription.

While NSAIDs and coxibs provide important analgesic and antiinflammatory benefits to millions of patients, the cardiovascular safety concerns have led many patients to discontinue their use. Reports about potential links between these agents and adverse cardiovascular outcomes have been presented by the media before publication in the peer-reviewed literature and have left treating doctors unsure whether yesterday’s recommended drug might be on today’s “blacklist.” The alphabet soup of trials—APPROVe, APC, PreSAP, ADAPT, and others—is dizzying, and without reporting of important details from these trials, rhetoric and finger-pointing have been more common than scientific debate.

Reviewing a subject that is a “moving target” is not without hazard. With this caveat in mind, this review will focus on the basic and clinical science underlying the current controversy regarding the cardiovascular safety of coxibs. First, I will summarize the proposed mechanisms underlying the relationship between thrombotic cardiovascular events and these medications. Second, I will examine the key randomized controlled trials that have been published or presented publicly and the relevant cardiovascular safety information. Finally, I will discuss the data from published observational epidemiologic studies. This review will concentrate on thrombotic cardiovascular outcomes, mainly acute myocardial infarction (MI) and ischemic cerebrovascular accident (CVA). Data on congestive heart failure will be noted when available, but few studies have addressed this outcome. There are scant data available on the cardiovascular safety of nonselective NSAIDs, and thus they will not be the focus of this review. Etoricoxib will not be included because safety data on this agent have not yet been published in the peer-reviewed literature.

Biologic plausibility

Early during the development and testing of the coxibs, studies in humans suggested that these agents may be associated with adverse cardiovascular events. Selective COX-2 inhibition had been heralded as a means of deriving analgesic and antiinflammatory benefit without the risk of bleeding. This assertion was based on the understanding that platelet-derived COX-1 is the major source of thromboxane (2). However, the origins of circulating prostacyclin, the other important vasoactive eicosanoid, were not as clear. The importance of prostacyclin lies partly in its reciprocal relationship with thromboxane—thromboxane critical for platelet aggregation and vasoconstriction, and prostacyclin for dampening the effects of thromboxane through fibrinolysis and vasodilatation.

Studies of healthy volunteers showed that urine
levels of a prostacyclin metabolite were reduced in subjects taking rofecoxib, suggesting that endovascular production of prostacyclin may be COX-2 mediated (3). Additional concerns were raised by studies in animal models showing that COX-2 is up-regulated in vascular segments under conditions of increased shear stress (4). One potential implication of these observations is that COX-2 plays a beneficial role in vascular health and that its inhibition may create an imbalance between thromboxane and prostacyclin, thereby favoring thrombosis and vasoconstriction. The potential risk of thrombosis based on these mechanisms was articulated in an early review of coxibs (5). Furthermore, this risk was highlighted in a report of a case series describing 4 patients with connective tissue disease who developed thrombotic complications (6). While the prothrombotic potential of coxibs does not seem to be relevant in most patients, results of a series of elegant animal studies using knockout mice suggest that an imbalance between prostacyclin and thromboxane may account for some of the cardiovascular risk associated with these agents (7,8). It is unclear whether this imbalance may also link NSAIDs with cardiovascular outcomes. Even nonselective NSAIDs are differentially selective for COX-1 and COX-2 (9) (Figure 1).

The vasoreactive potential of coxibs was further demonstrated in several studies in which flow-mediated dilatation in humans was measured. This test assesses the release of nitric oxide from the endothelium and has been established as an important surrogate measure of cardiovascular health, with reduced flow-mediated dilatation associated with a greater number of future cardiovascular events (10). Separate studies have tested the effects of rofecoxib and celecoxib on flow-mediated dilatation. Two small studies with celecoxib were both ≤2 weeks in duration (11,12); subjects were hypertensive patients without known coronary artery disease in 1 study and men with stable coronary artery disease in the other. Both of these studies demonstrated an improvement in flow-mediated dilatation while patients were taking celecoxib 200 mg twice daily compared with placebo. Two studies investigating effects of rofecoxib 25 mg daily in patients with known coronary artery disease showed no change in flow-mediated dilatation compared with placebo over the 2–6-month study periods (13,14).

Other possible mechanisms linking certain coxibs (and NSAIDs) to long-term cardiovascular outcomes include changes in blood pressure and possible oxidative modification of biologic lipids. The hypertensive potential of NSAIDs was recognized more than a decade ago, with some suggestion that certain agents had greater effects on blood pressure than others (15,16). Celecoxib and rofecoxib have been noted to have differing effects on blood pressure. Findings of a recent meta-analysis suggest that both agents are associated with elevations in blood pressure and that rofecoxib is associated with a 2.8–mm Hg greater rise in systolic blood pressure compared with celecoxib, but with no relative elevation in diastolic blood pressure (17). Much less is known about the effect of different agents on oxidative modification of lipids leading to atherosclerosis. Findings of 1 study have suggested that rofecoxib and etoricoxib, agents with a sulfone moiety, exhibit pro-oxidant activity leading to

Figure 1. Concentrations of selected nonsteroidal antiinflammatory drugs and coxibs required to inhibit 50% of the cyclooxygenase 1 (COX-1) and COX-2 enzymatic reactions in assays with whole human blood (50% inhibition concentrations [IC50]). Reproduced from ref. 9.

Figure 2. Thrombotic cardiovascular events experienced by patients in the VIGOR (Vioxx Gastrointestinal Outcomes Research) trial. Note that the event curves diverge after ~6 weeks. Vertical bars show the 95% confidence intervals (95% CIs). Reproduced from ref. 21.
Table 1. Cardiovascular events observed in long-term trials of coxibs and NSAIDs*

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<th>Trial (ref.)</th>
<th>Patient population</th>
<th>Median followup, months</th>
<th>Aspirin, %</th>
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<th>Comparator arm</th>
<th>Relative risk (95% CI)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>n</td>
<td>Dosage</td>
<td>Events Rate†</td>
<td>n</td>
</tr>
<tr>
<td>Rofecoxib</td>
<td>VIGOR (20)</td>
<td>RA</td>
<td>9</td>
<td>0</td>
<td>4,047</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>APPROVe (22)</td>
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<td>30</td>
<td>16</td>
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<td>46</td>
</tr>
<tr>
<td>Celecoxib</td>
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<td>OA and RA</td>
<td>9</td>
<td>22</td>
<td>3,987</td>
<td>34</td>
</tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>APC (26)</td>
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<td>30</td>
<td>685</td>
<td>16</td>
</tr>
<tr>
<td>PreSAP (28)</td>
<td>Adenomatous polyp</td>
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<td>16</td>
<td>933</td>
<td>20</td>
<td>0.72</td>
</tr>
<tr>
<td>AD 97-02-001 (28)</td>
<td>Miloid-to-moderate AD</td>
<td>12</td>
<td>NA</td>
<td>285</td>
<td>23</td>
<td>15</td>
</tr>
<tr>
<td>Valdecoxib</td>
<td>CABG I (29)</td>
<td>Post-CABG</td>
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<td>100</td>
<td>311</td>
<td>24</td>
</tr>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CABG II (30)</td>
<td>Post-CABG</td>
<td>1.3</td>
<td>100</td>
<td>555</td>
<td>11</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Luminarcoxib</td>
<td>TARGET (27)</td>
<td>OA</td>
<td>12</td>
<td>22</td>
<td>4,376</td>
<td>19</td>
</tr>
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<td></td>
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</tr>
</tbody>
</table>

* Some of the cardiovascular events were not analyzed in the references noted. When post-publication analysis changed numbers, the revised numbers from the Food and Drug Administration reviewer's reports (http://www.fda.gov/ohrms/dockets) were used. NSAIDs = nonsteroidal antiinflammatory drugs; 95% CI = 95% confidence interval; VIGOR = Vioxx Gastrointestinal Outcomes Research study; RA = rheumatoid arthritis; bid = twice a day; APPROVe = Adenomatous Polyp Prevention on Vioxx trial; CLASS = Celecoxib Long-term Arthritis Safety Study; OA = osteoarthritis; tid = 3 times a day; APC = Adenoma Prevention with Celecoxib trial; PreSAP = Prevention of Spontaneous Adenomatous Polyps study; AD = Alzheimer's disease; NA = not available; CABG = coronary artery bypass grafting; TARGET = Therapeutic Arthritis Research and Gastrointestinal Event Trial.
† Number of cardiovascular events per 100 person-years. The definition of events differed by study (see text).
‡ When not available, the relative risks were calculated as the crude event rate in coxib users divided by the crude event rate in the comparator group.
§ Same comparator group as shown immediately above for celecoxib 200 mg bid.
¶ Medications were administered intravenously (parecoxib) for the first 3 days and then orally for 11 more days in CABG I and for 7 more days in CABG II.
# Same comparator group as shown immediately above for parecoxib/valdecoxib 20 mg bid.
F$_2$-isoprostane formation; this activity is not associated with their COX activity and was not observed with other coxibs and NSAIDs (18).

No one mechanism linking coxibs (or NSAIDs) to cardiovascular events has been proven. However, several compelling possibilities have been described: thrombosis and vasoconstriction associated with an imbalance between thromboxane and prostacyclin, hypertension from inhibition of prostaglandin-dependent counterregulatory mechanisms, and COX-independent oxidative stress. These potential mechanisms could explain both short-term and long-term risks, similar to those suggested by the clinical trial and observational data.

Randomized clinical trial data

It is difficult to gain a complete picture of the cardiovascular safety of coxibs (or NSAIDs) based on the existing randomized clinical trial data. Several factors contribute to this characterization. Few trials were long in duration or enrolled enough subjects to observe adequate numbers of cardiovascular events. When cardiovascular events were recorded and evaluated, definitions of these outcomes were often created retrospectively and differed across studies. Except for several prevention studies, most recent trials have compared a coxib with an NSAID in patients with arthritis. While it is difficult to imagine conducting a placebo-controlled trial in patients with arthritis, the use of active NSAID comparators has created complexity in terms of interpreting the relative cardiovascular event rates, because these agents may also affect cardiovascular outcomes. Comparison of one coxib trial with another is also fraught with difficulty because of the different populations recruited across studies (19). Finally, none of the trials have been large enough to determine whether the risk of cardiovascular events associated with coxibs is concentrated in certain subgroups of patients. Of course, patients with many cardiovascular risk factors have larger cardiovascular event rates, but the relative risk of cardiovascular events may be higher in coxib users with fewer risk factors. The current data do not address the critical question of whether coxibs are “safe” from a cardiovascular standpoint in certain subgroups of patients—perhaps, for example, younger persons without known cardiovascular risk factors. However, patients in this subgroup rarely “require” the potential gastrointestinal (GI) safety afforded by coxibs.

Rofecoxib. Rofecoxib was withdrawn from the market by Merck on September 30, 2004, after it was determined in an adenomatous polyp prevention trial that long-term treatment with the drug at 25 mg/day was associated with a doubling of the risk for thrombotic cardiovascular events. However, the potential for an elevated risk of thrombosis with rofecoxib treatment was widely recognized 4 years earlier, with publication of the Vioxx Gastrointestinal Outcomes Research (VIGOR) trial (20). In that trial, event rates diverged after ~6 weeks, and the hazard ratio (the event rate in the rofecoxib users compared with the event rate in the naproxen users) did not appear proportional over the followup period (21) (Figure 2).

![Figure 2](image-url)

The Adenomatous Polyp Prevention on Vioxx (APPROVe) trial tested rofecoxib 25 mg daily against placebo among 2,586 patients with a prior adenomatous polyp (22) (Table 1). Seventeen percent reported daily aspirin use at the time of study enrollment. Patients in the rofecoxib arm experienced a 4.61-fold increase (95% confidence interval [95% CI] 1.50–18.83) in the risk of congestive heart failure, which became clear early during followup (Figure 3). Severe thrombotic cardiovascular events (acute MI, CVA, unstable angina, sudden cardiac death, transient ischemic attack, peripheral arterial thrombosis, peripheral venous thrombosis, and pulmonary embolus) were approximately twice as frequent in the rofecoxib arm than in the placebo arm. Divergence in these curves became apparent after 18 months of followup (Figure 4).

The VIGOR trial compared 50 mg daily of rofecoxib versus 500 mg twice daily of naproxen in 8,076 patients with rheumatoid arthritis (Table 1). Aspirin
users were excluded from this trial. While the patients in the rofecoxib arm experienced fewer adverse GI outcomes, they experienced acute MIs 5 times more frequently, and the relative risk for severe thrombotic cardiovascular events (acute MI, CVA, unstable angina, sudden cardiac death, transient ischemic attack, peripheral arterial thrombosis, peripheral venous thrombosis, and pulmonary embolus) was doubled. Several other trials with rofecoxib 25 mg had not demonstrated an increased cardiovascular risk, although these were generally of shorter duration (23).

Celecoxib. The CLASS (Celecoxib Long-term Arthritis Safety Study) compared celecoxib 400 mg twice daily with ibuprofen 800 mg 3 times daily or diclofenac 75 mg twice daily (24) (Table 1). Patients with osteoarthritis or rheumatoid arthritis were enrolled. At trial enrollment, 22% of the patients reported regular aspirin use. Rates of cardiovascular events (acute MI, CVA, or cardiovascular death) did not differ significantly between the celecoxib users and the combined NSAID user group. Some have suggested that cardiovascular event rates among the non–aspirin users were higher among celecoxib users than the NSAID comparators; however, this difference has not been shown to be statistically significant (25).

The Adenoma Prevention with Celecoxib (APC) trial demonstrated that high-dose, long-term celecoxib use was associated with an increase in the risk of thrombotic cardiovascular events (acute MI, CVA, or cardiovascular death) (26) (Table 1). In this chemoprevention trial, 2,035 patients with prior adenomatous polyps were randomized to receive either celecoxib 200 mg twice daily, celecoxib 400 mg twice daily, or placebo. At trial entry, 30% of patients reported aspirin use. Thirty-three months after its initiation the trial was halted by the National Cancer Institute because of an increase in the risk of cardiovascular events observed in both celecoxib arms (Table 1 and Figure 5). There did appear to be a dose-response effect, with the higher dosage of celecoxib associated with more frequent cardiovascular events. Congestive heart failure occurred in 2 subjects (0.3%) in the placebo arm versus 5 (0.4%) in both celecoxib arms.

A number of post hoc analyses of the APC data examined whether patient factors could identify those at particularly high or low risk for thrombotic cardiovascular events. While results of these analyses suggest that older age, male sex, and the presence of cardiovascular risk factors may identify different groups with a higher likelihood of cardiovascular events, none of the interaction terms (between treatment arm and patient factor) reached statistical significance. Importantly, the aspirin user subgroup (hazard ratio 3.8, 95% CI 0.9–16.6) did not experience a lower risk of cardiovascular events than those not using aspirin (hazard ratio 2.4, 95% CI 0.9–6.4). The wide and overlapping CIs around these estimates demonstrate that we have too little data to clarify whether aspirin will have a role as cotherapy with coxibs for cardioprotection. However, we do know that patients taking aspirin do not experience a reduced risk of GI toxicity while taking coxibs compared with nonselective NSAIDs (25,27).

A second adenomatous polyp prevention trial, the PreSAP (Prevention of Spontaneous Adenomatous
Polyps) study (28), also tested celecoxib versus placebo (Table 1). In that yet-to-be-published trial, 1,561 subjects were randomized to receive once-daily (not twice) dosing of celecoxib at 400 mg or placebo. After 33 months of followup, there was no increase in cardiovascular events (acute MI, CVA, or cardiovascular death) observed in the celecoxib arm versus the placebo arm.

The last celecoxib trial to be discussed was completed several years ago and has not been reported in the peer-reviewed literature, but it was presented at the Food and Drug Administration (FDA) Arthritis and Drug Safety and Risk Management Advisory Committee meeting in February 2005 (28) (Table 1). This trial enrolled 425 patients with mild-to-moderate Alzheimer’s disease; 285 received celecoxib 200 mg twice daily and 140 received placebo. Over the 52-week study, there was an increase in the occurrence of thrombotic cardiovascular events (acute MI, CVA, and peripheral thrombotic events) among patients taking celecoxib compared with placebo. However, the findings of the trial are difficult to interpret in light of substantial baseline imbalance between the 2 randomized groups: the rate of coronary artery disease at baseline was 4 times higher among patients in the celecoxib group than among those in the placebo group.

Valdecoxib. There have been several short-term studies of valdecoxib in patients with arthritis, but none have had adequate numbers of events to enable one to deem the medication safe or unsafe. However, the results of trials in patients undergoing coronary artery bypass grafting (CABG), the CABG I (29) and CABG II (30), have shown an increased risk of cardiovascular events (acute MI, CVA, congestive heart failure, thrombophlebitis, death) among patients taking valdecoxib or its intravenous formulation, parecoxib (Table 1). These trials were designed to determine whether valdecoxib might have a role in postoperative pain management. In CABG I, 462 patients were enrolled, in a 2:1 active treatment:placebo ratio; those in the valdecoxib arm (n = 311) received the intravenous formulation within 30 minutes after extubation. Intravenous or oral valdecoxib 20 mg twice daily was continued for 14 days. All types of serious adverse events occurred twice as frequently among patients in the valdecoxib arm (19%) than among those in the placebo arm (9.9%). Cardiovascular events (acute MI, CVA, congestive heart failure, thrombophlebitis, death) were also more common with valdecoxib treatment than with placebo.

In CABG II 1,671 patients were assigned to 1 of 3 treatment arms: intravenous parecoxib followed by valdecoxib, intravenous placebo followed by valdecoxib, or intravenous placebo followed by oral placebo. Intravenous treatment was continued for 3 days and then oral treatment for 7 days, both at 20 mg twice daily. The risk ratio for confirmed cardiovascular events (acute MI, unstable angina, sudden death) was 3.7 when the parecoxib/valdecoxib group was compared with the double-placebo group (Table 1).

Lumiracoxib. Lumiracoxib, a coxib not currently approved for use in the US, has a structure different from that of the other agents. It has a very short half-life but can be administered once daily because of its lipophilic nature. It has been approved by the European Medicines Agency for use at 60–120 mg daily by patients with osteoarthritis, rheumatoid arthritis, or acute gouty arthritis. The TARGET (Therapeutic Arthritis Research and Gastrointestinal Event Trial) compared 1-year therapy with lumiracoxib 400 mg once daily versus naproxen 500 mg twice daily or ibuprofen 800 mg 3 times daily (27). Osteoarthritis patients ages 50 and older were randomized and stratified by age and use of low-dose aspirin. The rates for the primary cardiovascular end point (acute MI, CVA, and cardiovascular death) were similar among lumiracoxib and NSAID users (Table 1). The comparisons between lumiracoxib and each NSAID (ibuprofen and naproxen) individually suggested some possible differences, but none were statistically significant.

Observational data

While randomized clinical trial data are the gold standard for judging a drug’s benefits, in many trials participants are not studied for more than several weeks, and relatively healthy subjects are preferentially enrolled. Thus, a drug’s true side effect profile is often not well understood prior to its marketing. After the coxibs were approved for marketing and their use began to grow, large-scale trials that would enroll enough patients at risk for cardiovascular events were slow to be organized. Eventually, such trials were mounted to test whether these drugs would be effective for other indications, such as adenomatous polypl prevention and Alzheimer’s disease. As discussed above, the cardiovascular adverse event analyses from trials such as APPROVe and APC enhanced our understanding of the coxibs’ cardiovascular risk profile.

Epidemiologic analyses of postmarketing data can also assist in detecting a potential adverse event “signal” and can be used to study specific questions that would be difficult to examine in trials. For example, rare events that might develop only in specific subgroups of patients (those taking concomitant medications or with
specific comorbid conditions) will always be difficult to detect in trials because of limited recruitment. Moreover, well-conducted epidemiologic studies with typical patients may reflect the actual (or “true”) adverse event experience more closely than the somewhat contrived setting of a randomized clinical trial. These pharmacoepidemiologic analyses would include patients with the full range of comorbid conditions, taking concomitant medications, and using the drug in question in typical dosages at usual intervals.

Several pharmacoepidemiologic analyses on coxibs and cardiovascular disease that have been published (Table 2). Ray and colleagues examined celecoxib and rofecoxib use among Medicaid beneficiaries in Tennessee (31). Subjects were new users of these agents and were compared with nonusers. Adjusted models used Poisson regression, which assumes a constant risk of outcomes over the entire period of followup; this is an assumption that appears not to be true based on the event curves from the VIGOR and APPROVe trials. The end point of interest was a composite of acute MI or cardiac death. The study revealed no increased risk with celecoxib and no increased risk with rofecoxib at $\leq 25$ mg/day, but a near-doubling of the risk with rofecoxib at $>25$ mg/day.

My colleagues and I conducted a similar analysis using data on low-income subjects from Pennsylvania or New Jersey who were Medicare beneficiaries and also had a drug benefit (32). We drew from a source population of $\sim 500,000$ persons. The primary analysis used 1999–2000 information. All analyses were prespecified, with the end point being acute MI. We compared coxib users with nonusers, as well as with each other and with NSAID users. Multivariate models included information on potential confounders that preceded use of the agents of interest. The primary analysis revealed an increased risk of acute MI among rofecoxib users compared with nonusers (Table 2) and with celecoxib users. There was a trend toward an increased risk with rofecoxib compared with NSAIDs, but this did not reach statistical significance. Prespecified dosage analyses with all comparators revealed a dose-response relationship between rofecoxib and acute MI. In addition, secondary analysis among new users revealed that the first 90 days comprised the period of highest risk associated with rofecoxib use (odds ratio 1.39), compared with later than 90 days (odds ratio 0.96). Celecoxib was not associated with an increased risk of acute MI in any of these analyses. While the potential effect of confounders was deemed minimal based on examination of an external data set (33), our analysis was limited by lack of information on some cardiovascular risk factors.

An FDA-sponsored epidemiologic analysis examined members of a large health maintenance organization (34). This analysis compared current use of coxibs and NSAIDs with remote use of these agents (>60 days prior). The end points included acute MI and sudden cardiac death. The primary analysis showed a statistically significant increase in risk with rofecoxib at daily dosages $>25$ mg (Table 2). At dosages of $\leq 25$ mg/day, the risk was elevated but did not reach statistical significance. Celecoxib was not associated with an increase in cardiovascular risk. Naproxen use was associated with a slight increase in risk. An important strength of this study was the use of patient surveys to augment cardiovascular risk factor data.

Kimmel and colleagues conducted an epidemiologic analysis in 36 hospitals located in 5 counties (35). They selected cases of acute MI from hospital discharge information. They then attempted to enroll patients who had had acute MI and controls from the same county. The response rate was 50–55% for each group. Drug exposure information was gathered as part of a detailed telephone survey conducted by trained interviewers. Modest response rates and potential recall bias limit this study, but the detailed collection of cardiovascular risk factor data and aspirin information is an important strength. The results of this study are consistent with those of other analyses (Table 2).

Two observational epidemiologic studies, from Ontario and Maryland, have shown no increased risk with any coxib (36,37) (Table 2). Each of these studies had potential limitations. The Canadian study (36) restricted the analysis to subjects who filled multiple prescriptions. Thus, events that occur with a first and only prescription may have been missed. In addition, these analyses may have overcontrolled for potential mediators of the coxibs’ effects on the cardiovascular system, since they included hypertension, congestive heart failure, and angina diagnosed after the initiation of treatment as adjusters in multivariate models. The methods used by the Maryland investigators (37) are complex, but a key question is whether use of coxibs and/or NSAIDs was required to overlap with the event dates. If drug exposure on the event date was not confirmed, it is impossible to draw any meaningful conclusions about the potential association between coxibs and cardiovascular events.

All epidemiologic studies have limitations that need to be described and their implications understood.
A presentation at the American College of Rheumatology 2004 annual scientific meeting described these potential methodologic problems (38). They include the study of prevalent (versus new) users (39), overcontrolling for factors on the causal pathway, and proportional hazards violations or assumptions that hazards were constant over time. Some of these issues may have been important limitations of the epidemiologic studies of coxibs. However, it is interesting to ponder whether the “signal” of adverse cardiovascular events found in several epidemiologic studies should have raised concerns sooner than September 2004, when the APPROVe trial results were announced.

Coxibs: are they all the same?

As long-term trial data have become available, the similarity in cardiovascular risk between agents has emerged. However, there may be a gradient of cardiovascular risk across coxibs. It is not uncommon for one member of a therapeutic class to show an increased risk of a specific adverse event. Two notable examples of this include bromfenac, an NSAID that was briefly marketed in the mid 1990s but withdrawn because of several early cases of liver failure (40), and cerivastatin, a lipid-lowering drug removed from the market because of an increased risk of rhabdomyolysis (41).

Coxibs in the US have different molecular structures and different potency for COX-2 inhibition. As noted above, there are data suggesting that they may interact with the vascular system in different ways. This may result in distinct patterns of nitric oxide release, blood pressure regulation, and oxidative stress. However, it may be that at certain daily or cumulative doses in certain people (due to interindividual pharmacogenomic differences), the agents may be more similar than different. These issues are summarized in Table 3.

The discrepancy between findings in the APC trial, in which a significantly increased cardiovascular risk caused by celecoxib was found (Table 1), and the observational data, with at least 6 studies showing no cardiovascular risk with this agent (Table 2), warrants inspection. While this difference may be due to methodologic limitations of all of the observational studies, it is more likely that real differences between the APC trial and the observational studies explain the variation. In the APC trial, subjects took celecoxib in high doses twice daily for many months. In typical practice, some patients use similar dosing schedules, but this is probably the exception. Most patients take no more than 200 mg per day, many take the drug once daily as opposed to twice, and few continue the drug as long as patients in the APC trial did. Thus, there may be “safer” coxib dosing schedules for the small group of people in whom therapy with these drugs is needed on a continuing basis.

### Table 2. Relative risk of cardiovascular events observed in epidemiologic studies of coxibs

<table>
<thead>
<tr>
<th>Author, year (ref.)</th>
<th>Patient population (mean age, years)</th>
<th>Coxib</th>
<th>Comparator‡</th>
<th>Group</th>
<th>Events†</th>
<th>Relative risk (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ray et al, 2002 (31)</td>
<td>Medicaid (62)</td>
<td>Rofecoxib, ≤25 mg</td>
<td>20,245</td>
<td>55</td>
<td>Nonuse§</td>
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<td>Rofecoxib, &gt;25 mg</td>
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<td>74</td>
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<td>Ontario (76)</td>
<td>Rofecoxib, any dose</td>
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<td>Nonuse 100,000</td>
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<td>Solomon et al, 2004 (32)</td>
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<td>Nonuse</td>
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<td>HMO (68)</td>
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<td>Remote use</td>
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<tr>
<td>Kimmel et al, 2005 (35)</td>
<td>Community (55)</td>
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<tr>
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<td>Celecoxib, any dose</td>
<td>507</td>
<td>NA</td>
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</table>

* HMO = health maintenance organization (see Table 1 for other definitions).
† See text for study-specific definitions of cardiovascular events.
‡ Some of the studies included multiple comparator groups. To simplify comparisons across studies, the number of comparators has been limited.
§ Nonuse refers to no NSAID or coxib use.
¶ Same comparator group as shown above for the given study.
Patient subgroups: identifying “low-risk” patients

The cardiovascular risk data (as well as the economic cost) suggest that coxib use should be limited to certain patient groups. It may be that coxibs are appropriate for a small group of patients based on risk/benefit considerations. How might we identify that group? It would be ideal from a clinical standpoint if patients who are at low risk, or alternatively, at high risk, for coxib-associated cardiovascular events could be identified. This would allow clinicians and patients to weigh the risk of GI toxicity from NSAIDs against the risk of cardiovascular events from coxibs.

Factors that might help to risk-stratify patients include clinical, demographic, and genetic data (42). The genetic markers may not be available immediately, but perhaps clinical and demographic factors can be used to risk-stratify. Indices for predicting cardiovascular risk, such as the Framingham Risk Score (43), are well accepted. Known cardiovascular risk factors can help identify patients at increased absolute risk of cardiovascular events. However, it is unclear whether these factors would help to identify groups of patients at an increased relative risk for coxib-associated events. Many investigators have suggested this, but currently, the value of risk-stratifying coxib users is unproven.

Conclusions

Development of safer analgesic treatments is important for patients. Investigators in the Nurses’ Health Study found that among women 52–77 years of age, 10.8% report using NSAIDs ≥6 days per week, and 26.7% on at least 1 day per week (44). While there has been recent evidence for a reduction in NSAID-associated GI morbidity, the Arthritis, Rheumatism, and Aging Medical Information System investigators suggested that ~16,500 deaths and 107,000 hospitalizations annually appear to be related to NSAID-associated GI toxicity (45,46). Since many rheumatic disease patients are older or have system inflammatory conditions that already place them at an increased risk for adverse cardiovascular outcomes, it may be possible to “trade” an increased risk of cardiovascular events for the potential of reduced GI toxicity.

The mechanisms underlying the increased risk of cardiovascular events with coxibs are not clear. An apparent short-term risk in some studies and a long-term risk in others is difficult to explain. However, it may be that at different dosages, with different dosing frequencies, in different populations, the dominant mechanisms linking coxibs to cardiovascular outcomes differ. Moreover, the degree of COX-2 selectivity that confers risk is unclear. As noted in Figure 1, the nonselective NSAIDs differentially inhibit COX-1 and COX-2. Diclofenac inhibits COX-2 almost to the same degree that celecoxib does. In several trials, the rate of cardiovascular events in the NSAID comparator arms was similar to that in the coxib arm (Table 1). Thus, an important question is whether some or all of the nonselective NSAIDs also confer cardiovascular risk.

The subgroup analyses from the APC trial suggest that aspirin may not abrogate the potential cardiovascular harm of coxibs. This point is also underscored by the fact that valdecoxib with concomitant aspirin treatment in the CABG I and CABG II trials was associated with an increased cardiovascular risk compared with placebo. Both of these findings provide evidence that the mechanism underlying the cardiovascular risk associated with coxibs is more complicated than a simple imbalance between COX-1 and COX-2 inhibition.

This review has not focused on the policy impli-
cations of the recent events regarding coxibs and NSAIDs, but several lessons are apparent. Politicians, regulators, physicians, and patients have realized that we often know less about the safety of marketed medications than we would suppose. This is not only true for coxibs and NSAIDs, but extends to other rheumatic disease drugs (e.g., biologic disease-modifying antirheumatic drugs) and more broadly. The recent FDA and European Medicines Agency proceedings have facilitated a more complete public disclosure of important data and focused attention on the postmarketing surveillance process. The options for analgesia are many, but coxibs and NSAIDs may have important roles for certain patient groups. These subgroups cannot be well defined based on the available data. Thus, as we grapple to formulate appropriate drug safety policy, research on patient safety must accelerate.

**Addendum.** On April 7, 2005 (after the current report was accepted for publication), the FDA requested that valdecoxib’s manufacturer withdraw the drug from the US market based on insufficient long-term cardiovascular safety data, evidence of an increased cardiovascular risk in short-term studies among patients undergoing heart surgery, the risk of life-threatening skin reactions, and no proven advantage over other NSAIDs. The manufacturer complied, and valdecoxib is no longer available in the US. In addition, the FDA has asked the manufacturers of all NSAIDs, including celecoxib, to include a “boxed warning” highlighting the potential cardiovascular risks of these agents (http://www.fda.gov/cder/drug/ advisory/COX2.htm; accessed April 12, 2005).

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SPECIAL ARTICLE

How Should Treatment Effect on Spinal Radiographic Progression in Patients With Ankylosing Spondylitis Be Measured?

Désirée van der Heijde, Robert Landewe, and Sjef van der Linden

Introduction

Ankylosing spondylitis (AS) is a chronic inflammatory disease characterized by pain, stiffness, and often, impaired mobility of the spine. The latter is due to spinal inflammation as well as the formation of erosions and vertebral syndesmophytes, and may result in complete ankylosis (“bamboo spine”). AS has a burden of illness comparable with that of rheumatoid arthritis (RA) (1–3). Recent research from our group has now convincingly shown that radiographic damage in AS interferes with long-term functioning, independent of actual disease activity (4), and therefore, radiographic damage is an important target for therapeutic intervention.

Previous experience in the field of RA may create the impression that the causal chain of inflammation leading to radiographic damage may hold for AS, as has been proven for RA. However, firm evidence that inflammation of the spine in AS actually precedes radiographic damage in a causal manner is currently lacking. To date, it has also not been possible to predict at an early stage which patients will develop significant radiographic damage of the spine over time and which patients will not. In the Outcome in Ankylosing Spondylitis International Study (OASIS) (5) (see below), the only variable with some prognostic ability regarding progression of damage appeared to be the presence of radiographic damage at baseline (6), which is obviously not a true predictor.

Radiographic progression in AS also differs from that in RA with respect to the rate at which it can be observed. Progression in AS is a slow process, often occurring over several years (5), whereas in some RA patients with a high level of disease activity, progression can be detected reliably as early as 3 months after the first radiograph (7). In AS, it takes ~2 years, using currently available radiographic scoring methods, to reliably distinguish true progression from background noise and measurement error (8).

The current status of therapy in AS

Until recently, AS patients were treated mainly with nonsteroidal antiinflammatory drugs (NSAIDs), which have been proven to be efficacious in alleviating signs and symptoms of the disease, and possibly in retarding radiographic progression (9). Other treatment modalities with proven efficacy with regard to signs and symptoms are physiotherapy, group exercise (10), and spa therapy (11).

A number of placebo-controlled studies have now shown that tumor necrosis factor (TNF)–blocking drugs are highly effective in improving signs and symptoms in patients with longstanding AS (12,13). There is also evidence that these improvements coincide with improvements in the activity score as measured by magnetic resonance imaging (MRI) of the spine (14). It is not known, however, whether TNF-blocking drugs may also retard structural damage of the spine, as measured by conventional radiography. Information about radiographic progression in AS is relevant because of its association with function independent of disease activity (4). Therefore, new therapies in AS should be assessed for their potential to influence radiographic...
progression, as was recognized at a consensus meeting to develop recommendations for clinical trials (15).

**Randomized clinical trials**

Analogous to the situation in RA, the gold standard for investigating the effects of drugs on radiographic progression in AS should be the randomized clinical trial (RCT), in which patients are allocated randomly to an active intervention group (e.g., a TNF-blocking drug) or a control group. The principal argument for the use of randomization in such studies is that all factors—either known or unknown—that may influence radiographic progression would be expected to be similarly divided between the 2 groups (the “prognostic similarity” principle) (16).

The active intervention group will then be treated with the drug under investigation, and the control group preferably with the drug that represents the current standard of treatment. In an RCT with radiographic progression as the primary outcome parameter, sample sizes will be chosen such that if there is a true difference in radiographic progression between the active treatment and control groups, the experiment should confirm this difference (statistical power).

There are, however, a number of specific difficulties related to AS clinical trials, which make a proper RCT with radiographic progression as the primary end point almost, if not completely, impossible. These features are 1) the low frequency and slow rate of radiographic progression in AS, resulting in the need for a minimum followup of 2 years, 2) the lack of an appropriate comparator drug that has efficacy with regard to the signs and symptoms of AS similar to that of the TNF-blocking drugs, and 3) the lack of appropriate prognostic factors with regard to radiographic progression. These difficulties will be discussed briefly below.

**Low frequency and slow rate of radiographic progression in AS**

There are 3 available methods to assess and follow up radiographic damage in AS: the Bath Ankylosing Spondylitis Radiology Index (17), the Stoke Ankylosing Spondylitis Spine Score (SASSS) (18), and a modification of the SASSS (mSASSS) (19). Recently, we compared the validity of these 3 methods for use in clinical trials (20) and evaluated their use in a clinical trial of NSAID treatment in AS (9). The mSASSS was found to be the preferred method, because with this method, the most progression in a 2-year period was identified and interreader variability of change scores was the smallest; in addition, this method assesses changes in both the cervical and the lumbar spine, thus adding to the face validity. Using the mSASSS, we investigated the sample sizes that would be needed in an RCT to provide sufficient statistical power to detect true differences in radiographic progression between patients treated with active drug and controls after 1 year and 2 years of followup (see below). Because of the low number of patients with actual progression, the number of patients needed for a 1-year trial was unacceptably high. A followup duration of ~2 years was necessary in order for a study to have a sufficiently large number of patients showing measurable progression.

Such a long duration of followup creates serious methodologic drawbacks. The most important one is probably bias due to crossover of treatment, i.e., patients in the placebo arm will start treatment from the comparator arm. A second drawback is concomitant treatment with other drugs that may influence radiographic progression, such as NSAIDs. Crossover will be the inevitable consequence of a placebo-controlled trial of TNF-blocking drugs if these drugs are registered and reimbursed for clinical use, which is already the case in several countries and will be the case soon in many others. It is unethical to expect that patients with active disease will continue to take placebo for 2 years when effective drugs are available. Patients with the most active disease will be the most likely to stop taking placebo, so the patient group that completes the 2-year followup with placebo will not be representative of the original control group. Moreover, patients in the placebo group will withdraw from the trial after a short period of followup because the effectiveness of TNF-blocking drugs is apparent within only 6 weeks in the majority of patients.

**Lack of an acceptable comparator drug in AS trials**

One of the issues complicating long-term comparative trials in AS is that, unlike RA, in which methotrexate is considered an acceptable anchor drug for control groups in RCTs, there is currently no acceptable alternative drug to TNF blockers. Conventional disease-modifying antirheumatic drugs (DMARDs) such as sulfasalazine (21) and methotrexate (22), as well as corticosteroids, are not effective in treating axial disease. This means that any RCT with radiographic progression as an end point should be placebo controlled. However, as noted above, 2-year placebo-
controlled trials in AS would be neither feasible nor considered ethical.

Lack of appropriate prognostic factors in AS

RCTs in RA usually include patients with a high level of disease activity, in order to create a trial population that is “prone to change” and has a high propensity for an unfavorable outcome (e.g., radiographic progression). In AS, however, in designing RCTs with radiographic progression as the primary end point, it is not known which patients to select. In a recent study of prediction of radiographic progression in the OASIS cohort, a prospectively followed cohort of consecutive patients with AS from 3 countries, we found that of >25 variables potentially related to radiographic progression, none had any predictive value except the baseline presence of damage itself (odds ratio 1.05 [95% confidence interval 1.03–1.07] per mSASSS unit of baseline damage) (6). The inability to define patients at risk for further radiographic progression deflates the sensitivity to change in an RCT, which additionally complicates the design of the trial.

Potential design of trials to assess radiographic progression in AS

Taking into account the above considerations, two possible designs for RCTs may appear attractive at first glance. The first would be a 2-year RCT comparing TNF-blocking drugs with placebo in patients with low levels of disease activity. Such a design is methodologically feasible but ethically disputable because of administration of potentially harmful drugs to patients who, according to international expert opinion (23), do not need them. Thus, such a trial would not be advocated by rheumatologists or by the pharmaceutical industry.

The second design would be a 2-year RCT comparing TNF-blocking drugs with NSAIDs in patients whose disease is already responding to NSAIDs. Such a design creates the same ethical concerns as the first because effective treatment (NSAIDs) would be replaced by TNF-blocking drugs in patients who do not a priori need the latter treatment. In addition, this design may increase the potential for Type II error because, as we have recently shown (9), NSAIDs alone can retard radiographic progression in AS. The consequence is that such a trial would have to include such a large number of patients that we consider it unfeasible.

In summary, both the low frequency/slow rate of measurable radiographic progression and the inability to predict further radiographic progression in AS make it necessary to design RCTs of at least 2 years’ duration. Because of the lack of acceptable (effective) comparator drugs, such trials can only be placebo controlled. The availability of highly effective TNF-blocking drugs for short-term relief of signs and symptoms means that such RCTs cannot be thoroughly conducted for a period of 2 years. Crossover and unbalanced concomitant interventions during followup (with patients in the placebo group more likely to take NSAIDs than those in the active treatment group) will compromise prognostic comparability with respect to radiographic progression, and devalue the trial results.

Because of the above issues, alternative solutions must be explored in order to obtain the most scientifically sound data regarding the ability of TNF-blocking agents to inhibit radiographic progression in AS. It should be noted that MRI of the spine is currently not a good alternative to conventional plain radiography, because scoring of structural damage observed by MRI is still entirely experimental (24), and we are only beginning to learn about the performance of existing scoring methods in comparison with radiography in assessing change over time. Data on sensitivity to change over time are not available. Another alternative to conventional radiography that could be considered is computed tomography, a modality known for good imaging of bone structures. However, its usefulness in the assessment of AS is completely unknown. Moreover, high costs, high radiation exposure, and lack of a scoring system limit the applicability of this method.

We present here an alternative design, using a historical control group consisting of patients who participated in a followup study on the natural course of AS with conventional treatment: the OASIS cohort. We propose using baseline and 2-year radiographs from the OASIS cohort (186 patients), mixing these sets of radiographs with sets of radiographs from patients in trials of TNF-blocking drugs, and offering these sets for scoring according to the mSASSS, by readers who are blinded with regard to the cohort from which the radiographs were derived, and the time sequence. As shown below, the OASIS is a representative cohort with respect to radiographic progression in AS patients; selection of only patients with high levels of disease activity is not necessary. Furthermore, the degree of change observed in the OASIS cohort is sufficient to create an experimental environment with adequate statistical power to demonstrate true inhibition of radiographic progression.
Table 1. Characteristics of the members of the Outcome in Ankylosing Spondylitis International Study cohort, assessed at baseline (n = 180)*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD/median (interquartile range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>44 ± 13/43 (34–53)</td>
</tr>
<tr>
<td>Sex, % male</td>
<td>68</td>
</tr>
<tr>
<td>Disease duration, years</td>
<td>11.4 ± 8.6/9.6 (4.5–15.2)</td>
</tr>
<tr>
<td>Age at onset of symptoms, years</td>
<td>32.4 ± 10.6/31.1 (24.2–40.5)</td>
</tr>
<tr>
<td>HLA–B27 positive, %</td>
<td>82</td>
</tr>
<tr>
<td>Hip involvement, %</td>
<td>24</td>
</tr>
<tr>
<td>History or presence of uveitis, %</td>
<td>15</td>
</tr>
<tr>
<td>History or presence of IBD, %†</td>
<td>7</td>
</tr>
<tr>
<td>History or presence of psoriasis, %</td>
<td>5</td>
</tr>
<tr>
<td>Presence of peripheral arthritis, %</td>
<td>20</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean ± SD/median (interquartile range).
† IBD = inflammatory bowel disease (Crohn’s disease or ulcerative colitis).

The OASIS cohort

The OASIS cohort consists of Dutch, French, and Belgian patients with AS who have been followed up since 1996 (5). There are no specific inclusion criteria, other than consecutive enrollment of patients who have AS according to the modified New York criteria (25) and are receiving care from a rheumatologist. As such, OASIS is a cross-sectional representation of AS patients seen in rheumatology practice (Table 1).

Since the OASIS cohort is an unselected cross-sectional representation of AS patients, disease activity at baseline could be expected to be lower compared with AS patients in clinical trials (Table 2). The mean baseline score on the Bath Ankylosing Spondylitis Disease Activity Index (26) in the OASIS cohort was <4, and thus below the level for inclusion in some RCTs of TNF-blocking drugs in AS. As expected, the mean erythrocyte sedimentation rate was also quite low.

Table 2. Patient-reported disease activity and levels of acute-phase reactants in the Outcome in Ankylosing Spondylitis International Study cohort, assessed at baseline

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SD/median (interquartile range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BASDAI</td>
<td>3.5 ± 2.1/3.3 (1.8–5.1)</td>
</tr>
<tr>
<td>Morning stiffness severity†</td>
<td>3.6 ± 2.9/2.7 (0.9–6.0)</td>
</tr>
<tr>
<td>Morning stiffness duration†</td>
<td>3.7 ± 3.1/3.0 (0.9–6.0)</td>
</tr>
<tr>
<td>Global assessment of disease activity†</td>
<td>3.8 ± 2.8/3.6 (1.2–5.6)</td>
</tr>
<tr>
<td>Back pain†</td>
<td>4.4 ± 2.7/4.5 (2.2–6.6)</td>
</tr>
<tr>
<td>BASFI</td>
<td>3.5 ± 2.6/3.3 (1.1–5.3)</td>
</tr>
<tr>
<td>C-reactive protein, mg/dl</td>
<td>1.6 ± 2.1/0.7 (0.6–1.6)</td>
</tr>
<tr>
<td>ESR, mm/hour</td>
<td>13.3 ± 14.3/10.0 (4.0–17.0)</td>
</tr>
</tbody>
</table>

† Assessed by the patient on a 10-cm visual analog scale.

Table 3. Treatment with nonsteroidal antiinflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs (DMARDs), and other agents in the Outcome in Ankylosing Spondylitis International Study cohort, assessed at baseline

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSAIDs</td>
<td>78</td>
</tr>
<tr>
<td>DMARDs</td>
<td>11*</td>
</tr>
<tr>
<td>Corticosteroids</td>
<td>3</td>
</tr>
<tr>
<td>Simple analgesics</td>
<td>12</td>
</tr>
<tr>
<td>NSAIDs + DMARDs</td>
<td>9</td>
</tr>
</tbody>
</table>

* Sulfasalazine (18 patients), methotrexate (2 patients), or intramuscular gold (1 patient).

Treatment in the OASIS was according to “pre-TNF” standards (Table 3). The majority of patients received NSAIDs, and only a few were treated with DMARDs (sulfasalazine). None of the patients was treated with TNF-blocking drugs or with other biologic agents during the first 4 years of followup. One of the aims of the OASIS was to explore radiographic progression of the axial disease, and for this purpose, radiographs of the cervical and lumbar spine, as well as of the pelvis, were obtained in every patient at baseline, at 1 year, at 2 years, and at 2-year intervals thereafter.

Radiographic progression in the OASIS cohort

Radiographic progression was assessed independently, using the mSASSS (scale of 0–72), by 2 readers who were not aware of the time sequence. The mean ± SD 2-year progression scores were 1.38 ± 2.86 mSASSS units for reader 1 and 1.05 ± 2.89 for reader 2. In order to test whether the OASIS is a representative cohort with respect to radiographic progression, we assessed radiographic progression under the same reading conditions in a different RCT comparing continuous NSAID use versus NSAID use on demand (9). Only reader 2 scored the radiographs in this trial, and the mean ± SD progression as scored by this reader was 0.91 ± 2.19 mSASSS units, which is close to the mean progression scored by the same reader in the OASIS cohort. These results indicate that radiographic progression measured in the OASIS is representative (and reproducible) of 2-year radiographic progression in AS.

We tested the influence of disease activity on radiographic progression in the OASIS cohort by comparing radiographic progression in patients who would have fulfilled the inclusion criteria in one of the RCTs with TNF-blocking drugs (the most strict inclusion criteria) versus that in patients not fulfilling these criteria
due to low disease activity. The results of this exercise are depicted as probability plots (Figure 1), as recently recommended by us (27). Briefly, a probability plot depicts the actual scores of all patients against their cumulative probability (scores are ordered from low to high, and are assigned a cumulative probability). The probability plots in Figure 1 show the following: 1) ~35% of all patients have some degree of radiographic progression (mSASSS >0) over a 2-year period; 2) “negative progression scores” occur in ~10% of the patients as a consequence of measurement error with blinded reading order; and 3) the 2 curves almost overlie one another, which means that selection of patients for high disease activity does not influence radiographic progression.

### Radiographic progression in the OASIS cohort as a benchmark

The question is now whether the level of radiographic progression as measured by the mSASSS over a 2-year period suffices to serve as a benchmark for the effects of new drugs, e.g., TNF blockers. The null hypothesis in such an experiment is that radiographic progression in both groups (active intervention and historical control) is similar, or that the effect of the new drug (TNF blocker) on radiographic progression is negligible. If the null hypothesis can be rejected by statistical testing, the new drug is considered to be better at inhibiting radiographic progression than the “usual treatment” in the historical control group, and it may be claimed that such a drug has the potential to influence structural damage. A useful means to explore the feasibility of such an approach, which does not differ fundamentally from sample size calculations in the design phase of an RCT, is performance of power calculations. Figure 2 shows the results of such power calculations for the OASIS cohort, presented as sample sizes (on the y-axis) against minimum detectable differences, depicted as treatment contrast (on the x-axis).

A noteworthy assumption is that rereading of the radiographs of patients in the OASIS cohort under blinded conditions, as described above, will result in a mean progression score of 1.05 mSASSS units (assay sensitivity). This number serves as a benchmark against

![Figure 1](image1.png)

**Figure 1.** Probability plot of patients in the Outcome in Ankylosing Spondylitis International Study cohort, stratified by eligibility for a randomized controlled trial of tumor necrosis factor (TNF)-blocking drugs based on inclusion and exclusion criteria. mSASSS = modified Stoke Ankylosing Spondylitis Spine Score.

![Figure 2](image2.png)

**Figure 2.** Sample size calculations (“power curves”) that describe the relationship between treatment contrast and standard deviation, and the number of patients required, in a trial aimed at detecting a contrast in radiographic progression between an “untreated” control group (e.g., the Outcome in Ankylosing Spondylitis International Study [OASIS] cohort) and an active intervention group (e.g., patients treated with tumor necrosis factor [TNF]-blocking drugs). Treatment contrasts and SDs are chosen based on observations in the OASIS cohort. Statistical power is fixed at 0.80. The probability of a Type I (alpha) error is set at 0.05.
which different putative progression scores derived from the trial with the TNF-blocking drug (but scored in the same reading session) are compared. The difference between the benchmark (1.05 mSASSS units) and the putative score from the trial with the new drug is called the contrast, or, as in RCTs, the treatment effect. A second assumption is that true negative progression scores do not exist in AS, so the treatment effect will be 1.05 mSASSS units at most. Repeated t-test–based sample size calculations were performed to construct the power curves in Figure 2, which reflect the relationship between required sample sizes and minimum detectable difference, for different levels of the standard deviation of the mean progression score. The curves show that studies with 130–150 patients per treatment group provide sufficient statistical power to detect differences of 0.8–1.0 mSASSS units in radiographic progression between treatment groups. This number of patients is feasible in the context of the OASIS cohort, which includes 2-year radiographic data from 186 AS patients. It should be noted that the t-test–based sample size calculation may have caused an overestimation of the actual required number, because of the skewness of the radiographic data. Usually, nonparametric statistical testing or parametric statistical testing after a data normalization procedure increases statistical power.

In summary, we have shown here that the OASIS cohort is a representative cohort of AS patients in terms of radiographic progression, that the level of radiographic progression in patients in the OASIS cohort is not dependent on the level of disease activity. Therefore, radiographic findings in this group can appropriately be used as a background against which the effects of (TNF-blocking) drugs can be tested.

Conclusions

The advantages of adopting a study design with a historical control group should be weighed against the disadvantage of inability to perform an RCT due to feasibility and ethical considerations. An advantage of a historical control group design is that it eliminates the need for 2-year placebo-controlled trials of TNF-blocking drugs, and the effect of TNF-blocking drugs on radiographic progression can be assessed without delay, instead of after 2 years. The major disadvantage of this design is potential prognostic dissimilarity between the OASIS cohort and the efficacy trial. The question thus becomes whether this type of prognostic dissimilarity is so relevant that it outweighs the advantages.

First, prognostic similarity, which is expected “by definition” with the use of a randomization procedure, is important in that differences at baseline determine to some extent the outcome of interest. As noted above, we could not identify variables that had prognostic impact on radiographic progression in the OASIS, the only exception being the presence of radiographic damage itself. The absence of known predictors of radiographic progression does not preclude the existence of yet-unknown predictors (e.g., geographically determined), but unknown prognostically relevant predictors would be important only if they are unequally distributed among the treatment groups. Furthermore, all phase 3 trials of TNF-blocking drugs in AS that are currently being performed include patients from both the US and Europe. Unexpected geographic differences with respect to radiographic progression can easily be traced (and adjusted for) in the analysis.

The second and more important argument favoring the use of a historical control group over an RCT design in this specific context is the above-mentioned bias that will occur in a 2-year placebo-controlled RCT, due to treatment crossover and concomitant interventions. This kind of bias, which has the same consequences regarding comparability of groups as does prognostic dissimilarity at baseline (16), can be avoided entirely by use of a historical control group design. It is not reasonable to assume that the potential prognostic dissimilarity at baseline in the historical control group design outweighs the longitudinal bias that will compromise the RCT design.

A third argument moderating the importance of the RCT design in this setting is that the actual acquisition of the data, i.e., the reading of radiographs, will occur concurrently and under strictly blinded conditions in both groups. Looking at treatment effects in terms of biases and error (noise), one can distinguish noise generated by prognostic dissimilarity (baseline and/or longitudinal) and noise generated by measurement error (radiographic scoring). We do not know how these sources of error quantitatively relate to one another, but we do know that the signal of interest (radiographic progression) is rather low, and that reading variation should be kept as low as possible in order to pick up the signal. Presumably, optimization of the quality of the reading procedure greatly outweighs optimization of the trial design, and we therefore recommend thorough training of the readers in order to improve reliability, and use of readers who are familiar with the types of abnormalities often seen in AS.

Taken together, these arguments provide evidence in favor of the use of historical control patients
rather than designing a 2-year placebo-controlled RCT in order to investigate whether TNF-blocking drugs inhibit radiographic progression in AS. The OASIS cohort can be considered an appropriate example of a feasible historical cohort that can be used as a control group.

REFERENCES

Risk and Case Characteristics of Tuberculosis in Rheumatoid Arthritis Associated With Tumor Necrosis Factor Antagonists in Sweden


Objective. Because treatment with tumor necrosis factor (TNF) antagonists may increase the risk of tuberculosis (TB), and because knowledge of the risk of TB in rheumatoid arthritis (RA) not treated with biologics is scarce and of uncertain generalizability to low-risk populations, this study sought to determine the risk of TB among Swedish patients with RA.

Methods. Using data from Swedish nationwide and population-based registers and data from an ongoing monitoring program of TNF antagonists, the relative risks of TB in patients with RA (versus the general population) and of TB associated with TNF antagonists (versus RA patients not treated with biologics) were determined by comparing the incidence of hospitalization for TB in 3 RA cohorts and 2 general population cohorts from 1999 to 2001. We also reviewed the characteristics of all reported cases of TB in RA patients treated with TNF antagonists in Sweden and calculated the incidence of TB per type of TNF antagonist between 1999 and 2004.

Results. During 1999–2001, RA patients who were not treated with TNF antagonists were at increased risk of TB versus the general population (relative risk 2.0, 95% confidence interval [95% CI] 1.2–3.4). RA patients treated with TNF antagonists had a 4-fold increased risk of TB (relative risk 4.0, 95% CI 1.3–12) versus RA patients not treated with TNF antagonists. The reported TB cases during 1999–2004 in RA patients exposed to TNF antagonists (9 infliximab, 4 etanercept, 2 both) were predominantly pulmonary. TB occurred up to 3 years following the start of treatment.

Conclusion. Irrespective of whether TNF antagonists are administered, Swedish patients with RA are at increased risk of TB versus the general population (relative risk 2.0, 95% CI 1.2–3.4). RA patients treated with TNF antagonists had a 4-fold increased risk of TB (relative risk 4.0, 95% CI 1.3–12) versus RA patients not treated with TNF antagonists. The reported TB cases during 1999–2004 in RA patients exposed to TNF antagonists (9 infliximab, 4 etanercept, 2 both) were predominantly pulmonary. TB occurred up to 3 years following the start of treatment.

Tumor necrosis factor (TNF) plays a major role in host defense against tuberculosis (TB) (1). The frequencies of TB in phase III trials of TNF antagonists (infliximab [2] and adalimumab [3]) have suggested that treatment with TNF antagonists (infliximab in particu-
lar) may indeed increase the risk of TB, and these observations have been followed by numerous spontaneous reports to regulatory agencies (4–6). Judgments on the significance of these descriptive data, however, require information on the incidence of TB in well-defined cohorts of patients with rheumatoid arthritis (RA) treated with TNF antagonists, in whom the risk of TB relative to the expected incidence of TB in contemporary RA patients not treated with biologics should be calculated.

Our current understanding of the relative risk of TB associated with RA per se and associated with TNF antagonists in routine care is scarce and can be derived from data on Spanish and US populations. In Spain, a 4-fold risk of TB in RA on the basis of 7 cases (7) and a further 12–20-fold increase in risk associated with TNF antagonists on the basis of 17 cases (8) were reported. In the US, a nonelevated risk of TB in RA in 1 case, but an increased incidence of TB in RA associated with TNF antagonists based on 4 cases (9), were reported. These cases of TB all occurred while the patients were receiving infliximab, and were characterized by a seemingly high proportion (50%) of extrapulmonary TB.

Of note, active TB in patients exposed to TNF antagonists mainly appears to constitute reactivation of latent TB (10), and is therefore a reflection of the pattern of infection many years previously. Accordingly, it is far from certain that the relative risks reported in Spain (8) or in ethnically heterogeneous populations in the US (9) are at all applicable to low-incidence areas such as Sweden or selected low-risk populations in the US. Indeed, the current incidence of TB in Spain (20 cases per 100,000 population) exceeds that in Sweden (5 cases per 100,000 population) and that in the non-Hispanic white population in the US (11). A proper judgment requires more data from populations with different background prevalences of TB. Likewise, although many case reports and case series (4,6) have described the characteristics of TB occurring in TNF antagonist–treated patients (proportionally more occurring among those receiving infliximab compared with etanercept [12]), its origin in spontaneous reporting systems is obscured by underreporting and uncertainties regarding the underlying duration (person-time) of these drug regimens, thus limiting inferences and generalizability. To fully capture pretreatment risk factors for and characteristics of TB in TNF antagonist–treated individuals, data on consecutive cases of TB occurring in well-defined population-based cohorts of patients treated with TNF antagonists are needed.

In Sweden, high-quality nationwide health and census registers offer the unique potential to assess comorbidity rates through register linkage, which, at the time of this study, provided register data through 2001. Ongoing programs that monitor patients treated with TNF antagonists offer well-defined cohorts of such patients. We performed a cohort study to assess the relative risk of hospitalization for TB in patients with RA not treated with biologics, and the relative risk of hospitalization for TB in RA associated with TNF antagonists, from 1999 through 2001. In addition, we reviewed the medical files on all reported cases of TB among patients treated with TNF antagonists in Sweden during 1999–2004 and calculated drug-specific incidences of TB in that time period.

**PATIENTS AND METHODS**

**Cohorts.** We assembled 5 different cohorts for the study, as described below.

**RA cohort from the Inpatient Register.** Swedish inpatient care is public and population-based, with referrals based on geography rather than financial capacity or health insurance. The Swedish Inpatient Register contains individual-based information on inpatient care for each county since 1964, with nationwide coverage since 1987. Discharge diagnoses (main diagnosis and up to 5 contributory diagnoses) for each discharge are coded according to the International Classification of Diseases (ICD) revisions 7–10 (13). We identified all individuals older than age 16 years who were discharged from inpatient care and identified as having RA at discharge between 1964 and 2001 (and not at any other point in time); patients discharged with a diagnosis of psoriatic arthritis, systemic lupus erythematosus, or ankylosing spondylitis were excluded. For each individual, we collected information on sex, year of birth, county of residence during the year of first discharge listing RA (Table 1).

**General population reference cohort from the Population Register.** For each of the individuals in the Swedish Inpatient Register RA cohort, we randomly selected 2 reference individuals from the Register of Total Population (the Swedish census register), who were matched to the RA patients by sex, year of birth, county of residence during the year of first discharge listing RA, and marital status (married, unmarried, widow). This reference cohort reflected the true background population.

**General population reference cohort from the Inpatient Register.** The RA patients constituting the Inpatient Register RA cohort were identified on the basis of at least one overnight hospital stay listing RA between 1964 and 2001. To assess whether any increased risk in this cohort was due to the hospitalization rather than to the RA itself, we assembled a second reference cohort by randomly selecting, for every individual in the Inpatient Register RA cohort, 4 reference individuals from the entire Inpatient Register who had been hospitalized due to any medical diagnosis during the same year as that of the first discharge listing RA in the corresponding
been set up, and although many patients might have had a
quently, guidelines for pretreatment TB screening had not yet
published in October 2001 [4]). Conse-
clinical attention. (The first major report of TNF antagonist–
assessed relative risks of hospitalization for TB, the awareness
Table 1. Characteristics of the Swedish patients with rheumatoid arthritis (RA) followed up for tuberculosis (TB), 1999–2001

<table>
<thead>
<tr>
<th></th>
<th>Inpatient register cohort</th>
<th>Early arthritis cohort</th>
<th>Anti-TNF cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall no.</td>
<td>31,185</td>
<td>2,430</td>
<td>2,500</td>
</tr>
<tr>
<td>No. male/no. female</td>
<td>8,149/23,036</td>
<td>724/1,706</td>
<td>665/1,835</td>
</tr>
<tr>
<td>Age at start of followup, no.</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0–39 years</td>
<td>5,949</td>
<td>384</td>
<td>460</td>
</tr>
<tr>
<td>40–59 years</td>
<td>12,724</td>
<td>933</td>
<td>1,236</td>
</tr>
<tr>
<td>60–79 years</td>
<td>11,195</td>
<td>1,011</td>
<td>763</td>
</tr>
<tr>
<td>80+ years</td>
<td>1,317</td>
<td>102</td>
<td>41</td>
</tr>
<tr>
<td>Mean DAS28*</td>
<td>–</td>
<td>3.6</td>
<td>5.8</td>
</tr>
<tr>
<td>Mean HAQ score*</td>
<td>–</td>
<td>0.8</td>
<td>1.5</td>
</tr>
<tr>
<td>Diabetes mellitus, %†</td>
<td>6.8</td>
<td>2.7</td>
<td>4.7</td>
</tr>
<tr>
<td>Hip/knee prosthesis, %†</td>
<td>23/16</td>
<td>3/0/17</td>
<td>23/12</td>
</tr>
<tr>
<td>Person-years of followup</td>
<td>77,431</td>
<td>7,600</td>
<td>4,000</td>
</tr>
<tr>
<td>TB cases, no.</td>
<td>27</td>
<td>2</td>
<td>4</td>
</tr>
</tbody>
</table>

* In the early arthritis cohort, the 28-joint Disease Activity Score (DAS28) and Health Assessment Questionnaire (HAQ) score reflect the mean values at 6 months after diagnosis of RA. In the anti–tumor necrosis factor (anti-TNF) cohort, the DAS28 and HAQ score reflect the mean values at start of treatment.
† Defined as hospitalization for either diagnosis between January 1, 1964 and December 31, 2001.

Inpatient Register RA patient. The reference subjects were further matched by sex, year of birth, and county of residence. This second reference cohort thus reflected the ever-hospitalized subset of the general population.

**Early RA cohort.** A cohort of patients with early RA consisted of all individuals with newly diagnosed RA (<1 year) that had been diagnosed at either of the participating rheumatology outpatient clinics/units or departments in Sweden since the mid-1990s, with a geographically varying but increasing coverage of the estimated numbers of cases of incident RA (40–100% in different regions, overall ~70%) (15). For each individual, date of birth, detailed medical information on the RA diagnosis, date of RA diagnosis, sex, and national identification number were collected (Table 1).

**TNF antagonist RA cohort.** Within the context of an ongoing Swedish comprehensive postmarketing surveillance program (Anti-rheumatic Treatment in Sweden, or ARTIS), we assembled a cohort of 2,500 RA patients who were treated with etanercept (983 subjects, 1,722 person-years) or infliximab (1,565 subjects, 2,050 person-years) between 1999 and December 31, 2001 (Table 1). Details and patient identification methods have been described elsewhere (16). Briefly, patients were identified through the Swedish Medical Products Agency in collaboration with the Swedish Society for Rheumatology, and through regional surveillance programs of patients treated with TNF blockade. For each individual, we collected information on date of birth, sex, the national registration number, type of TNF antagonist, and start and discontinuation dates of treatment (Table 1).

During 1999–2001, i.e., the period during which we assessed relative risks of hospitalization for TB, the awareness of TB risks associated with TNF antagonists had received little clinical attention. (The first major report of TNF antagonist–associated TB was published in October 2001 [4]). Consequently, guidelines for pretreatment TB screening had not yet been set up, and although many patients might have had a pretreatment chest radiograph performed for various reasons, no routine and specific search for radiologic TB lesions was done, nor was there any routine recording of a history of TB. Performance of a purified protein derivative test was uncommon among these patients.

**Followup and occurrence of TB.** Each individual in the 5 cohorts was linked to the Swedish Register of Total Population and Population Changes, 1969–2001, and to the Swedish Cause of Death Register, 1964–2001. Through this linkage, we collected information on the vital status of each individual from date of first discharge with RA until date of death/emigration or December 31, 2001. Through linkage of all individuals to the Swedish Inpatient Register, 1987–2001, we identified all discharges listing TB (ICD9 codes 010–019, ICD10 codes A15–A19). Each individual was counted only once, irrespective of the number of discharges listing TB.

**Statistical analysis.** To assess the relative risk of TB in patients with RA, we compared the incidence of hospitalization for TB in the Inpatient Register RA cohort with that in its 2 reference cohorts. First, to maximize statistical power, we compared incidences of hospitalization for TB during the followup period, 1987–2001. Thereafter, we restricted followup to 1999–2001 and included only those individuals in the Inpatient Register RA cohort who were hospitalized with RA from 1964 to 1997 and who did not have a hospitalization listing TB before January 1, 1999. This was done to mirror the period for which we had register-based followup data on the TNF antagonist cohort, and to minimize selection bias for inclusion in the Inpatient Register RA cohort due to hospitalization because of incipient TB. Irrespective of restriction, we kept the matched design intact. Values for relative risk were estimated using Cox regression.

To assess the relative risk of TB in RA associated with TNF antagonists, we compared the incidence of hospitalization for TB in the TNF antagonist cohort during 1999–2001 with that in the above-described Inpatient Register RA cohort.
followed up during 1999–2001, as well as with that in the Early RA cohort during 1999–2001. Individuals in the TNF antagonist cohort who appeared in either of the other 2 cohorts were censored from the latter cohorts on the date of entry into the TNF antagonist cohort. Relative risks in this cohort were estimated as age- and sex-adjusted incidence rate ratios.

Case review and incidence of TB following TNF antagonist treatment, 1999–2004. In the second part of our study, we reviewed all reported cases of TB in RA patients treated with TNF antagonists in Sweden from 1999 through 2004. This review thus included the 4 cases involving hospitalization found in the register-based followup of 1999–2001 and all cases, irrespective of hospitalization, reported thereafter. Cases were reported to the Medical Products Agency pharmacovigilance database, to the national TNF antagonist surveillance database, or both. Scrutiny of the mandatory reports of TB cases to the Swedish Institute for Infectious Disease Control yielded 1 additional patient in whom TNF antagonist treatment was mentioned.

To estimate the incidence of reported TB during 1999–2004 among RA patients exposed to TNF antagonists, we divided the reported TB cases that occurred within the ARTIS program by the total and drug-specific number of person-years of followup in the same program.

RESULTS

Relative risk of hospitalization for TB in patients with RA versus general population, 1987–2001 and 1999–2001. In analyses of the 1987–2001 followup period, the Inpatient Register RA cohort comprised 62,321 patients followed up for 467,770 person-years. During followup, 230 individuals in this cohort were hospitalized with TB, which, compared with the reference cohort from the Population Register, corresponded to a 4-fold increase in risk (relative risk 3.9, 95% confidence interval [95% CI] 3.1–5.0). When the Inpatient Register RA cohort was compared with the reference cohort drawn from the Inpatient Register, the risk of TB in RA was increased 60% (relative risk 1.6, 95% CI 1.3–1.9). These relative risks were essentially similar to those found in analyses restricted to 1999–2001 (Table 2), in which the Inpatient Register RA cohort encompassed 31,185 individuals (Table 1) and its reference cohort from the Inpatient Register encompassed 83,007 subjects, of whom 33 were hospitalized with TB.

Relative risk of hospitalization for TB in TNF antagonist–treated RA versus other RA, 1999–2001. When the TNF antagonist–treated RA cohort was compared with either of the 2 cohorts of RA patients who were not treated with TNF antagonists, the 4 cases of hospitalization for TB in the TNF antagonist cohort corresponded to a 4-fold increased risk of TB (e.g., relative risk 4.0, 95% CI 1.3–12 versus Inpatient Register RA cohort not treated with biologics) (Table 2).

Case characteristics and incidence of TB associated with TNF antagonists, 1999–2004. Between 1999 and September 2004, 15 cases of TB in RA patients treated with TNF antagonists were reported (Table 3). All but 1 case occurred during treatment with a TNF antagonist. Eleven patients had been treated with infliximab and 6 with etanercept (2 patients had received both; 1 of these 2 patients also received adalimumab).

The mean duration of TNF antagonist treatment was 10 months (median 8 months). Ten patients were receiving concomitant treatment with methotrexate, and 8 with corticosteroids. Ten patients (67%) had pulmonary TB, 2 had disseminated (fatal) TB, and 3 had musculoskeletal TB. Review of the medical records did not reveal any known case of latent TB at the time of treatment initiation. None of the patients had received prophylactic treatment. Three patients originated from TB-endemic countries.

Overall, the incidence of reported TB in RA treated with TNF antagonists during 1999–2004 was 118 (95% CI 58–210) per 100,000 person-years among RA patients during treatment, and 105 (95% CI 56–180) per 100,000 person-years among RA patients who had ever been treated with a TNF antagonist. Among RA patients who had received only infliximab the incidence (during treatment) of TB was 145 (95% CI 58–299) per 100,000 person-years; among RA patients who had received only etanercept the incidence was 80 (95% CI 16–232) per 100,000 person-years, and among patients who had received both infliximab and etanercept the incidence was 129 (95% CI 3.3–719) per 100,000 person-years. In comparing these incidences, the relative risk associated with etanercept was 0.5 (95% CI 0.1–2.4), which changed little after adjustment for age.

DISCUSSION

In our register-based assessment, patients with RA who were not treated with biologics were indeed at

Table 2. Relative risk of TB in RA cohorts compared with reference cohorts in Sweden, 1999–2001*

<table>
<thead>
<tr>
<th>RA cohort, comparator cohort</th>
<th>Relative risk of TB (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inpatient Register RA</td>
<td></td>
</tr>
<tr>
<td>Population Register reference</td>
<td>3.7 (1.7–8.1)</td>
</tr>
<tr>
<td>Inpatient Register reference</td>
<td>2.0 (1.2–3.4)</td>
</tr>
<tr>
<td>Anti-TNF–treated RA — no biologics</td>
<td>4.0 (1.3–12)†</td>
</tr>
<tr>
<td>Early arthritis — no biologics</td>
<td>4.1 (0.6–21)†</td>
</tr>
</tbody>
</table>

* 95% CI = 95% confidence intervals (see Table 1 for other definitions).
† Age and sex adjusted.
increased risk of TB compared with the general population, and TNF antagonist treatment was associated with a further 4-fold increased risk during 1999–2001. Characterization of all reported cases of TB occurring in RA patients treated with TNF antagonists in Sweden revealed that TB may occur not only shortly after the start of treatment, but also years thereafter. Moreover, the results suggested a higher incidence of TB associated with infliximab than with etanercept, although this was based on only a few cases.

The introduction of TNF antagonists has highlighted the need for appropriate data on comorbidity in treated conditions. Specifically, many adverse events that may limit the use of these agents may be difficult to detect and quantify using data from short-term clinical trials, which often encompass subjects and conditions substantially different from those encountered during provision of routine care. Pharmacovigilance based on spontaneous reporting is limited by underreporting, an unknown propensity among physicians to report adverse events and to select the cases reported, and uncertainties with respect to the sample size corresponding to the reported number of adverse events (17,18). With respect to TB, it is unclear whether RA in conjunction with common treatments is, in itself, a risk factor. Indeed, it is likely that the incidence of TB in RA differs from that in otherwise-healthy individuals. In low-incidence populations, most cases of TB occur in individuals with particular risk factors (19). Therefore, national TB rates may not be suitable as comparator rates, especially not in ethnically heterogeneous populations (9,11). Studies of TB are hindered by yet another methodologic complication, with implications for generalizability: since TNF-associated TB largely represents reactivation of latent infection (10), the burden of TB attributable to TNF antagonists may be a function of past prevalence of natural infection with TB in the birth cohorts that typically harbor patients with RA.

The relative risks of TB associated with RA in our study were more precise, but in the same order of magnitude as those reported from Spain (8). In contrast to the findings reported in the US cohort (9), our results suggest that RA patients are at increased risk of TB even in the absence of TNF antagonists. The further 4-folding of the risk among RA patients treated with TNF antagonists is lower than the 12–20-fold increased risk reported during the same period in Spain (8), and may be lower than the risk increase observed in the US (9). Both the Spanish and the US studies were, however, restricted to infliximab-treated RA patients.

Apart from the different populations covered, the methodologic differences between the present study, the Spanish study, and the US study are substantial. The Spanish study was based on a cohort of infliximab-treated RA patients, the coverage of which was not reported, and TB was assessed through physician’s reporting of culture-positive TB. National TB rates and rates obtained in a TNF antagonist-naive RA cohort

Table 3. Characteristics of TB occurring among anti-TNF–treated RA patients in Sweden between 1999 and September 2004*

<table>
<thead>
<tr>
<th>Sex/age, years</th>
<th>RA duration, years</th>
<th>Time receiving etanercept, months</th>
<th>Time receiving infliximab, months</th>
<th>TB localization</th>
<th>TB diagnostics</th>
<th>DMARD</th>
<th>Prednisone</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/61</td>
<td>1</td>
<td>–</td>
<td>12</td>
<td>Pulmonary</td>
<td>Culture</td>
<td>MTX</td>
<td>+</td>
</tr>
<tr>
<td>F/64</td>
<td>10</td>
<td>–</td>
<td>1.5</td>
<td>Pulmonary</td>
<td>Culture</td>
<td>MTX</td>
<td>+</td>
</tr>
<tr>
<td>M/68</td>
<td>31</td>
<td>–</td>
<td>7.5</td>
<td>Pulmonary</td>
<td>Culture</td>
<td>MTX</td>
<td>–</td>
</tr>
<tr>
<td>F/75</td>
<td>14</td>
<td>–</td>
<td>3</td>
<td>Fatal/diss.†</td>
<td>AFB†</td>
<td>AZA</td>
<td>+</td>
</tr>
<tr>
<td>F/49</td>
<td>16</td>
<td>9</td>
<td>–</td>
<td>Pulmonary</td>
<td>Culture</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F/60</td>
<td>7</td>
<td>–</td>
<td>6</td>
<td>Pulmonary</td>
<td>Culture</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>F/78</td>
<td>&gt;30</td>
<td>–</td>
<td>0.5</td>
<td>Fatal/diss.†</td>
<td>AFB†</td>
<td>MTX</td>
<td>+</td>
</tr>
<tr>
<td>F/94</td>
<td>6</td>
<td>5</td>
<td>–</td>
<td>Wrist/hand</td>
<td>Culture</td>
<td>MTX</td>
<td>+</td>
</tr>
<tr>
<td>F/21</td>
<td>2.5</td>
<td>–</td>
<td>21</td>
<td>Chest tumor</td>
<td>Culture</td>
<td>MTX</td>
<td>+</td>
</tr>
<tr>
<td>F/62</td>
<td>18</td>
<td>–</td>
<td>36</td>
<td>Pulmonary</td>
<td>Culture</td>
<td>MTX</td>
<td>–</td>
</tr>
<tr>
<td>F/75</td>
<td>18</td>
<td>4</td>
<td>11</td>
<td>TB arthritis</td>
<td>Culture</td>
<td>ADA/MTX‡</td>
<td>–</td>
</tr>
<tr>
<td>M/60</td>
<td>2</td>
<td>–</td>
<td>8</td>
<td>Pulmonary</td>
<td>Previous TB§</td>
<td>MTX</td>
<td>–</td>
</tr>
<tr>
<td>F/80</td>
<td>40</td>
<td>29</td>
<td>–</td>
<td>Pulmonary</td>
<td>Culture</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>F/72</td>
<td>23</td>
<td>1</td>
<td>1</td>
<td>Spondylitis</td>
<td>Culture</td>
<td>MTX</td>
<td>+</td>
</tr>
<tr>
<td>F/18</td>
<td>NA</td>
<td>3¶</td>
<td>–</td>
<td>Pulmonary</td>
<td>Culture</td>
<td>NA</td>
<td>NA</td>
</tr>
</tbody>
</table>

* DMARD = disease-modifying antirheumatic drug; MTX = methotrexate; AZA = azathioprine; ADA = adalimumab; NA = not available (see Table 1 for other definitions).
† For patients with fatal/disseminated TB, autopsy indicated miliary TB, with acid-fast bacilli (AFB) present.
‡ Treated with ADA (Humira) for 2 months prior to the TB diagnosis.
§ Personal history of culture-positive TB many years previously. Currently, resolution of TB pleurisy was achieved upon anti-TB treatment.
¶ Stopped 12 months prior to TB.
were used as reference. In the US study, RA patients were selected from the practices of some 900 rheumatologists, and were followed up through regular contacts rather than through register linkage. TB was identified on the basis of patient self-report, which was subsequently validated. US rates of TB were used as reference. In our study, the Inpatient Register RA cohort was identified through prospectively recorded data on discharge diagnoses. Validation of these RA diagnoses suggests that almost 90% of them reflect RA as defined by the American College of Rheumatology (formerly, the American Rheumatism Association) criteria (20), with the remainder chiefly being other rheumatic conditions (21).

We estimate that ~50% of all patients with RA in Sweden in 2001 were included in our cohort according to our inclusion criteria. To circumvent biases related to hospitalization, one of our general population reference cohorts was also identified in the Inpatient Register, and thus these patients were also required to have at least one overnight hospital stay, irrespective of diagnosis. Based on sales statistics, we estimate the coverage of the TNF antagonist cohort to be ~80% (higher for etanercept than infliximab). Accordingly, we consider it unlikely that the Inpatient Register RA cohort or the Early RA Register cohort would harbor unidentified cases of TNF antagonist–associated TB, especially since the relative risks of TB in RA during 1987–2001 and 1999–2001 were similar.

In the assessment of relative risk of TB during 1999–2001, we recorded hospitalizations for TB and had no way to validate the diagnoses made, except for the TB cases occurring in the TNF antagonist cohort and in the Early RA cohort, in whom all cases of hospitalized TB during 1999–2001 also reflected true TB. Although hospitalization is not mandatory for TB treatment, the general tendency toward hospitalization may be higher in Sweden than in the US. Moreover, hospitalization is common during the clinical examination (e.g., because of unexplained inflammation/malaise or to obtain findings on bronchoscopy), thus leading to TB diagnosis. Accordingly, 12 of the 15 patients (80%) reviewed for TB had been hospitalized because of their TB. The incidence of TB hospitalization in our general population reference cohort was 10 cases per 100,000 population, which is similar to the national Swedish notification rates of 4–14 cases per 100,000 population in the corresponding age groups (19), and the incidence of hospitalization for TB following TNF antagonist treatment in the register-based part of the study was of similar magnitude as the incidence of TB based on the reported and reviewed TB cases.

A comparison of the distribution of ICD codes (A15.0–A19.9) defining the TB cases in the general population and Inpatient Register RA cohorts revealed similar relative risks for each code (e.g., A15.0) and a typical (19) mix of 75% pulmonary TB and 25% extrapulmonary TB, which provides further evidence against systematic differences in the diagnostic processes and coding between the cohorts under study. Of note, we used the same definition and method of ascertainment of TB (which was independent of drug exposure and devoid of recall bias) in all cohorts. Therefore, even if our method of TB ascertainment in 1999–2001 may not be 100% sensitive and may not provide accurate estimates of incidence (which we therefore do not report), the corresponding relative risks should remain valid. In the event that the propensity for hospitalization were higher in the TNF antagonist cohort than in, e.g., the general population cohort, the relative risks would, however, be biased (upwards), but such bias does not preclude our conclusion of a lower relative risk of TB following TNF antagonist treatment than has been previously reported.

The incidence of reported TB in RA patients treated with TNF antagonists provides us with the opportunity to compare the incidence of TB following treatment with etanercept with that following infliximab within the same country. Although, based on a few cases, the higher incidence of TB following treatment with infliximab in our study supports previous observations of a higher number of reported cases of TB following infliximab treatment, our results nevertheless suggest that the difference in risk between the 2 drugs may not be as marked as previously thought. Our review of TNF antagonist–treated TB cases indicated several cases of extrapulmonary TB. Importantly, in addition to immediate TB cases, our series also included cases occurring years after the start of TNF antagonist treatment, which may indicate that the risk of reactivation of TB following TNF antagonist treatment is not transient.

ACKNOWLEDGMENTS

We thank the ARTIS study group on biologics use in RA in Sweden, the Swedish RA Register, and all doctors and health professionals involved in reporting patients within the framework of these structured programs. We also thank Maud Rütting, Medical Products Agency, for help with the retrieval of medical files of the TB cases, and the National Board of Health and Welfare.
REFERENCES


Association of the Lymphoid Tyrosine Phosphatase R620W Variant With Rheumatoid Arthritis, but Not Crohn’s Disease, in Canadian Populations

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Objective. A single-nucleotide polymorphism in the PTPN22 gene encoding the lymphoid protein tyrosine phosphatase (Lyp) has recently been identified as a functional variant associated with susceptibility to rheumatoid arthritis (RA), type 1 diabetes, and systemic lupus erythematosus. To determine whether association of this variant (PTPN22 1858T) with RA is reproducible and is also observed in another autoimmune condition, Crohn’s disease, we investigated the association between the PTPN22 1858T allele and RA and Crohn’s disease in a Canadian population.

Methods. Two RA case–control cohorts representing a total of 1,234 patients and 791 healthy controls as well as a cohort of 455 patients with Crohn’s disease and 190 controls were genotyped for the PTPN22 C1858T polymorphism, and genotype frequencies were compared between patients and controls.

Results. Significant association of the PTPN22 1858T allele with RA was detected in both the Toronto-based RA cohort (P = 1.6 × 10^{-6}, odds ratio [OR] 1.8) and the Halifax-based RA cohort (P = 9.4 × 10^{-4}, OR 1.94). Association of the risk allele with RA was not affected by sex, age at disease onset, or the presence of either rheumatoid factor or rheumatoid nodules. No association between the PTPN22 risk allele and Crohn’s disease was detected.

Conclusion. These observations confirm the association of RA susceptibility with the PTPN22 1858T allele. However, the data also reveal a lack of association between this variant and Crohn’s disease, suggesting that the PTPN22 1858T allele is a risk allele for multiple, but not all, autoimmune diseases.

Rheumatoid arthritis (RA) is one of the most common systemic autoimmune disorders, affecting an estimated 0.5–1% of the population. The disease is characterized by peripheral synovial joint inflammation, which leads to cartilage and bone damage and, ulti-
mately, joint destruction. The etiology of RA is complex and multifactorial, but family aggregation and twin concordance data indicate a significant causal role for genetic factors, with heritability being estimated at 60% (1).

Much of the research involving RA susceptibility factors has focused on the major histocompatibility complex (MHC) region on chromosome 6p21, which has been shown to account for approximately one-third of the genetic risk for RA (2). More recently, linkage data from genome-wide screens of multicase RA families also identified a number of non-MHC chromosomal regions as possible RA susceptibility loci (3–7), and analyses of selected candidate genes within such regions revealed several gene variants as being associated with RA in some populations (8–10). These latter variants include a single-nucleotide polymorphism (SNP) in the PTPN22 gene encoding a cytosolic protein tyrosine phosphatase, Lyp, which in mice was shown to interact with the negative regulatory kinase, Csk, thereby inhibiting T lymphocyte activation (11–13). This PTPN22 variant (1858C→T) is a particularly credible susceptibility allele for RA because it engenders a substitution in Arg620Trp that disrupts Lyp binding to Csk (14) and would thereby predictably reduce or abrogate the inhibitory effects of the Lyp–Csk interaction on T cell activation. In addition, prior to its identification as a risk allele for RA, the PTPN22 1858T variant was shown to be associated with type 1 diabetes (14) and as such may be of etiologic relevance in multiple autoimmune diseases.

Although the capacity of the PTPN22 1858T variant to impair Lyp function strongly supports its relevance to RA susceptibility, genetic association data derived from a single population/study are not always reproducible in other populations/studies (15,16). To address this issue and to ascertain whether the PTPN22 1858T variant might also be etiologically relevant to another autoimmune disease, Crohn’s disease, we investigated the association of this PTPN22 allele with RA and Crohn’s disease in the Canadian population.

**PATIENTS AND METHODS**

**Subjects.** For the RA patient cohorts, patients were recruited from 2 sites (Toronto and Halifax) in conjunction with the Mount Sinai Hospital/Ellipsis Biotherapeutics Corporation Rheumatoid Arthritis Genetics Study involving referrals from rheumatologists affiliated with the University of Toronto and Dalhousie University (Halifax). All patients with RA met the American College of Rheumatology (formerly, the American Rheumatism Association) 1987 revised criteria for RA (17), and none had a family history of RA. Patients with Crohn’s disease were recruited in Toronto via the Mount Sinai Hospital Inflammatory Bowel Disease Genetics Project. The diagnosis of Crohn’s disease was based on standard clinical, endoscopic, radiologic, or histologic criteria. Control subjects (matched only for ethnicity and geographic location) for this study included 793 Toronto-based (603 for the RA study and 190 for the Crohn’s disease study) and 188 Halifax-based healthy anonymous volunteers with no history of inflammatory arthritis or bowel disease. All study participants were white (Caucasian).

**Genotyping.** Genotyping of the PTPN22 C1858T SNP (rs2476601) was performed using the MassARRAY matrix-assisted laser desorption ionization–time-of-flight mass spectrometry system (Sequenom, San Diego, CA). The primer pairs were 5’-ACGGTTGGATGAACGTATCCACAGCATTCC-3’ (forward) and 5’-ACGGTTGGATGAGATGAGAGAAATCCCCCTC-3’ (reverse), and the extension primer was 5’-AAATATGATTCCAGGTTGCC-3’. Allele-specific extension products were plated onto a SpectroCHIP (Sequenom), subjected to mass spectrometric analysis, and the genotypes were identified using SpectroCALLER software (Sequenom). Genotype assignment was successful in >95% of samples tested.

**Statistical analysis.** Association and Hardy-Weinberg equilibrium were evaluated using the chi-square test. Relative risk/odds ratios (ORs) were calculated using standard logistic regression methods and were processed for the analyses using SAS version 8.2 software (Cary, NC). A Fisher’s exact test was used to determine significance in any instance in which the number of individuals in any cell was less than 5, and a nonparametric Mann-Whitney test was used for assessing clinical similarities between patient cohorts.

### RESULTS

**Association of the PTPN22 1858T variant with RA.** To determine whether association of the PTPN22 1858T variant with RA occurs in RA cohorts other than those used initially to identify this association, the frequency of this variant was investigated in 2 independently collected Canadian RA patient and control cohorts. These cohorts included 906 unrelated patients with sporadic RA and 603 controls from the Toronto area, and 328 unrelated patients with sporadic RA and 188 controls from Halifax, Nova Scotia. As shown in Table 1, the 2 patient cohorts were similar with respect

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Toronto cohort</th>
<th>Halifax cohort</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female sex, %</td>
<td>76.0</td>
<td>70.8</td>
</tr>
<tr>
<td>Mean age at study entry, years</td>
<td>59.3</td>
<td>61.7</td>
</tr>
<tr>
<td>Mean age at disease onset, years</td>
<td>43.1</td>
<td>47.6*</td>
</tr>
<tr>
<td>Rheumatoid factor positive, %</td>
<td>69.4</td>
<td>73.3</td>
</tr>
<tr>
<td>Erosive disease, %</td>
<td>86.4</td>
<td>73.4*</td>
</tr>
<tr>
<td>Presence of nodules, %</td>
<td>31.3</td>
<td>31.9</td>
</tr>
</tbody>
</table>

* P = 0.028 by nonparametric Mann-Whitney test. † P = 0.0009 by Pearson’s chi-square test.
to sex, age at study entry, rheumatoid factor (RF) positivity, and the presence of nodules. However, the mean age at disease onset was significantly lower and the percentage of patients with erosive disease was significantly higher in the Toronto cohort compared with the Halifax cohort.

Genotyping of the 2 cohorts revealed that allele frequencies for the PTPN22 SNP were in Hardy-Weinberg equilibrium among both RA patient and control populations. Analysis of the Toronto cohort revealed that the frequency of the PTPN22 risk allele (1858T) was significantly different in patients (0.140) compared with controls (0.088, respectively) reported by Begovich et al (for cases, OR 1.49–2.53). Similarly, analysis of the Halifax cohort revealed that the risk for RA conferred by heterozygosity for the PTPN22 risk allele was only marginally lower than that conferred by the combined heterozygous and homozygous genotype. Results of likelihood ratio tests were also consistent with additive (P = 0.90) or dominant (P = 0.21) modes of inheritance and rejected a recessive inheritance model (P < 0.0001).

Regression analysis was also used to evaluate possible correlates between the PTPN22 risk allele and the specific demographic and clinical characteristics shown in Table 1. Results of these analyses revealed that the PTPN22 association with disease was unaffected by sex, age at disease onset, and the presence of erosions or rheumatoid nodules. Effects of RF status on this association were also evaluated by comparing PTPN22 risk allele frequencies between RF-positive and RF-negative patient subgroups. Among the 483 patients in the Toronto cohort who were RF positive, 10, 120, and 353 carried the TT, TC, and CC genotypes, respectively.

The relevance of the risk allele copy number to disease susceptibility was also investigated using standard logistic regression analysis. This analysis, however, was limited by the paucity of risk allele homozygotes among both patients and controls. The Toronto cohort, for example, included only 13 individuals homozygous for the risk allele (1.4%), and the association between RA and PTPN22 1858T homozygosity did not reach a level of significance in this group (Table 2). However, as is consistent with a dominant effect on risk, the risk for disease conferred by heterozygosity for the PTPN22 1858T allele (χ² = 23.58, P = 1.2 × 10⁻⁶, OR 1.93, 95% CI 1.48–2.54) was identical to the combined risk conferred by PTPN22 1858T heterozygous and homozygous genotypes (χ² = 24.72, P = 6.6 × 10⁻⁷, OR 1.94, 95% CI 1.49–2.53).

Regression analysis was also used to evaluate possible correlates between the PTPN22 risk allele and the specific demographic and clinical characteristics shown in Table 1. Results of these analyses revealed that the PTPN22 association with disease was unaffected by sex, age at disease onset, and the presence of erosions or rheumatoid nodules. Effects of RF status on this association were also evaluated by comparing PTPN22 risk allele frequencies between RF-positive and RF-negative patient subgroups. Among the 483 patients in the Toronto cohort who were RF positive, 10, 120, and 353 carried the TT, TC, and CC genotypes, respectively.

Table 2. Association of RA with PTPN22 genotypes

<table>
<thead>
<tr>
<th>Cohort, genotype</th>
<th>No. (%) in RA patients</th>
<th>No. (%) in controls</th>
<th>χ²</th>
<th>P†</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Toronto</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>664 (73.3)</td>
<td>508 (84.3)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CT</td>
<td>229 (25.3)</td>
<td>90 (14.9)</td>
<td>23.58</td>
<td>1.2 × 10⁻⁶</td>
<td>1.93 (1.48–2.54)</td>
</tr>
<tr>
<td>TT</td>
<td>13 (1.4)</td>
<td>5 (0.8)</td>
<td>1.73</td>
<td>0.188</td>
<td>1.98 (0.70–5.59)</td>
</tr>
<tr>
<td>TT + CT</td>
<td>242 (26.7)</td>
<td>95 (15.8)</td>
<td>24.72</td>
<td>6.6 × 10⁻⁷</td>
<td>1.94 (1.49–2.53)</td>
</tr>
<tr>
<td><strong>Halifax</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CC</td>
<td>230 (70.1)</td>
<td>153 (81.4)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CT</td>
<td>84 (25.6)</td>
<td>34 (18.1)</td>
<td>4.78</td>
<td>0.028</td>
<td>1.64 (1.05–2.57)</td>
</tr>
<tr>
<td>TT</td>
<td>14 (4.3)</td>
<td>1 (0.5)</td>
<td>6.74</td>
<td>0.0094</td>
<td>9.31 (1.21–71.56)</td>
</tr>
<tr>
<td>TT + CT</td>
<td>98 (29.9)</td>
<td>35 (18.6)</td>
<td>7.92</td>
<td>0.0049</td>
<td>1.86 (1.20–2.88)</td>
</tr>
</tbody>
</table>

* Odds ratios (ORs) were calculated using CC as the reference genotype. RA = rheumatoid arthritis; 95% CI = 95% confidence interval.
† Pearson’s chi-square test was used for all comparisons (with CC serving as the reference genotype), except for one test in which Fisher’s exact test was used because the number of individuals within one cell was <5.
controls studied here (0.067; (0.075) was no different from that observed in the
PTPN22
Results of this analysis revealed that the frequency of the
ported) and 190 controls from the Toronto area (18).
demographic characteristics have been previously re-
455 patients with Crohn’s disease (whose clinical/
autoimmune disease, Crohn’s disease, by genotyping of
1858T variant might also predispose to another
crohn’s disease.
We investigated the possibility that the
Crohn’s disease.
increased risk for Crohn’s disease.
to those observed in controls is strong evidence that the
risk allele in patients with Crohn’s disease
(0.075) was no different from that observed in the
controls studied here (0.067; \( \chi^2 = 0.46, P = 0.50 \)) or in
the subjects used as controls for analysis of the RA
patient cohort (for Crohn’s disease patients, \( \chi^2 = 0.38, P = 0.54 \); for Crohn’s disease controls, \( \chi^2 = 1.01, P = 0.31 \)). Frequencies of homozygosity and heterozygosity
for the
PTPN22
risk allele were also no different in
patients with Crohn’s disease compared with controls.
Given these sample sizes, this study had 80% power to
detect, with 5% significance, a difference between pa-
tients and controls if the frequency of
PTPN22
alleles
was at least 0.117 (corresponding to an OR of 1.58) in
the Crohn’s disease patients and controls or at least
0.107 (corresponding to an OR of 1.32) in the RA
controls. The fact that
PTPN22
allele frequencies ob-
served in the patients with Crohn’s disease were similar
to those observed in controls is strong evidence that the
PTPN22
1858T allele does not confer any substantially
increased risk for Crohn’s disease.

DISCUSSION

In the current study, 1,234 unrelated patients
with RA, representing 2 independently collected RA
patient cohorts, and 455 patients with Crohn’s disease
were genotyped for a functional variant in the
PTPN22
gene (1858T) that was recently reported to be associated
with RA in the US population (10). The data revealed
that the
PTPN22
risk allele was associated with RA in
both of the patient cohorts studied here but showed no
significant association with Crohn’s disease. These find-
ing therefore replicate the previously reported association
of the
PTPN22
1858T allele with RA and indicate
that this allele contributes significantly to the risk of RA
but not to the risk of Crohn’s disease. Because cases of
familial RA were specifically excluded in this study but
were not excluded in the initial study showing RA
association with this
PTPN22
variant, these data also
serve to establish the relevance of
PTPN22
1858T to
susceptibility in cases of sporadic RA.

The frequencies of the
PTPN22
risk allele among
Toronto patients with RA and controls in the present
study were similar to those previously reported for a
US-based Caucasian RA case–control cohort (10). How-
ever, despite the similar ethnic origins of the Toronto
and Halifax populations (primarily the British Isles), the
frequency of the
PTPN22
risk allele was slightly higher in
Halifax-based patients and controls compared with that
in the Toronto and US RA patient cohorts. This discrep-
ancy may relate to differences in patient selection,
particularly in view of the later age at disease onset and
less frequent occurrence of erosive disease manifested
by the Halifax patients compared with the Toronto
patients. However, given that we also observed a slight
increase in risk allele frequency in the Halifax control
population, these findings more likely reflect ethnic
disparity between the populations, or stochastic chance.

The data reported here, showing that the associ-
ation of the
PTPN22
risk allele with disease is unaffected
by RF status, are discrepant from those in the study by
Begovich et al, who reported that this association was
not detected in RF-negative patients (10). The basis for
this discrepancy is unclear, because the 2 studies are very
similar in relation to the sample sizes analyzed and the
confidence intervals for risks conferred by the
PTPN22
risk allele in RF-negative patients. Resolution of this
issue will likely require the analysis of patient cohorts
including larger numbers of RF-negative patients.

A role for the
PTPN22
risk variant in RA is highly
consistent with the predicted effects of this variant on
the function of the
PTPN22-encoded Lyp enzyme. Al-
though the biologic roles of Lyp are not well character-
ized, Lyp and its murine ortholog (PEST domain-
enriched tyrosine phosphatase [PEP]) have been shown
to play a major role in inhibiting antigen receptor-
induced T cell activation (12,13,19,20). This effect of
Lyp/PEP reflects, at least in part, its capacity to interact
with the Csk protein tyrosine kinase (14,21), an associ-
aton that enables both effectors to function synergisti-
cally so as to deactivate the major Src family protein
tyrosine kinases required for T cell antigen receptor
signaling, Lck and Fyn (12,13). In contrast, the Lyp
variant (620Trp) encoded by the
PTPN22
1858T allele
has been shown to bind less efficiently to Csk (10,14),
and its expression would therefore predictably engender
increases in spontaneous and/or inducible T cell activa-
tion that may favor triggering or maintenance of aber-
rant autoimmune responses. Disruption of the Lyp–Csk
interaction may also predispose to autoimmunity by
virtue of alterations in T cell development/maturation,
memory T cell compartment in PEP-deficient mice (19) or, alternatively, by altering behavior of other hematopoietic cell lineages, as suggested by the capacity of Lyp to modulate Bcr–Abl signaling in myeloid cells (22). Although these issues require further investigation, the available data concerning the functions of Lyp and the Lyp620Trp variant strongly support involvement of this variant in RA susceptibility.

Initially identified as a susceptibility allele for type 1 diabetes (14), the PTPN22 1858T variant has now been implicated in the genetic etiology of RA and, most recently, systemic lupus erythematosus (10,23). The sharing of this risk allele among these autoimmune diseases is consistent with longstanding epidemiologic data showing familial clustering of autoimmune conditions (24,25), with genetic data revealing considerable overlap among susceptibility loci identified for different autoimmune diseases (26,27), and with very recent data identifying variants in the CARD15 and SLC22A4/SLC22A5 genes as susceptibility alleles for both Crohn’s disease and psoriatic arthritis (28–31).

Taken together, these observations provide compelling evidence of the existence of pleiotropic autoimmune genes that confer susceptibility to multiple autoimmune conditions. Interestingly, the Crohn’s disease–associated CARD15 and SLC22A4/SLC22A5 alleles do not appear to confer risk for RA, at least in Caucasian populations (32,33), and the data reported here suggest that the PTPN22 1858T variant also is not involved in Crohn’s disease susceptibility. These findings raise the possibility that Crohn’s disease and psoriatic arthritis are genetically similar conditions, while RA is genetically distinct from these disorders and instead has genetic overlap with other conditions, such as lupus and type 1 diabetes. Although evaluation of this hypothesis requires further investigation into the spectrum of diseases associated with each gene variant, the current data strongly support the designation of PTPN22 1858T as a variant involved in the predisposition to RA, and suggest that definition of the molecular pathways coupling this variant to disease will provide significant insights into the pathogenesis of RA and other autoimmune diseases.

ACKNOWLEDGMENTS

We thank the many rheumatologists and RA patients who assisted with and participated in this study.

REFERENCES

Invasiveness of Fibroblast-like Synoviocytes Is an Individual Patient Characteristic Associated With the Rate of Joint Destruction in Patients With Rheumatoid Arthritis

Tanja C. A. Tolboom, Annette H. M. van der Helm-van Mil, Rob G. H. H. Nelissen, Ferdinand C. Breedveld, René E. M. Toes, and Tom W. J. Huizinga

Objective. Rheumatoid arthritis (RA) is characterized by inflammation and destruction of synovial joints. Fibroblast-like synoviocytes (FLS) harvested from synovial tissue of patients with RA can invade normal human cartilage in severe combined immunodeficient (SCID) mice and Matrigel basement membrane matrix in vitro. This study was undertaken to investigate the association of these in vitro characteristics with disease characteristics in patients with RA.

Methods. Synovial tissue samples from 72 RA and 49 osteoarthritis (OA) patients were obtained. Samples of different joints were collected from 7 patients with RA. The FLS invasiveness in Matrigel was studied, and the intraindividual and interindividual differences were compared. From the patients with FLS who exhibited the most extreme differences in in vitro ingrowth (most and least invasive FLS), radiographs of the hands and feet were collected and scored according to the Sharp/van der Heijde method to determine the relationship between in vitro invasion data and estimated yearly joint damage progression.

Results. FLS from patients with RA were more invasive than FLS from patients with OA ($P < 0.001$). The mean intraindividual variation in FLS invasion was much less than the mean interindividual variation (mean $\pm$ SD $1,067 \pm 926$ and $3,845 \pm 2,367$ for intraindividual and interindividual variation, respectively; $P = 0.035$), which shows that the level of FLS invasion is a patient characteristic. The mean $\pm$ SEM Sharp score on radiographs of the hands or feet divided by the disease duration was $4.4 \pm 1.1$ units per year of disease duration in patients with the least invasive FLS ($n = 9$), which was much lower compared with the $21.8 \pm 3.1$ units per year of disease duration in patients with the most invasive FLS ($n = 9$) ($P < 0.001$).

Conclusion. The ex vivo invasive behavior of FLS from RA patients is associated with the rate of joint destruction and is a patient characteristic, given the much smaller intraindividual than interindividual FLS variation.

Rheumatoid arthritis (RA) is an autoimmune disease that predominantly targets the synovial joints and ultimately leads to joint destruction. The destructive process is suggested to be mediated, at least in part, by fibroblast-like synoviocytes (FLS) from the synovium because in a SCID mouse coinfection model, it was shown that FLS from RA patients attach to and invade normal cartilage (1). Moreover, others have observed that in RA, FLS show characteristics of transformed cells, such as anchorage-independent growth (2), insensitivity to apoptosis, and increased proliferation.

Processes that are associated with the change in FLS from normal to aggressive behavior are phosphorylation of the STAT-3 protein and elevated levels of the prioncogene c-myc (3). It is not yet known whether these features are noncausal associations or a causal factor. FLS in culture express large amounts of proteinases that can degrade extracellular matrix components such as collagens. One family of proteinases expressed by FLS are the matrix metalloproteinases (MMPs). FLS express MMPs 1, 3, 9, and 10, and the expression of
In this study, we investigated the association between in vitro characteristics of FLS and disease characteristics of RA patients. We addressed whether the degree of FLS invasion was comparable in different joints of the same patient (i.e., is FLS invasiveness a characteristic that occurs in multiple joints of the same patient or is it a random process), and whether the degree of in vitro invasion correlated with the degree of radiologic destruction.

**MATERIALS AND METHODS**

**Patients and synovium.** Synovial tissue was obtained from 121 patients (72 with RA and 49 with osteoarthritis [OA]) at joint replacement surgery or synovectomy. Sixty-nine percent of the patients with RA were women and the mean ± SD age was 60 ± 14 years. Samples were obtained from knees (53 patients), elbows (17 patients), shoulders (13 patients), hips (26 patients), ankles (7 patients), wrists (4 patients), and feet (1 patient). All patients with RA met the criteria of the American College of Rheumatology (formerly, the American Rheumatism Association) (8). Tissue was harvested and tested for invasiveness, as previously described (4). RA FLS were significantly more invasive than OA FLS (n = 9) were scored according to the Sharp/van der Heijde method (9). The person who scored the radiographs (AHH) was unaware of the clinical data and the study question. The total erosion and joint space narrowing scores, as well as the total Sharp/van der Heijde scores, were divided by the disease duration from the date of diagnosis to determine the yearly progression of radiologic joint damage (10).

**Statistical analysis.** Results are expressed as the mean ± SD. Differences in invasiveness of FLS between patients, differences between intra-individual and inter-individual variation, and the radiographic scores of the patients with the most and least invasive FLS were compared by the Mann-Whitney U test. P values less than 0.05 were considered significant.

**RESULTS**

FLS were isolated from the tissues and cultured. When the cells had grown to confluence, the cells were harvested and tested for invasiveness, as previously described (4). RA FLS were significantly more invasive than OA FLS in this study (mean ± SD 2,884 ± 2,326 and 4,573 ± 2,502 for OA and RA FLS, respectively; P < 0.001) (Figure 1). These results are consistent with those of previous studies (1,4).

We then studied whether the invasiveness of FLS...
obtained at different times from different joints exhibited the same invasive characteristics. Two different samples were obtained from 2 different joints of 7 patients with RA. The mean differences in FLS invasiveness of different samples from an individual patient were significantly less than the mean differences between patients (mean $\pm$ SD 1,067 $\pm$ 926 and 3,845 $\pm$ 2,367 for intra- and interindividual variation, respectively; $P = 0.035$) (Figure 2). Thus, the variation within patients was smaller than the variation between patients. From the group of OA patients, 3 patients were operated upon twice. This group was too small for an analysis of the differences between intraindividual and interindividual variation.

Next, we addressed whether the in vitro invasiveness of FLS was correlated with radiologic joint destruction in RA patients. It has been shown that the estimated annual rate of destruction obtained by dividing the Sharp/van der Heijde score per radiographs of hands and feet by the disease duration correlates well with the destruction rate reported in observational studies using multiple measurements (10). We assessed the association between the invasiveness of FLS and radiologic joint destruction.

Sharp/van der Heijde scores from the 9 patients with the most invasive FLS and the 9 patients with the least invasive FLS were determined, and the estimated yearly destruction rates were compared (Figure 3). The patients with the most invasive FLS, the mean $\pm$ SEM total Sharp/van der Heijde score per year of disease duration was 21.8 $\pm$ 3.1, with a mean $\pm$ SEM erosion score of 13.3 $\pm$ 1.7 and a narrowing score of 8.5 $\pm$ 1.6. For patients with the least invasive FLS, the mean $\pm$ SEM total Sharp/van der Heijde score per year of disease duration was 4.4 $\pm$ 1.1, with an erosion score of 2.5 $\pm$ 0.7 and a narrowing score of 1.9 $\pm$ 0.5. No difference in disease duration was observed between patients with the most and the least invasive FLS (mean $\pm$ SEM 15.9 $\pm$ 11.5 versus 15.0 $\pm$ 7.3 years, respectively; $P = 0.85$). These results show a strong association between invasiveness of FLS and radiologic joint destruction.
cally estimated yearly rate of joint destruction ($P < 0.001$).

**DISCUSSION**

Our findings show that in RA, the intraindividual variation in FLS invasiveness is much lower than the interindividual variation, indicating that the invasive behavior of FLS is a characteristic of individual RA patients. Furthermore, this study is the first to show that in vitro FLS invasiveness is associated with radiologic joint destruction. Patients with the least invasive FLS had significantly lower Sharp/van der Heijde scores per year of disease duration than did patients with the most invasive FLS. This suggests that the invasive behavior of FLS is relevant to the pathogenesis of RA.

The finding of a rather large variation in the rate of invasion of FLS between patients implies that the mechanism or processes underlying the invasive behavior of FLS differs between individuals. The mechanism that leads to the transformation of FLS is not fully understood.

Previous studies have shown a myriad of alterations in the behavior of FLS in RA. One very striking change in FLS is the expression of oncogenes (11). Oncogenes that are up-regulated in RA are c-myc (12,13), ras (5), p53, and others. Inhibition of the Ras pathway reduced expression of MMP-1 and MMP-3. Inhibition of both the Ras and c-Myc pathways also reduced invasion into normal human cartilage in the SCID mouse coimplantation model (14). It has also been shown that down-regulation of p53 influences the proliferation and invasion of RA FLS (15,16). Transformation of FLS is different in different individuals, suggesting that a genetic component plays a role.

This study shows that the transformed behavior of FLS in patients with RA is a patient characteristic and is strongly associated with clinical joint destruction. We used FLS from passages 1 and 2 in this study. Although no macrophages were detected in these samples by light microscopy and Giemsa staining, and no CD14-positive cells were detected in a randomly chosen subset of the samples, we cannot exclude unambiguously that macrophages contaminated the cells. However, the experiments in which FLS from different passages and from different donors were compared yielded similar levels of invasiveness, which is evidence against major artifacts from macrophages. Further research should elucidate the exact mechanism of transformation of FLS and the role of a genetic component.

**REFERENCES**

Intracellular Free Radical Production in Synovial T Lymphocytes From Patients With Rheumatoid Arthritis

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Objective. To investigate the cellular and molecular sources of oxidative stress in patients with rheumatoid arthritis (RA) through analysis of the production of reactive oxygen species (ROS) in synovium.

Methods. Cytochemical procedures based on the 3,3′-diaminobenzidine (DAB)–Mn2+ deposition technique were used on unfixed cryostat sections of synovium from RA patients and rheumatic disease controls. For immunophenotyping, sections were incubated, fixed, and stained with fluorescein isothiocyanate–labeled antibodies. Fluorescence-activated cell sorter analysis of the ROS-reactive dye 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate-di(acetoxymethyl ester) was used to measure intracellular ROS in T lymphocytes from peripheral blood and synovial fluid. To determine which enzymes produced ROS, different inhibitors were tested.

Results. Large quantities of DAB precipitated in the majority of RA synovial T lymphocytes, indicative of intracellular ROS production. These ROS-producing T lymphocytes were observed throughout the synovium. Polymerization of DAB was observed to a lesser extent in other forms of chronic arthritis, but was absent in osteoarthritis. DAB staining of cytospin preparations of purified RA synovial fluid T cells confirmed the presence of ROS-producing cells. One of the ROS involved appeared to be H2O2, since catalase suppressed intracellular ROS production. Superoxide dismutase, which uses superoxide as a substrate to form H2O2, diphenyleneiodonium (an inhibitor of NADPH oxidase), N(G)-monomethyl-l-arginine (an inhibitor of nitric oxide synthesis), nordihydroguaiaretic acid (an inhibitor of lipoxygenase), and rotenone (an inhibitor of mitochondrial ROS production) failed to suppress ROS production.

Conclusion. Our findings show that chronic oxidative stress observed in synovial T lymphocytes is not secondary to exposure to environmental free radicals, but originates from intracellularly produced ROS. Additionally, our data suggest that one of the intracellularly generated ROS is H2O2, although the oxidase(s) involved in its generation remains to be determined.

Reactive oxygen species (ROS) play an important role in a variety of pathologic conditions, such as ischemia-reperfusion, carcinogenesis, acquired immunodeficiency syndrome, and aging. In rheumatoid arthritis (RA), oxidative stress has been described as an important mechanism that underlies destructive proliferative synovitis (1,2). In addition, oxidative stress was found to influence functional characteristics of synovial T lymphocytes, with critical implications for proximal and distal T cell receptor (TCR) signaling events (3–6). Chronic oxidative stress in synovial fluid (SF) T lymphocytes inhibits TCR-dependent phosphorylation of pivotal signaling molecules required for efficient T cell proliferation, thus contributing to severe hyporesponsiveness of these cells to antigenic stimulation. Oxidative stress in RA SF lymphocytes also plays a role in NF-κB-dependent gene transcription, resulting, for example, in the up-regulation of tumor necrosis factor α and interleukin-1 (7).

Several sources of ROS in the synovial joint that could lead to the disturbed redox homeostasis in SF T lymphocytes have been proposed. These include exo-
sure to free radicals liberated by activated phagocytic cells at the site of inflammation (8), ischemia-reperfusion–compromised oxygen radical tension in the inflamed joint (9), and generation of hydroxyl radicals by Fe$^{2+}$ released from dying cells (10). Recent evidence, however, suggests that T lymphocyte oxidative stress originates from intracellular enzyme activity controlled by the small GTPases Ras and Rap1 (11).

The specific detection of free radicals is hampered by several methodologic problems due to the high reactivity and short life of free radicals. Ongoing oxidative stress is therefore generally analyzed by measurement of secondary products, such as oxidized proteins, peroxidized lipids and their breakdown products, or oxidized DNA. These methods, however, give only limited information on the cellular source(s) of ROS production in situ. Therefore, we attempted to identify the source(s) of synovial oxidative stress using a cytochemical technique based on the principles described for the histochemical localization of ROS production in polymorphonuclear leukocytes (developed by Karnovsky 1993), using 3,3′-diaminobenzidine (DAB) and manganese ions (12). Free radicals directly react with DAB, forming an insoluble DAB polymer in a reaction catalyzed by the presence of Mn$^{2+}$. This technique has been successfully used to identify ROS-producing sites (13–16).

**PATIENTS AND METHODS**

**Reagents.** KCN, Na$_3$As, and CoCl$_2$ were obtained from Merck (Darmstadt, Germany). Polyvinyl alcohol (M, 70,000–100,000), menadione, catalase, diphenyleleniodionium (DPI), rotenone, and MnCl$_2$.4H$_2$O were purchased from Sigma (St. Louis, MO). DMSO was obtained from PGM Chemicals (New Germany, South Africa), DAB from Fluka (Buchs, Switzerland), Cu/Zn superoxide dismutase (SOD), nordihydroguaiaretic acid (NDGA) from Alexis Biochemicals (Breda, The Netherlands), and N$^\bullet$-methyl-L-arginine, acetate salt (L-NMA) from Molecular Probes (Leiden, The Netherlands).

**Patients and tissue processing.** Synovial tissue samples were obtained from patients during knee arthroscopy, which was performed under local anesthesia. These included 30 patients with established RA (mean ± SD disease duration 14 ± 11 years), 3 patients with early (joint symptoms ≤3 months), disease-modifying antirheumatic drug (DMARD)–naïve RA, and 25 control patients (7 with psoriatic arthritis, 8 with reactive arthritis, and 10 with osteoarthritis [OA]). Tissue fragments were snap frozen by immersion in methylbutane (−80°C) and stored in liquid nitrogen until used.

Peripheral blood (PB) and SF T cells from 5 RA patients (mean ± SD disease duration 6 ± 16 years) were purified from mononuclear cells using a negative isolation procedure (T Cell Negative Isolation kit; Dynal, Oslo, Norway), which resulted in a >90% CD3$^+$ cell population. Purified T cells were subsequently spun onto slides using a cytospin centrifuge (Shandon, Frankfurt, Germany) for histochemical ROS staining or for ROS detection using a FACSscan (Becton Dickinson, Bithoven, The Netherlands).

**Histochemical procedures.** Cryostat sections (8 μm thick) were freshly cut at a cabinet temperature of −25°C. The sections were placed on Star Frost adhesive slides (Optic Labor, Friedrichsdorf, Germany) and immediately used for staining. Before use, sections were air dried for 3 minutes at room temperature. The incubation medium contained 10% weight/volume polyvinyl alcohol (PVA), dissolved in 100 mM Tris maleate buffer (pH 8.0). Sodium azide (5 mM) was added to inhibit endogenous myeloperoxidase activity. The following components were added shortly before incubation of the cryostat sections: 0–12.5 mM DAB, 0–6.5 mM MnCl$_2$, and 0–100 mM CoCl$_2$ (1). All the compounds were added in strict order and thoroughly mixed from stock solutions into the PVA-containing medium. After incubation for 60 minutes at 37°C, sections were washed in distilled water to stop the reaction and then mounted in glycerol for light microscopy.

To further characterize DAB$^+$ cells, immunohistochemical staining was performed using a double-staining immunofluorescence procedure following incubation in DAB-Mn$^{2+}$ solution. The synovial cryostat sections were subsequently washed, air dried, and fixed with 4% paraformaldehyde. Sections were then incubated for 30 minutes at 4°C with unconjugated mouse anti-human monoclonal antibodies against CD3, CD15, CD19, and CD68 (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), or the appropriate isotype control antibodies. Fluorescein isothiocyanate (FITC)–labeled sheep anti-mouse secondary antibodies (PharMingen, Erembodegem, Belgium; 1:1,000 dilution) were used for visualization.

Tissue sections were scored independently by 2 observers (PHJR and TJMS) for the presence of DAB precipitate in T cells, immunohistochemical procedures were performed using a double-staining immunofluorescence procedure following incubation in DAB-Mn$^{2+}$ solution. The synovial cryostat sections were subsequently washed, air dried, and fixed with 4% paraformaldehyde. Sections were then incubated for 30 minutes at 4°C with unconjugated mouse anti-human monoclonal antibodies against CD3, CD15, CD19, and CD68 (Central Laboratory of The Netherlands Red Cross Blood Transfusion Service, Amsterdam, The Netherlands), or the appropriate isotype control antibodies. Fluorescein isothiocyanate (FITC)–labeled sheep anti-mouse secondary antibodies (PharMingen, Erembodegem, Belgium; 1:1,000 dilution) were used for visualization.

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**RESULTS**

**Production of free radicals in rheumatoid synovium.** DAB precipitates in the presence of free radicals to form an insoluble final reaction product that is visible as a brown DAB polymer. Manganese ions can be added to catalyze DAB polymerization, since Mn$^{2+}$ is oxidized to Mn$^{3+}$ by O$_2$·$, enabling Mn$^{3+}$ to oxidize DAB. In
synovial tissue from the 30 patients with established RA, a large number of DAB+ cells were detectable perivascularly, in leukocyte aggregates, and in the intimal and subintimal synovial lining (Figure 1). The presence of DAB in the incubation medium led to strong formation of the insoluble final reaction product (Figure 1A). Addition of Mn$^{2+}$ only marginally enhanced the amount of DAB precipitate, without affecting the localization pattern of the precipitate (Figure 1B). The highest amounts of final reaction product were found when the incubation medium contained 12.5 mM DAB and 2.5 mM MnCl$_2$. This composition was then used for all further experiments. Addition of cobalt ions to DAB- or DAB-Mn$^{2+}$–containing media leads to the formation of a blue final reaction product. (Original magnification × 200.)

Strong staining intensity did not allow recognition of the shape of the nuclei when counterstained with hematoxylin or Giemsa (results not shown) for the differentiation between polymorphonuclear cells and mononuclear cells. To further characterize the cellular sources of ROS, cryostat sections of synovium were incubated with monoclonal antibodies against CD3, CD15, CD19, and CD68. Within the synovial CD15$^+$ population (neutrophils), 36 ± 19% (mean ± SD) of the cells contained intracellular DAB final reaction product. Additionally, 67 ± 22% of the CD3$^+$ cells (T lymphocytes) also contained high levels of intracellular DAB precipitate (Figure 2). No DAB precipitate was found in B cells (CD19) or macrophages (CD68) (results not shown).

To eliminate possible effects of DMARDs and to investigate whether intracellular oxidation of DAB in T cells also occurs in early arthritis, we performed the DAB staining on sections from 3 patients with recent-onset, DMARD-naive RA. ROS staining of cryostat sections from these patients (Figure 3B) was indistin-

**Figure 1.** Staining of rheumatoid arthritis synovial tissue with 3,3′-diaminobenzidine (DAB). DAB precipitates in the presence of free radicals to form an insoluble final reaction product, which is visible as a brown DAB polymer. **A**, A large number of DAB+ cells are seen. **B**, Addition of Mn$^{2+}$ only marginally enhances the amount of DAB precipitate, without affecting the localization pattern of the precipitate. **C**, Addition of Co$^{2+}$ to DAB- or DAB-Mn$^{2+}$–containing media leads to the formation of a blue final reaction product. (Original magnification × 200.)

**Figure 2.** Combined 3,3′-diaminobenzidine (DAB) and CD3 immunofluorescence of rheumatoid arthritis synovial tissue. CD3$^+$ cells contained high levels of intracellular DAB precipitate. **Left,** Fluorescence microscopy image. **Center,** Corresponding light microscopy image. **Right,** Combined fluorescence and brightfield image. (Original magnification × 400.)

**Figure 3.** Staining of synovial tissue sections from **A,** osteoarthritis, **B,** early rheumatoid arthritis (RA), and **C,** established RA patients with 3,3′-diaminobenzidine (DAB). Tissue sections from patients with early RA and established RA both stained equally positive for DAB. (Original magnification × 200.)
Figure 4. Staining of osteoarthritis (OA) and psoriatic arthritis synovial tissue sections with 3,3’-diaminobenzidine (DAB). DAB precipitate was observed rarely and only in CD15+ neutrophils. Although a minimum of 50 CD3+ cells per patient were counted, no DAB+ T lymphocytes were found in OA patients. Top, OA tissue with a significant number of CD3+ lymphocytes (arrow). Middle, All DAB+ cells in OA tissue corresponded to CD15+ neutrophils. Bottom, CD3+, DAB+ lymphocytes in tissue from a psoriatic arthritis patient. Left, Fluorescence microscopy images. Center, Corresponding light microscopy images. Right, Combined fluorescence and brightfield images. (Original magnification × 400.)

Figure 5. Staining of cytospin preparations of purified peripheral blood (PB) T cells and synovial fluid (SF) T cells with 3,3’-diaminobenzidine (DAB). PB T cells (PBTC) and SF T cells (SFTC) were incubated for various periods, as indicated. As shown in the SF samples incubated for 15 minutes, DAB precipitation in SF T cells originates in the cytoplasm. After longer incubation periods (30–60 minutes), there is a time-dependent increase in intracellular DAB final reaction product. (Original magnifications are indicated at left.)
guishable from that of sections from patients with established RA (Figure 3C).

To examine the disease specificity of ROS production by synovial T lymphocytes, cryostat sections of RA and non-RA synovial tissue were compared for DAB precipitation products. No polymerization of DAB was observed in any of the T cells in OA tissue samples (n = 10) (Figure 4, top). A minimum of 50 CD3+ DAB– cells were detected in each of the OA tissue samples. Rare DAB+ cells were identified as CD15+ neutrophils (Figure 4, middle). In 1 of the 8 patients with reactive arthritis and 3 of the 7 patients with psoriatic arthritis, DAB precipitation was observed in 73 ± 3% (mean ± SD) and 48 ± 12% of the CD3+ cells, respectively (Figure 4, bottom). There was no correlation between DAB precipitation and disease activity, disease duration, rheumatoid factor positivity, disease pattern, or erosiveness.

**ROS production in RA SF T cells.** When DAB staining was performed on cytospin preparations of purified paired PB and SF T cells, DAB precipitate was not found in any of the PB T lymphocytes (Figure 5, top), but was easily detectable in T cells from SF (Figure 5, middle and bottom). The formation of ROS-dependent DAB polymerization in synovial T lymphocytes is likely due to intracellular enzymatic activity. First, DAB polymerization was not affected by preincubation of cytospin preparations or cryostat sections in aqueous medium at 37°C, but was completely inhibited by preincubation for 10 minutes at 100°C. Second, prefixation with 4% paraformaldehyde also abolished the formation of final reaction product, as did overnight air-drying at room temperature. Third, the amount of DAB polymerization increased with increasing incubation times, from 15 minutes to 60 minutes. On cytospin preparations, the initial DAB precipitate (15 minutes) in T lymphocytes was detected in the cytoplasm but not the nucleus of the SF T cells. With increasing incubation periods, DAB precipitate could be detected throughout the cell.

**Catalase-sensitive generation of H2O2 in synovial T lymphocytes.** Because it is difficult to quantitate DAB precipitation, we measured intracellular ROS production in purified SF and PB T cells from 5 RA patients, using FACs analysis of the ROS-reactive dye 6-carboxy-DCF. SF T cells displayed a higher basal rate of ROS production than did PB T cells (Figure 6A). To determine which oxidants and enzymes are responsible for the free radical production, inhibitors of the different enzyme systems proposed to regulate ROS production in T lymphocytes were tested (Figure 6B). The addition of exogenous catalase, which uses H2O2 as substrate, clearly diminished the DAB polymerization in SF T cells, reducing basal ROS production in SF T cells to near the levels observed in PB T cells. A majority of intracellular H2O2 originates from the dismutation of O2•− by SODs. However, when SOD was added to the incubation medium, no significant inhibition of ROS production was observed. Also, addition of DPI (an NADPH oxidase inhibitor), L-NMA (a nitric oxide synthetase inhibitor), or NDGA (a lipoxygenase inhibitor) did not result in significant inhibition of intracellular ROS production. Addition of rotenone (the mitochondrial complex I inhibitor) also did not inhibit increased ROS production in SF T cells.
DISCUSSION

T lymphocytes are considered to play a key role in the pathogenesis and perpetuation of RA. Synovial T lymphocytes display features of severe oxidative stress, which results in a number of proliferative and signaling abnormalities (2–5). While there is consensus about the presence of oxidative stress in synovial T cells, the origin of this oxidative stress is unknown.

Our present data demonstrate that chronic oxidative stress observed in synovial T cells mainly originates from free radicals generated by intracellular sources. This conclusion is at odds with suggestions that exposure of synovial T cells to environmental free radicals or proximity to ROS-producing neutrophils and/or macrophages is responsible for oxidative stress in synovial T cells. Moreover, perivascular T cells, which have recently entered the synovial milieu, as well as T cells distributed in the intima, the subintima, and in lymphocyte clusters all produced ROS. This suggests that acquisition of intracellular ROS production is a very early event following extravascularization of T lymphocytes into synovial tissue and, again, indicates that in synovial T cells, free radicals originate from intracellular sources rather than environmental free radicals. Although DAB+ cells were clearly identified as T lymphocytes, not all synovial T lymphocytes contained DAB precipitate, which suggests that only a specific subpopulation of synovial T cells is under oxidative stress. The presence of DAB precipitate in more than 90% of SF T cells on cytopsin preparations versus 68% on tissue sections could be due to a difference in CD3 subset composition between SF and synovial tissue, or it could be due to technical issues. Further analysis of specific T cell subpopulations within synovial fluid and tissue should provide insight into these possibilities.

The presence of ROS-producing T cells was not entirely specific for RA, since synovial tissue specimens from a proportion (5 of 15) of patients with other forms of chronic arthritis also contained DAB+ synovial T cells. No DAB precipitate was found in synovial tissue from OA patients.

During the last decade, reduction–oxidation (redox) reactions that generate ROS have been identified as important chemical mediators in the regulation of signal transduction processes (14). In particular, ROS appears to play a central role in the balance between cell growth, survival, and apoptosis. The specific cellular response is dependent on the species of oxidants produced, their subcellular source and localization, the kinetics of production, and the quantities produced. Therefore, the identification of intracellular free radicals in synovial T cells may not only provide an explanation for the altered behavior of synovial T cells, but also prove a pivotal hallmark in understanding the underlying pathophysiologic mechanisms in RA.

Jackson et al (17) recently identified 3 different ROS-producing events following T cell receptor activation: first, a rapid H$_2$O$_2$ production independent of Fas or NADPH oxidase; second, a sustained H$_2$O$_2$ production dependent on both Fas and NADPH oxidase; and third, a delayed superoxide production that was dependent on Fas ligand and Fas, yet independent of NADPH oxidase. Our results favor the first oxidase as the primary source in synovial T lymphocytes, since the intracellular ROS production was catalase-dependent and DPI-independent. Under physiologic conditions, however, intracellular ROS production is a transient phenomenon, occurring only up to 15 minutes after T cell stimulation. Therefore, the sustained intracellular ROS production in SF T cells might be a hallmark for chronic arthritis, which is not found in PB T cells isolated from RA patients or healthy controls. Identifying the oxidase responsible for the intracellular ROS in SF T cells, as well as the proteins that regulate the oxidase, could provide new therapeutic targets in RA.

There is a growing body of evidence demonstrating the critical role of ROS in the regulation of mitogenesis, differentiation, and apoptosis in physiologic and pathophysiologic conditions. Our present results indicate that the chronic oxidative stress observed in synovial T lymphocytes from RA patients originates from intracellularly generated free radicals, rather than environmental influences.

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Retroviral Gene Transfer of an Antisense Construct Against Membrane Type 1 Matrix Metalloproteinase Reduces the Invasiveness of Rheumatoid Arthritis Synovial Fibroblasts

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Objective. Membrane type 1 matrix metalloproteinase (MT1-MMP) is expressed prominently in rheumatoid arthritis synovial fibroblasts (RASFs), but the specific contribution of MT1-MMP to fibroblast-mediated destruction of articular cartilage is incompletely understood. This study used gene transfer of an antisense expression construct to assess the effects of MT1-MMP inhibition on the invasiveness of RASFs.

Methods. Retroviral gene transfer of a pLXIN vector-based antisense RNA expression construct (MT1-MMP/H9251S) to MT1-MMP was used to stably transduce RASFs. Levels of MT1-MMP RNA and protein were determined by quantitative polymerase chain reaction, Western blotting, and immunocytochemistry in MT1-MMP/H9251S-transduced RASFs as well as in control cells, with monitoring for 60 days. The effects of MT1-MMP inhibition on the invasiveness of RASFs were analyzed in the SCID mouse coimplantation model of RA.

Results. MT1-MMP/H9251S–transduced RASFs produced high levels of antisense RNA that exceeded endogenous levels of MT1-MMP messenger RNA by 15-fold and resulted in a down-regulation of MT1-MMP at the protein level. Inhibition of MT1-MMP production was maintained for 60 days and significantly reduced the invasiveness of RASFs in the SCID mouse model. Whereas prominent invasion into cartilage by nontransduced and mock-transduced RASFs was observed (mean invasion scores 3.0 and 3.1, respectively), MT1-MMP/H9251S–transduced cells showed only moderate invasiveness (mean invasion score 1.8; P < 0.05).

Conclusion. The data demonstrate that an antisense RNA expression construct against MT1-MMP can be generated and expressed in RASFs for at least 60 days. Inhibition of MT1-MMP significantly reduces the cartilage degradation by RASFs.

Membrane-type matrix metalloproteinases (MT-MMPs) are cell membrane–anchored MMPs that have been associated with both normal tissue remodeling and various diseases. Six different MT-MMPs have thus far been described, of which membrane type 1 MMP (MT1-MMP) has been studied most intensively. MT1-MMP (also called MMP-14) has a specific structural organization that is characterized by a hydrophobic transmembrane domain and a short cytoplasmic tail. It also contains a recognition site for furin-like proprotein convertases, which, by furin-dependent cleavage, activate MT1-MMP intracellularly (1). MT1-MMP digests interstitial collagens as well as other extracellular matrix components, including fibronectin, laminin, aggregan,
and gelatin (2). In addition, MT1-MMP has been identified as an important activator of other MMPs, such as MMP-2 and MMP-13 (3,4).

MT1-MMP has been shown to be indispensable for normal growth and development, in that MT1-MMP–deficient mice are known to exhibit a short life span and a variety of pathologic abnormalities in the connective tissue that ultimately lead to the development of arthritis, dwarfism, and premature death (5). Although these findings point to a role for MT1-MMP in normal development, its contribution to pathologic development in adults is also evident. High levels of MT1-MMP have been detected in a number of malignant cells, and the importance of MT1-MMP in tumor cell migration and invasion is well established.

Interestingly, high expression of MT1-MMP has also been found in nonmalignant diseases that are associated with progressive matrix degradation, such as aseptic prosthesis loosening (6) and rheumatoid arthritis (RA) (7). However, the degree to which MT1-MMP contributes to the progressive destruction of articular cartilage in RA is poorly understood. Herein we studied the effects of retroviral gene transfer of an antisense RNA expression construct against MT1-MMP (MT1-MMPαS) on the long-term production of MT1-MMP in RA synovial fibroblasts (RASFs), and investigated whether inhibition of MT1-MMP affects the invasiveness of RASFs into human articular cartilage in vivo.

**PATIENTS AND METHODS**

**Isolation and culture of RASFs.** Synovial tissues were obtained at joint replacement surgery (at the Schulthess Clinic, Zurich, Switzerland and Department of Orthopedic Surgery, University Hospital, Magdeburg, Germany) from 5 patients with active RA according to the revised criteria of the American College of Rheumatology (formerly, the American Rhematism Association) (8). RASFs were obtained by enzymatic digestion as previously described (9) and grown in Dulbecco’s modified Eagle’s medium (Biochrom, Berlin, Germany) with 10% fetal calf serum (Biochrom) in a humidified atmosphere containing 5% CO2 for 4–6 passages, before being used in the experiments.

**Generation of the MT1-MMP antisense construct and retroviral gene transfer.** A 502-bp MT1-MMP–specific complementary DNA (cDNA) fragment with known binding capability to MT1-MMP messenger RNA (mRNA) (7) was amplified from RASFs by polymerase chain reaction (PCR) and cloned into the Hpa I site of the retroviral pLXIN vector. Following transformation of competent Escherichia coli, individual clones were selected, and the retrieved plasmids were analyzed for identity and orientation of the inserts by automated sequencing. PT67 amphotropic packaging cells (Clontech, Heidelberg, Germany) were transfected with the retroviral vector carrying the MT1-MMP fragment in antisense orientation (MT1-MMPαS) by lipofection (SuperFect; Roche Diagnostics, Mannheim, Germany) and selected with G418. RASFs were transduced by incubation with the retroviral supernatants for 12 hours in the presence of 8 μg/ml polybrene. The procedure was repeated after an interval of 12 hours to increase retroviral infection. Successfully transduced RASFs were selected with G418 (800 μg/ml; Clontech) for at least 10 days. As controls, mock transduction with the empty pLXIN vector as well as the pLEIN retroviral vector expressing enhanced green fluorescent protein were used, along with nontransduced RASFs.

**Analysis of MT1-MMP production.** Levels of MT1-MMP RNA were analyzed by quantitative real-time PCR using a fluorogenic 5′-nuclease assay (TaqMan; Applied Biosystems, Weiterstadt, Germany) on an ABI Prism 7900 HT Sequence Detection system. For each experiment, total RNA was extracted from 10^5 cells using the RNeasy system (Qiagen, Hilden, Germany) and reverse transcribed using random hexamer primers. For quantitative PCR, the primers and FAM-TAMRA–labeled probes were as follows: MT1-MMP forward primer, 5′-TGG-AGG-AGA-CAC-CCA-CTT-TGA-3′, MT1-MMP reverse primer, 5′-GCC-ACC-AGG-AAG-ATG-TCA-TTT-C-3′, MT1-MMP TaqMan probe, 5′-FAM-5′-ACT-GAT-CCA-AGG-CTC-GGC-AGA-3′-TAMRA. Primers and probes were designed to detect both endogenous MT1-MMP mRNA and RNA derived from the MT1-MMPαS construct. MT1-MMP expression was determined relative to the 18S ribosomal RNA that was coamplified as an internal standard, with expression levels calculated with the ∆∆Ct method as previously described (10).

The expression of MT1-MMP protein was analyzed by Western blotting. Cells (5 × 10^5) were lysed with lysis buffer and protein concentrations were determined with a commercially available protein quantification kit (Uptima Interchim, Montluçon, France). Identical amounts of protein (50 μg) were loaded on a 10% sodium dodecyl sulfate–polyacrylamide gel and transferred onto a polyvinylidene difluoride membrane (Amersham Biosciences, Freiburg, Germany). The membrane was probed with rabbit anti-human MT1-MMP polyclonal antibodies (1:1,000; Dianova, Hamburg, Germany) for 1 hour at room temperature. Detection was carried out using horseradish peroxidase–conjugated donkey anti-rabbit (IgG) secondary antibodies and an enhanced chemiluminescence kit (Amersham Biosciences). Blots were stripped and reprobed with antibodies to β-actin (Abcam, Cambridge, UK) as loading control.

Long-term expression of MT1-MMP was monitored by indirect immunofluorescence (11). Briefly, RASFs were fixed in acetone/methanol (1:1) and incubated with a monoclonal antibody to MT1-MMP (clone 114-6G6; Oncogene Research Products, Cambridge, MA) at a dilution of 1:200 for 1 hour at room temperature. Cy3-conjugated sheep anti-mouse sera (Dianova) were used as secondary antibodies.

**SCID mouse coimplantation experiments and histologic evaluation.** Four-week-old SCID mice were provided by Charles River (Sulzfeld, Germany). Normal human articular cartilage was obtained from patients undergoing joint replacement surgery at the Department of Orthopedic Surgery, University Hospital, Tübingen, Germany. Implantation of RASFs together with normal human cartilage was performed as described previously (10). After 60 days, the implants were
removed at the time of death and the tissues were embedded into paraffin. Five animals were used per group (MT1-MMPαS, mock, and nontransduced), resulting in a total of 15 mice. Hematoxylin and eosin staining of the sections was carried out and histologic evaluation was performed using semiquantitative scores for invasion as previously described (10).

**Statistical analysis.** Results are expressed as the mean and SEM. Differences between groups were tested for statistical significance using the Mann-Whitney U test. Differences were considered statistically significant at a P value less than 0.05.

**RESULTS**

**Reduction of MT1-MMP by an MT1-MMP antisense RNA expression construct in RASFs.** The expression of MT1-MMP RNA was analyzed by quantitative real-time PCR using a combination of primers and probes that detected both endogenous MT1-MMP mRNA and RNA derived from the MT1-MMPαS construct. Stable transduction of RASFs with the MT1-MMPαS construct resulted in a 15-fold increase in MT1-MMP RNA as compared with that in both nontransduced and mock-transduced cells (Figure 1A), indicating a significant excess of MT1-MMP antisense RNA over endogenous MT1-MMP mRNA.

In the next step, Western blotting was performed to analyze the expression of MT1-MMP at the protein level. As shown in Figure 1B, both the 63-kd proMT1-MMP and the 60-kd active MT1-MMP forms (12) were found prominently in the nontransduced and mock-transduced RASFs. In contrast, RASFs that were stably transduced with MT1-MMPαS exhibited a markedly reduced expression of MT1-MMP. Immunocytochemistry demonstrated that the decreased expression of MT1-MMP was maintained over at least 60 days (Figure 1C). When compared with both nontransduced and mock-transduced RASFs, a markedly reduced staining for MT1-MMP was observed in MT1-MMPαS–transduced cells at 60 days after the establishment of stable cell cultures. The expression of other MMPs (MMP-2, MMP-3, and MMP-13) was not changed by MT1-MMPαS, as determined by quantitative PCR and enzyme-linked immunosorbent assay (results not shown).

**Decreased invasiveness of RASFs by gene transfer of MT1-MMPαS in the SCID mouse in vivo model of RA.** To investigate the effects of MT1-MMP inhibition on the invasiveness of RASFs, we used the SCID mouse coimplantation in vivo model that has been described in detail before. For this purpose, RASFs were coimplanted together with normal human articular cartilage into SCID mice for 60 days. It has been shown in a number of studies that RASFs deeply invade the coimplanted cartilage in the absence of human inflammatory
cells in this model, whereas normal, non-RA synovial fibroblasts do not exhibit such behavior.

Similar to the results in previous studies, non-transduced and mock-transduced RASFs showed deep cartilage invasion, with mean ± SEM invasion scores of 3.0 ± 0.03 and 3.1 ± 0.08, respectively (Figure 2). Stable retroviral expression of MT1-MMPαS significantly reduced the invasiveness of the RASFs (mean invasion score 1.8 ± 0.62;  P < 0.05). Although MT1-MMPαS failed to protect the cartilage completely, the highest invasion score in the MT1-MMPαS group was only 2.5, which was clearly below that seen in the mock-transduced and nontransduced groups (maximum invasion scores of 4.0 and 3.5, respectively).

**DISCUSSION**

MMPs are critically involved in both normal tissue remodeling and the degradation of extracellular matrix under disease conditions. Among the MMPs, the membrane-type MMPs are of special importance. They act on the surface of cells, which results in a close association of MT-MMP effects with cellular function. In addition, MT-MMPs contribute not only to the degradation of extracellular matrix, but also to the activation of other MMPs. MT1-MMP is the best-characterized member of the MT-MMP family and has been associated with the invasiveness of cancer cells (13,14).

To date, most studies using antisense strategies to inhibit MT1-MMP have been performed in conditions involving malignancies and have focused on MMP activation as well as cell trafficking. Monea and coworkers found that HT-1080 cells transfected with antisense cDNA failed to activate proMMP-2, which was accompanied by a considerable suppression of the 60-kd and 58-kd active forms of MT1-MMP in the antisense transfectants (15). On the basis of the hypothesis that furin is essential for proMT1-MMP activation, Sato and coworkers used antisense oligonucleotides against furin in human fibrosarcoma HT-1080 cells, human uterine cervical fibroblasts (HUCFs), and rabbit dermal fibroblasts (RDFs); they found that furin antisense constructs inhibited the concanavalin A–induced activation of proMMP-2 in HUCFs but not in RDFs, suggesting that there are different mechanisms of proMT1-MMP activation (16). Focusing on cell migration, Udayakumar et al demonstrated that MT1-MMP antisense oligonucleotides inhibited the migration of Du-145 prostate carcinoma cells, which was dependent on the cleavage of laminin-5 by MT1-MMP (17). These studies have clearly demonstrated a functional involvement of MT1-MMP in tumor progression and metastasis.

It has also been demonstrated that MT1-MMP is expressed at elevated levels in the RA synovial membrane and particularly at sites of joint destruction (7,18,19). In addition, data obtained by Honda and coworkers (20) have shown convincingly that proinflammatory cytokines such as interleukin-1β up-regulate the expression of MT1-MMP in RASFs, thus potentially contributing to MT1-MMP–mediated matrix degradation in RA. However, the specific contribution of MT1-MMP to direct, fibroblast-mediated destruction of articular extracellular matrix is incompletely understood (21).

In the present study we used gene transfer of an MT1-MMP antisense expression construct to study its effect on the invasiveness of RASFs into cartilaginous matrix. Retroviral gene transfer of MT1-MMPαS resulted in a sustained overexpression of MT1-MMP antisense RNA that down-regulated MT1-MMP production for at least 60 days. This was seen most clearly in Western blot analysis and immunocytochemistry, in
which MT1-MMP was found in nontransduced and mock-transduced RASFs but could be detected in only negligible amounts in MT1-MMPαS–transduced cells. Of interest, inhibition of MT1-MMP production in RASFs was accompanied by a significant reduction of their invasiveness in the SCID mouse model. Whereas prominent invasion into cartilage was observed with nontransduced and mock-transduced RASFs, cartilage destruction was reduced significantly in MT1-MMPαS–transduced cells.

It needs to be emphasized that our data do not allow us to distinguish between direct and indirect effects of MT1-MMP inhibition, i.e., between the inhibition of MT1-MMP–mediated matrix degradation per se and the reduced activation of other disease-relevant MMPs such as MMP-2 and MMP-13. However, our results do show clearly that MT1-MMP is involved functionally in the direct destruction of articular cartilage in RA. Although there is evidence that MT1-MMP is indispensable for normal embryonic development (5), it is difficult to predict whether inhibition of MT1-MMP will lead to unwanted effects in adults that may hamper the use of approaches that involve MT1-MMP inhibition. In this context, our data also demonstrate that viral gene transfer of antisense expression constructs may inhibit MMPs over extended periods of time, and thus constitutes a feasible tool for studying the individual contributions of MMPs (including MT-MMPs) to cartilage destruction in RA. Consequently, targeted inhibition of MT1-MMP production in RASFs may constitute a promising approach to inhibit joint destruction in RA.

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Effects of Doxycycline on Progression of Osteoarthritis

Results of a Randomized, Placebo-Controlled, Double-Blind Trial

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Objective. To confirm preclinical data suggesting that doxycycline can slow the progression of osteoarthritis (OA). The primary outcome measure was joint space narrowing (JSN) in the medial tibiofemoral compartment.

Methods. In this placebo-controlled trial, obese women (n = 431) ages 45–64 years with unilateral radiographic knee OA were randomly assigned to receive 30 months of treatment with 100 mg doxycycline or placebo twice a day. Tibiofemoral JSN was measured manually in fluoroscopically standardized radiographic examinations performed at baseline, 16 months, and 30 months. Severity of joint pain was recorded at 6-month intervals.

Results. Seventy-one percent of all randomized subjects completed the trial. Radiographs were obtained from 85% of all randomized subjects at 30 months. Adherence to the dosing regimen was 91.8% among subjects who completed the study per protocol. After 16 months of treatment, the mean ± SD loss of joint space width in the index knee in the doxycycline group was 40% less than that in the placebo group (0.15 ± 0.42 mm versus 0.24 ± 0.54 mm); after 30 months, it was 33% less (0.30 ± 0.60 mm versus 0.45 ± 0.70 mm). Doxycycline did not reduce the mean severity of joint pain, although pain scores in both treatment groups were low at baseline and remained low throughout the trial, suggesting the presence of a floor effect. However, the frequency of followup visits at which the subject reported a ≥20% increase in pain in the index knee, relative to the previous visit, was reduced among those receiving doxycycline. In contrast, doxycycline did not have an effect on either JSN or pain in the contralateral knee. In both treatment groups, subjects who reported a ≥20% increase in knee pain at the majority of their followup visits had more rapid JSN than those whose pain did not increase.

Conclusion. Doxycycline slowed the rate of JSN in knees with established OA. Its lack of effect on JSN in the contralateral knee suggests that pathogenetic mechanisms in that joint were different from those in the index knee.

In the present report, we describe the results of a randomized, placebo-controlled, double-blind trial of the tetracycline antibiotic, doxycycline, in subjects with knee osteoarthritis (OA). Selection of doxycycline as a potential disease-modifying OA drug was based not on the premise that OA is an infectious disease, but rather on results of in vitro studies showing 1) that doxycycline inhibited the degradation of type XI collagen, one of the minor collagens of articular cartilage, by 72-kd gelati-
nase (1); 2) that the presence of doxycycline during activation of procollagenase resulted in generation of low molecular weight, catalytically inactive fragments and marked reduction in the levels of active enzyme (2); and 3) that doxycycline inhibited messenger RNA for inducible nitric oxide synthase, an enzyme present in large quantities in OA cartilage, the activity of which results in secretion of matrix metalloproteinases (MMPs) by the chondrocyte (3, 4).

With this background, combined with evidence from Golub et al (5) that oral administration of tetracycline prevented periodontal disease in diabetic rats via inhibition of gingival collagenase, investigators in our group undertook in vivo studies of the effect of doxycycline in a canine cruciate-deficiency model of OA. These studies showed that this drug significantly reduced levels of active and total gelatinase and collagenase in extracts of the OA cartilage (6). Similar reductions in cartilage collagenase and gelatinase were obtained after administration of doxycycline to humans undergoing total joint arthroplasty (7). Furthermore, doxycycline administration markedly reduced the incidence and progression of joint pathology in the above-mentioned canine model of OA (6), which was consistent with evidence that it reduced the severity of spontaneous OA in the Hartley-Dunkin strain of guinea pig (8) and that treatment with a chemically modified tetracycline reduced the severity of joint pathology in a surgically induced model of OA in rabbits (9).

The primary outcome measure in this trial was the rate of joint space narrowing (JSN) in the medial tibiofemoral compartment. Based in part on epidemiologic evidence from the Chingford Health Study (10), a purportedly high-risk target population was selected (i.e., obese, middle-aged women with unilateral knee OA viewed on the conventional standing anteroposterior [AP] radiograph) that permitted co–primary comparisons of the treatment groups with respect to JSN in the index knee (which exhibited radiographic evidence of established OA at baseline) and in the contralateral knee (in which radiographic evidence of tibiofemoral OA in the standing AP view was absent at baseline). Secondary outcome measures included knee pain and function.

**PATIENTS AND METHODS**

The procedures, benefits, risks, and safeguards in this trial were approved by the Institutional Review Boards affiliated with the 6 participating Clinical Research Centers (Indiana University—Purdue University at Indianapolis, Northwestern University, University of Alabama at Birmingham, Arthritis Research Center Foundation, University of Arizona, and University of Pittsburgh). A data and safety monitoring board convened semiannually throughout the study.

**Subjects.** All subjects were obese women ages 45–64 years with unilateral radiographic knee OA according to the criteria of the American College of Rheumatology (11), with Kellgren/Lawrence (K/L) grade 2 or 3 changes in one knee (the index knee) and grade 0 or 1 changes in the contralateral knee (12) in a conventional standing AP view. All subjects were in the upper tertile of the age- and race-adjusted norms for body mass index (BMI) in women (13).

Exclusion criteria included posttraumatic or any other form of secondary knee OA, the presence of inflammatory arthritis, comorbidity that would confound measurement of knee pain and/or function, menopausal/contraceptive status that did not preclude pregnancy, and a history of tetracycline allergy. Subjects were also ineligible for enrollment if they received an intraarticular injection of hyaluronan within the previous 6 months or of corticosteroid within the previous 3 months.

**Run-in period.** Prior to randomization, all eligible subjects received a single-blind, 30-day supply of placebo capsules and underwent a 4-week, single-blind run-in test (14, 15) (Figure 1), which was designed to exclude from randomization subjects who were incapable of adhering (or unwilling to adhere) to a twice-daily dosing regimen and/or who could not/would not keep scheduled appointments. To “pass” the run-in test, subjects were required to keep 2 biweekly appointments at week –2 and week 0 and to maintain ≥80% adherence to a twice-daily dosing regimen over the entire 4-week period (as indicated by the dates on times at which each dose was taken) as recorded in the memory of an electronic dosing monitor (Medication Event Monitoring System; AARDEX, Union City, CA) contained in the cap of the medication vial (16, 17). The requirements for the run-in test (and the consequences of failing) were disclosed to the subject prior to screening, at the time informed consent was obtained.

**Double-blind phase.** Subjects who satisfied the requirements for the run-in test were randomly assigned to receive 100 mg doxycycline or matched placebo twice a day. Within each clinical center, subjects were allocated randomly to treatment groups in blocks of 6. Subjects were permitted to continue taking any prescription or over-the-counter pain medication for their knee OA throughout the trial, except during scheduled washout periods (see below). The double-blind phase entailed a baseline visit and 15 bimonthly followup visits, during each of which adherence to the treatment regimen (therapeutic coverage) and the occurrence of adverse events were recorded. Adherence was quantified cumulatively, with the subject receiving credit for 16 hours of coverage following each dose of study drug recorded by the electronic monitor. At the screening visit and every 6 months after randomization, blood and urine samples were obtained for the performance of laboratory tests for safety, which included measurement of serum levels of alkaline phosphatase, aspartate aminotransferase, alanine aminotransferase, bilirubin, and creatinine, as well as a complete blood cell count with differential and a urinalysis.

**Radiographic studies.** In addition to the conventional standing AP view that was used to determine eligibility, a fluoroscopically standardized semiflexed AP view (18), a supine lateral view of each knee, and skyline (Hughston) (19) views of the patellofemoral joints were obtained at baseline.
and 16 months and 30 months later. A conventional standing AP radiograph was also obtained at the final study visit.

The primary outcome measure was JSN in the medial tibiofemoral compartment, a surrogate for thinning of articular cartilage. The minimum joint space width (JSW) in the medial compartment was measured manually in the semiflexed AP view, according to the method of Lequesne (20), using the points of a screw-adjustable compass and a graduated magnifying lens. Measurements were made by an observer (SAM) who was blinded to the treatment group assignment of the subject. Measurements of JSW were adjusted for radiographic magnification, based on measurement of the image projected onto the film by a metallic ball (6.35 mm in diameter) that was affixed to the skin over the head of the fibula during the examination. The intra- and interreader reproducibilities of repeated measurements of JSW in a random sample of 30 radiographs (on which all identifying information was masked) were excellent (intraclass correlation coefficients of 0.99 and 0.96, respectively) (21).

Although the procedure for measurement of JSW was not blinded to the sequence of the radiographs (i.e., baseline, month 16, month 30), given that 1) each estimate of magnification-corrected JSW was a compound measurement of minimum interbone distance and the diameter of the magnification marker, 2) the Lequesne method (20) separates the recording of distances to be measured (which are obtained as pinpricks on a blank sheet of paper) from the actual measurement of those distances, and 3) the reader was blinded to the treatment group, it is highly unlikely that the absence of blinding to sequence permitted any bias regarding the comparison of active treatment and placebo groups with respect to JSN.

Clinical assessments. The clinical severity of knee OA was assessed every 6 months after a washout (5 half-lives) of all nonsteroidal antiinflammatory drugs (NSAIDs) and analogs. Subjects were permitted to take acetaminophen, as needed, up to 4 gm/day during all but the final 24 hours of the washout period. The clinical assessment included administration of the Likert version of the pain and physical functioning
scales of the Western Ontario and McMaster Universities Osteoarthritis Index (WOMAC) (22). Knee pain after a 50-foot walk was measured on a 10-cm visual analog scale (VAS) (0 = no pain; 10 = extreme pain). The subject and the investigator rated global disease activity on a 5-point Likert scale (1 = none; 2 = mild; 3 = moderate; 4 = severe; 5 = extreme). With the exception of the WOMAC physical functioning scale, all elements of the clinical assessment were recorded separately for the index and contralateral knees.

**Statistical analysis.** To assess the effectiveness of randomization, baseline demographic, clinical, and radiographic variables were compared between groups using $t$-tests for continuous measures, Wilcoxon rank sum tests for ordinal variables, and chi-square tests for nominal characteristics. The primary analysis for this study was the comparison of JSN in the doxycycline and placebo treatment groups. Group comparisons were performed using all subjects who underwent followup assessment, regardless of whether they had discontinued the study drug prematurely or completed the trial per protocol.

The null hypothesis of no effect was tested separately in the index and contralateral knees using a mixed-effects linear model (i.e., repeated-measures) approach with the critical value for statistical significance set at 0.05. The model included a random subject effect and fixed effects for treatment group, clinical center, visit (i.e., a class variable with two levels, 16 months or 30 months), the center–treatment group and visit–treatment group interactions, and baseline JSW. The model for each knee also included covariates representing demographic, clinical, or radiographic variables significantly related to JSN (e.g., pain at baseline). Comparisons between treatment groups at 16 months and 30 months were not adjusted for multiple tests because they were performed to describe the time course of the treatment effect rather than to test discrete hypotheses. To account for any possible effect of variation in timing of the 16- and 30-month visits, parallel mixed-effect linear model analyses were also performed with followup time as a continuous measure.

A mixed-effects linear model approach was also used to compare treatment groups with respect to secondary outcomes (i.e., mean scores for knee pain, function, global assessments), which were measured every 4–6 months throughout the double-blind phase. For the global assessments, which are ordinal measures, a rank transformation within visit (but across treatment) was implemented prior to fitting the mixed model.

Because a threshold level of knee pain was not among the inclusion criteria for this study, there was no assurance that pain scores would be sufficiently high to permit detection of a significant effect of doxycycline on mean pain scores. Therefore, a priori comparisons of treatment groups with respect to mean pain scores were supplemented with post hoc group comparisons of the frequency with which subjects reported clinically significant increases in knee pain across successive semianual pain assessments. For each subject, we tallied the number of pain assessments during which knee pain was increased ≥20% (with a minimum increase of 1 cm on the VAS) compared with that reported 6 months earlier. Student’s $t$-test was used to compare treatment groups with respect to the mean frequency (i.e., percentage of up to 5 followup pain assessments) with which ≥20% increases in knee pain were reported.

Changes in concomitant medications for OA pain, including the start and stop dates and daily doses of all prescription and over-the-counter NSAIDs and analgesic drugs (acetaminophen and opioid analogues), were documented at each visit. Treatment groups were compared over time with respect to the types of pain medications taken for OA pain (by chi-square test) and the strength of NSAID dose, expressed as the percentage of the minimum daily dose recommended for OA pain in the Physicians’ Desk Reference (23). Repeated-measures analysis of variance (ANOVA) was used to compare those who took NSAIDs in the 2 treatment groups with respect to the strength of dose.

The frequencies of adverse events in the 2 groups were compared using Fisher’s exact test. Even though relatively few subjects were lost to followup, we evaluated the possible effects of missing data on the results. First, to determine whether missing data could be considered to be missing completely at random, demographic and baseline clinical characteristics of subjects who underwent their 30-month radiographic examination were compared with those of subjects who were lost to followup at 30 months, using $t$-tests and chi-square tests. Second, JSN data for the index knee were further examined for evidence of nonignorable dropout, using an informative censoring modeling approach (24,25) that assumes that serial measurements of JSW follow a linear regression, with a random slope and an intercept for each subject. This method yields a test of the assumption of nonignorable dropout, in which a significant result indicates the missing data cannot be ignored. If the hypothesis is not rejected, the missing data can be considered to be missing at random and the mixed-model approach outlined above is appropriate.

**RESULTS**

Between May 1997 and May 2000, 1,975 volunteers underwent clinical and radiographic screening (Figure 1). Clinical eligibility and radiographic evidence of unilateral knee OA on the standing AP radiograph were confirmed in 489 of these volunteers (25%), 463 of whom agreed to take the run-in test. Of these, 431 subjects (93%) passed the run-in test and were randomly assigned to receive 100 mg doxycycline (n = 218) or placebo (n = 213) twice a day.

The 2 treatment groups were equivalent at baseline with respect to all demographic variables, BMI, K/L grade of radiographic severity, minimum JSW in the medial compartment, knee pain and function, and the types of drugs taken for OA pain (Table 1). Among the subjects who were randomized, 307 (71%) completed the 30-month trial per protocol (149 still taking doxycycline, 158 still taking placebo). Among the 124 who discontinued the study drug prematurely, 47 (38%) did so because of the burden of participation, 35 (28%) because of a possible adverse effect of doxycycline, 25 (20%) because of an adverse effect unrelated to doxycycline, and 17 (14%) because they moved away (Figure 1). Compared with subjects who completed the trial per
protocol, dropouts had significantly higher mean baseline scores for WOMAC pain (both knees) and physical functioning (P < 0.05 in each case).

Sixty of the 124 treatment dropouts (48%) returned for a knee radiograph at 30 months, resulting in an overall rate of loss to followup of only 14.8% and enabling an analysis of 30-month outcomes in 85% of all randomized subjects. Despite the difference noted above between dropouts and completers with respect to pain and function at baseline, informative censoring models for the index knee showed that the data could be treated as missing at random (P = 0.82). The data were therefore analyzed with mixed-effect linear models. This method uses all available data and yields unbiased results similar to those obtained with methods involving the imputation of missing data (24,25). The mean therapeutic coverage was very high in both treatment groups: 91.8% among subjects who completed the study per protocol and 81.5% among those for whom 30-month radiographic data were available (including the interval during which dropouts were not taking the study drug).

At the end of the study, radiographs of the index knees of 2 subjects in the placebo group indicated the presence of Paget’s disease of bone (in 1 subject) and osteonecrosis (in 1 subject) that was not apparent in the baseline examination. Data from these subjects were excluded from the efficacy analyses. Similarly, efficacy

### Table 1. Characteristics of subjects at baseline*

<table>
<thead>
<tr>
<th></th>
<th>Combined (n = 431)</th>
<th>Doxycycline (n = 218)</th>
<th>Placebo (n = 213)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>54.9 ± 5.6</td>
<td>54.8 ± 5.5</td>
<td>55.0 ± 5.8</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>36.7 ± 6.2</td>
<td>36.8 ± 6.3</td>
<td>36.5 ± 6.0</td>
</tr>
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<td>White, %</td>
<td>81</td>
<td>82</td>
<td>79</td>
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<tr>
<td>Overall severity of tibiofemoral OA, index knee, %†</td>
<td>59</td>
<td>62</td>
<td>56</td>
</tr>
<tr>
<td>K/L grade 2</td>
<td>41</td>
<td>38</td>
<td>44</td>
</tr>
<tr>
<td>Overall severity of tibiofemoral OA, contralateral knee, %†</td>
<td>68</td>
<td>68</td>
<td>68</td>
</tr>
<tr>
<td>K/L grade 0</td>
<td>32</td>
<td>32</td>
<td>32</td>
</tr>
<tr>
<td>Minimum medial JSW, mm‡</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Index knee</td>
<td>3.62 ± 1.17</td>
<td>3.64 ± 1.15</td>
<td>3.60 ± 1.19</td>
</tr>
<tr>
<td>Contralateral knee</td>
<td>3.94 ± 0.88</td>
<td>3.91 ± 0.90</td>
<td>3.96 ± 0.86</td>
</tr>
<tr>
<td>Index knee pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WOMAC pain score, 5–25</td>
<td>10.8 ± 4.2</td>
<td>10.8 ± 4.0</td>
<td>10.9 ± 4.3</td>
</tr>
<tr>
<td>50-foot walk pain, 0–10-cm VAS</td>
<td>2.1 ± 2.4</td>
<td>2.1 ± 2.4</td>
<td>2.0 ± 2.5</td>
</tr>
<tr>
<td>Contralateral knee pain</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WOMAC pain score, 5–25</td>
<td>9.0 ± 4.1</td>
<td>8.8 ± 4.0</td>
<td>9.2 ± 4.1</td>
</tr>
<tr>
<td>50-foot walk pain, 0–10-cm VAS</td>
<td>1.4 ± 2.2</td>
<td>1.4 ± 2.1</td>
<td>1.5 ± 2.3</td>
</tr>
<tr>
<td>WOMAC function score, 17–85</td>
<td>38.3 ± 13.0</td>
<td>38.5 ± 12.1</td>
<td>38.2 ± 13.8</td>
</tr>
<tr>
<td>Global disease activity, index knee, median (range)§</td>
<td>2 (1–5)</td>
<td>2 (1–5)</td>
<td>2 (1–5)</td>
</tr>
<tr>
<td>Patient’s rating</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physician’s rating</td>
<td>2 (1–5)</td>
<td>2 (1–4)</td>
<td>2 (1–5)</td>
</tr>
<tr>
<td>Drugs used for OA pain, subjects taking, %¶</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nonselective NSAID</td>
<td>60</td>
<td>63</td>
<td>56</td>
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<tr>
<td>Acetaminophen</td>
<td>32</td>
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<td>30</td>
</tr>
<tr>
<td>Opioid analgesic</td>
<td>7</td>
<td>8</td>
<td>6</td>
</tr>
<tr>
<td>COX-2 selective inhibitor</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>None</td>
<td>26</td>
<td>24</td>
<td>27</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean ± SD. BMI = body mass index; OA = osteoarthritis; K/L = Kellgren/Lawrence; JSW = joint space width; WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index; VAS = visual analog scale; NSAID = nonsteroidal antiinflammatory drug; COX-2 = cyclooxygenase 2.
† Graded in the standing anteroposterior (AP) view.
‡ Measured in the semiflexed AP view.
§ Five-point scale (1 = none; 2 = mild; 3 = moderate; 4 = severe; 5 = extreme).
¶ Percentages total >100% because some subjects took >1 pain medication.
data obtained after surgery from 4 subjects (2 in each group) who underwent total knee arthroplasty during the study were not included in the outcomes analyses, although they were included in the safety evaluation.

Quantitative measurement of JSN. Index knee. Preliminary analyses identified index knee pain at baseline as a significant covariate of JSN in that joint ($P < 0.001$ for WOMAC pain; $P < 0.05$ for 50-foot walk pain). Because these two pain scales were highly correlated, the former was used as a covariate in the initial repeated-measures model. The interaction terms (treatment group–clinical center, treatment group–visit) were not statistically significant in the mixed model. The 2 treatment groups differed significantly from each other with respect to overall rate of JSN ($P = 0.009$), visit ($P < 0.001$), clinical center ($P = 0.002$), baseline JSW ($P < 0.001$), and baseline pain ($P < 0.001$). Deviations in the timing of radiographic examinations around the 16-month and 30-month due dates were not significant; the results were unchanged when follow-up time was included as a continuous variable.

At both 16 months and 30 months, the mean rate of loss of JSW in the contralateral knee in the placebo group was comparable in magnitude and variability with that in the index knee (Table 2). When the subjects were divided into tertiles on the basis of baseline JSW in the contralateral knee, it was apparent that even among those in whom JSW at baseline was smallest (and whose contralateral knees therefore most closely resembled their index knees in this respect), doxycycline did not affect the rate of JSN (data not shown).

Secondary outcomes. From a clinical perspective, mean baseline scores for the secondary outcome measures indicated that the OA in these subjects was mild (Table 1), with little room for improvement in the double-blind phase. This is not surprising, since the subjects were recruited chiefly from the general population rather than from a clinic they were attending because of knee pain, and the inclusion criteria did not require that they exceed a threshold level of joint pain.

Three hundred eighty-three subjects underwent at least 1 followup clinical evaluation (WOMAC, VAS for 50-foot walk pain, and global assessments). For both knees, mean scores for WOMAC pain, 50-foot walk pain, and WOMAC function and median scores for the patient’s and physician’s global assessments were similar in the 2 treatment groups at baseline and throughout the double-blind phase. Nonetheless, the treatment groups differed in the frequency with which clinically important increases in pain were observed across successive semiannual assessments. The mean percentage of pain assessments in which subjects in the placebo group reported a ≥20% increase in knee pain (WOMAC pain...
score), compared with that reported 6 months earlier, was 27% greater than that in the doxycycline group (mean \( \pm /H11006 \) 30.2 \( /H11006 \) 21.1% versus 23.8 \( /H11006 \) 22.7%; \( /H11005 P = 0.004 \)). Comparable treatment group differences were found in 50-foot walk pain, for which a change of at least 1 cm on the 10-cm VAS was required in order to be considered clinically significant (24.3 \( /H11006 \) 23.2% in the placebo group versus 19.4 \( /H11006 \) 21.7% in the doxycycline group; \( /H11005 P = 0.032 \)). In contrast, treatment did not have a significant effect on the frequency of increases in WOMAC pain or 50-foot walk pain in the contralateral knee.

Relationship of JSN to frequency of increases in knee pain. In both knees, the percentage of semiannual assessments in which 50-foot walk pain was \( \geq 20\% \) more severe than that reported 6 months previously was directly related to the rate of JSN over 30 months (Figure 2). One-way ANOVA and post hoc analyses showed that the rate of JSN in both knees was significantly more rapid in patients reporting \( \geq 20\% \) increases in 50-foot walk pain during the majority (60–100%) of their followup visits than in patients who reported no increase in pain (\( /H11005 P = 0.05 \) for each knee). Similar patterns were seen for WOMAC pain scores for the contralateral knee (data not shown).

Effects of intraarticular injections and nutritional supplements. As noted above, subjects were permitted to follow the recommendations of their physicians regarding treatment of OA during the trial, including intraarticular injection of corticosteroids or hyaluronic acid (HA), both of which have been suggested to affect the rate of cartilage loss in knee OA (26–28). Subjects were also free to take nutritional supplements that have been associated with slower rates of progression of knee OA in epidemiologic studies (i.e., vitamin C, vitamin D) (29,30) or clinical trials (i.e., glucosamine) (31,32). Notably, neither injectable therapeutic agents nor nutritional supplements confounded the effects of doxycycline on JSN. Only 5 subjects (1 in the doxycycline group, 4 in the placebo group) received a corticosteroid injection during the trial; 1 of the 4 subjects in the placebo group also received a series of HA injections.

The 2 treatment groups were nearly identical with respect to the percentage of subjects who took vitamin C (54% in each group), vitamin D (49% in the placebo group and 54% in the doxycycline group), or glucosamine (24% in each group) at any time during the trial. Notably, JSN in the index knee of subjects who took glucosamine for any duration during the trial (with or without chondroitin sulfate) was more rapid than that in subjects who took no glucosamine (0.21 mm/year versus 0.13 mm/year; \( /H11005 P < 0.05 \) by Student’s \( t \)-test). The rate of JSN among subjects who took only glucosamine (0.24 mm/year) was similar to that in subjects who took glucosamine and chondroitin sulfate (0.20 mm/year). These observations must be viewed with caution, however, because exposure to glucosamine was uncontrolled, and glucosamine use may have been related to the severity of baseline knee pain, which was shown to be a significant baseline covariate for the rate of JSN in the index knee (see above).

Effects of analgesics/NSAIDs taken for OA pain. As shown in Table 1, 26% of subjects took no pain medication at baseline despite the presence of knee OA. Nonselective NSAIDs were the most commonly used pain medications (60% of all subjects). The small number of individuals taking cyclooxygenase 2 (COX-2) selective inhibitors at baseline (6 subjects [1%]) reflects the fact that most subjects were enrolled prior to the availability of COX-2 selective inhibitors at the end of 1999. By the end of the study, only 10 subjects (3% of completers) were not taking pain medication. Increases compared with baseline were observed in the frequency of use of all classes of analgesics (acetaminophen, opioid analgesics, NSAIDs)—most notably in the frequency of use of COX-2 selective inhibitors (23% of completers). The dosing of NSAIDs remained fairly constant over the course of the trial (on average, \( \sim 113–118\% \) of the recommended minimum daily dose for OA pain). However, the 2 treatment groups did not differ with respect to the types of drugs taken for OA pain, the frequency of
dosing, or the daily NSAID dose. Moreover, after controlling for baseline JSW, baseline pain, and treatment group, the frequency and dose of NSAIDs were unrelated to the rate of JSN in the index knee.

Adverse events. The frequencies of specific adverse events reported by ≥5% of subjects during the double-blind phase, regardless of severity, are shown in Table 3. Adverse events that occurred significantly more frequently (P < 0.05) in the doxycycline group than in the placebo group were restricted to recognized side effects of doxycycline (i.e., monilial vaginitis, sun sensitivity, nonspecific gastrointestinal [GI] symptoms). However, only a small proportion of subjects reporting doxycycline-related side effects discontinued the study medication prematurely (Table 3). Subjects in the active treatment group reported fewer urinary tract infections (P < 0.05), and there was a trend toward fewer upper respiratory tract infections in the doxycycline group than in the placebo group (P = 0.09).

Any adverse event resulting in death, hospitalization, prolongation of hospitalization, or development of a life-threatening or debilitating condition was categorized as a “serious adverse event.” Sixty subjects (31 in the doxycycline group and 29 in the placebo group) reported a total of 82 serious adverse events, none of which was attributable to doxycycline. No deaths occurred during the study.

DISCUSSION

Our decision to recruit for this trial obese women ages 45–64 years who had radiographic evidence of unilateral knee OA on the standing AP radiograph was based on data from a population-based study indicating that 47% of women with these characteristics developed radiographic evidence of incident OA in the contralateral knee 24 months later (10). It should be noted, however, that the incidence of OA in that study was based primarily on osteophytosis; JSN was factored into the grading of OA progression, but was scored semi-quantitatively with the use of a validated atlas and was not measured quantitatively, as in the present study.

Therefore, when this trial was implemented, we anticipated that we would be able to evaluate the effects of doxycycline on the progression of established OA in the index knee and on incident OA in the contralateral knee. However, during the course of the study, we found that although the contralateral knee is radiographically normal in a conventional standing AP view, in most cases it exhibits evidence of OA in a lateral, semiflexed

Table 3. Adverse events reported by ≥5% of randomized subjects*

<table>
<thead>
<tr>
<th>Adverse event</th>
<th>Subjects reporting adverse events, by treatment group</th>
<th>Led to discontinuation</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Combined (n = 431)</td>
<td>Doxycycline (n = 218)</td>
<td>Placebo (n = 213)</td>
</tr>
<tr>
<td>Nausea</td>
<td>76 (18)</td>
<td>56 (26)</td>
<td>20 (9)</td>
</tr>
<tr>
<td>Vomiting</td>
<td>44 (10)</td>
<td>26 (12)</td>
<td>18 (8)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>58 (13)</td>
<td>31 (14)</td>
<td>27 (13)</td>
</tr>
<tr>
<td>Dyspepsia</td>
<td>36 (8)</td>
<td>26 (12)</td>
<td>10 (5)</td>
</tr>
<tr>
<td>Heartburn/reflux</td>
<td>28 (6)</td>
<td>20 (9)</td>
<td>8 (4)</td>
</tr>
<tr>
<td>Monilial vaginitis</td>
<td>97 (23)</td>
<td>59 (27)</td>
<td>38 (18)</td>
</tr>
<tr>
<td>Sun sensitivity</td>
<td>33 (8)</td>
<td>31 (14)</td>
<td>2 (1)</td>
</tr>
<tr>
<td>Urinary tract infection</td>
<td>50 (12)</td>
<td>18 (8)</td>
<td>32 (15)</td>
</tr>
<tr>
<td>Upper respiratory tract infection</td>
<td>262 (61)</td>
<td>124 (57)</td>
<td>138 (65)</td>
</tr>
<tr>
<td>Elevated ALT/AST levels</td>
<td>58 (13)</td>
<td>31 (14)</td>
<td>27 (13)</td>
</tr>
<tr>
<td>Back pain</td>
<td>50 (12)</td>
<td>28 (13)</td>
<td>22 (10)</td>
</tr>
<tr>
<td>Elevated blood pressure</td>
<td>34 (8)</td>
<td>18 (8)</td>
<td>16 (8)</td>
</tr>
<tr>
<td>Dizziness</td>
<td>26 (6)</td>
<td>14 (6)</td>
<td>12 (6)</td>
</tr>
<tr>
<td>Fall</td>
<td>26 (6)</td>
<td>17 (8)</td>
<td>9 (4)</td>
</tr>
<tr>
<td>Chest pain</td>
<td>24 (6)</td>
<td>13 (6)</td>
<td>11 (5)</td>
</tr>
<tr>
<td>Mood disturbance</td>
<td>22 (5)</td>
<td>12 (6)</td>
<td>10 (5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Values are the number (%) of patients. Concurrent nonspecific gastrointestinal side effects (e.g., nausea and vomiting) were often cited as the reason for discontinuation of study medications. ALT = alanine aminotransferase; AST = aspartate aminotransferase.
AP and/or patellofemoral view (33). Hence, this study does not assess the effect of doxycycline on incident OA in the contralateral knee but rather on the progression of relatively mild OA in that joint, which was not apparent in the baseline standing AP view.

While the primary outcome variable in this trial was JSN in the medial tibiofemoral compartment, it should be noted that the eligibility criteria did not preclude enrollment of subjects whose index knee showed JSN in the lateral compartment at baseline. In fact, 10% of index knees exhibited isolated lateral JSN in the baseline semiflexed AP view. Lateral JSN in the index knee progressed in 7% of subjects in the doxycycline group (and in 4% of subjects in the placebo group) and did not confound our ability to detect a significant effect of doxycycline on medial JSN in the index knee. In addition, 5% of subjects in the doxycycline group and 4% of subjects in the placebo group exhibited progression of lateral JSN in the contralateral knee, in which there was no indication at baseline of involvement of the lateral compartment. Therefore, the failure of doxycycline to slow the mean rate of medial JSN in the contralateral knee was not due to lateral compartment narrowing with concomitant widening of medial compartment JSW.

After oral administration of a single 100-mg dose of doxycycline, the serum half-life of the drug in normal subjects is ~16 hours (34). Selection of the dose used in the present study, 100 mg twice a day, was based on findings in humans with OA undergoing joint replacement surgery: when administered for 5 days prior to surgery, doxycycline at this dose significantly reduced the levels of total and active collagenase and gelatinase in extracts of the OA cartilage, whereas this effect was not achieved with other dosing regimens (7). In vitro, 10 μM and 30 μM doxycycline inhibited cartilage gelatinase activity by 44% and 82%, respectively (1). Degradation of embryonic avian tibias was inhibited by exposure in vitro to a similarly low concentration of the drug, following which collagenase and 62-kd gelatinase were no longer detectable in the spent culture medium (35).

The feasibility of the protocol employed in this trial is evidenced by the fact that 30-month radiographs were obtained for 85% of all subjects randomly assigned to treatment and by the high mean rate of adherence to the dosing regimen (~90% among completers and >80% among all subjects for whom 30-month radiographic data were available). Furthermore, 71% of all patients randomly assigned to treatment were still taking doxycycline or placebo 30 months later. The approaches we used to optimize adherence to the dosing regimen and minimize the dropout rate are described in detail elsewhere (36).

It was an advantage for this trial that doxycycline is generally well tolerated and has a long track record of relative safety and well-recognized adverse effects (e.g., sun sensitivity, monilial vaginitis, nonspecific GI symptoms). Only these 3 types of adverse effects occurred significantly more frequently in the active treatment group than in the controls, and no serious adverse events were attributed to doxycycline. On the one hand, the fact that subjects were permitted to take prescription and over-the-counter NSAIDs/analgesics throughout the trial (except during washout periods preceding clinical evaluations) presumably also contributed to the low dropout rate and high level of adherence to the dosing regimen. On the other hand, continued use of NSAIDs/analgesics undoubtedly contributed to some of the nonspecific GI symptoms and to the dropout rate in both treatment groups. Although the emergence of features of the MMP-inhibitor syndrome (e.g., shoulder periarthritis, Dupuytren’s contracture) (37) was not actively sought, these were not detected by the adverse event reporting mechanism employed.

In both knees and both treatment groups, the mean rate of JSN at 30 months was about twice as great as that at 16 months (Table 2). In the index knee, doxycycline reduced the mean rate of JSN at 30 months by 33%; notably, a clear difference between doxycycline and placebo was already apparent in the 16-month data (40% less rapid JSN in the doxycycline group than in the placebo group; \( P = 0.027 \)). However, the drug had no effect on progression of the K/L grade in either knee.

At 16 months, the rate of JSN in the contralateral knee was identical in the 2 treatment groups; at 30 months, the rate was numerically greater in the doxycycline group than in the placebo group, but the difference was not statistically significant (Table 2). Although the possibility cannot be excluded that the difference might have reached significance if the sample size had been larger, the lack of evidence that doxycycline slowed the rate of JSN in the contralateral knee may have been due to the fact that some matrix degradation pathways are more important in the later stages of OA than in the earlier stages. Doxycycline may have interfered with processes driving cartilage breakdown in the index knee that were not (yet) operative in the contralateral knee.

In support of this possibility, synthesis of type II collagen and turnover of aggrecan were found to be increased in overt focal lesions in femoral articular cartilage of older humans, thus resembling changes in
established OA, but no up-regulation of synthesis accompanied earlier lesions (38). Messenger RNA for type II and type III collagen, biglycan, and MMPs 2, 11, and 13 was detected only in cartilage from patients with advanced OA, while the gene for MMP-3 was up-regulated only in samples of cartilage with structural evidence of early OA, but was undetectable in cartilage with more advanced disease (39).

It should be noted, however, that doxycycline had a protective effect in animal models of OA when given prophylactically (6,8), suggesting that in these models it is active in the very early stages of OA. The difference between the effects of doxycycline on early OA in animals and in humans may be due to differences among species in the relative contributions of various mediators or enzymes to articular cartilage pathology (40).

Doxycycline treatment did not result in a change in clinical outcomes that fulfilled recently proposed responder criteria for OA (41). Those criteria, however, were evaluated in OA patients in whom the baseline level of symptoms was relatively high, and they have not been adequately tested in patients with less severe symptoms, as in the present study. Even though doxycycline did not alter mean scores for joint pain, it significantly decreased the frequency with which subjects reported ≥20% increases in knee pain in successive semiannual visits. Our requirement that the increase in pain on a 10-cm VAS be ≥1 cm in magnitude corresponds closely to the definition of a minimum clinically important difference in patients with OA (42). Notably, the effect of doxycycline on symptoms was not as apparent in the contralateral knee, in which the drug had no effect on JSN and no clinical benefit.

In both knees, the rate of JSN was more than twice as rapid in subjects who reported frequent increases in knee pain as in those with a stable pain score, appearing to validate the clinical importance of retardation of articular cartilage loss. Joint pain may possibly serve as an indicator of synovitis that leads to cartilage destruction. Clinically unrecognized synovitis has been detected arthroscopically in up to one-third of patients with radiographic OA. Sites of synovial inflammation may abut cartilage lesions (43), suggesting that synovitis may cause localized chondropathy. On the other hand, destruction of joint cartilage, perhaps driven by the abnormal mechanical environment of the OA joint, may be the cause of synovitis.

Finally, some limitations of this study should be noted. The sample size was relatively small and the duration of followup was relatively brief, considering that changes in joint pain, function, and structure in patients with knee OA typically occur over many years. Furthermore, our patients were highly selected with respect to sex, BMI, age, and discordance in the severity of OA pathology in their two knees, limiting generalizability of the study results. It cannot be assumed that the protective effect of doxycycline noted in the index knee would occur at other joint sites (e.g., hips or hands), in subjects of all age groups, or in men. Finally, given the higher rate of adverse reactions in the doxycycline group than in the placebo group, the subjects may not have been completely blinded to their treatment assignments. However, although it is possible that unblinding, if it did occur, may have had some influence on symptomatic outcomes, it is unlikely to have affected the progression of structural damage in the OA joint.

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Weight Loss Reduces Knee-Joint Loads in Overweight and Obese Older Adults With Knee Osteoarthritis

Stephen P. Messier,1 David J. Gutekunst,1 Cralen Davis,1 and Paul DeVita2

Objective. To determine the relationship between change in body mass and knee-joint moments and forces during walking in overweight and obese older adults with knee osteoarthritis (OA) following an 18-month clinical trial of diet and exercise.

Methods. Data were obtained from 142 sedentary, overweight, and obese older adults with self-reported disability and radiographic evidence of knee OA who underwent 3-dimensional gait analysis. Gait kinetic outcome variables included peak knee-joint forces and peak internal knee-joint moments. Mixed regression models were created to predict followup kinetic values, using followup body mass as the primary explanatory variable. Baseline body mass was used as a covariate, and thus followup body mass was a surrogate measure for change in body mass (i.e., weight loss).

Results. There was a significant direct association between followup body mass and peak followup values of compressive force ($P = 0.001$), resultant force ($P = 0.002$), abduction moment ($P = 0.03$), and medial rotation moment ($P = 0.02$). A weight reduction of 9.8N (1 kg) was associated with reductions of 40.6N and 38.7N in compressive and resultant forces, respectively. Thus, each weight-loss unit was associated with an ~4-unit reduction in knee-joint forces. In addition, a reduction in body weight of 9.8N (1 kg) was associated with a 1.4% reduction (0.496 Nm) in knee abduction moment.

Conclusion. Our results indicate that each pound of weight lost will result in a 4-fold reduction in the load exerted on the knee per step during daily activities. Accumulated over thousands of steps per day, a reduction of this magnitude would appear to be clinically meaningful.

The precise etiology of osteoarthritis (OA) is unknown; however, several risk factors have been identified, including age (1,2), female sex (3), and both occupational (4,5) and sports-related joint stress (6–9). The most important modifiable risk factor for the development and progression of OA is obesity (1,10–17). Weight loss reduces the risk of symptomatic knee OA (13), and for obese patients with knee OA, weight loss and exercise are recommended by both the American College of Rheumatology and the European League Against Rheumatism (18,19). We have shown that an average weight loss of 5% over 18 months in overweight and obese adults with knee OA results in an 18% improvement in function. When dietary changes are combined with exercise, function improves 24% and is accompanied by a significant improvement in mobility (20).

Although obesity is strongly associated with knee OA, some obese but otherwise healthy adults adapt to their excessive weight and subsequently reduce knee-joint torques and possibly knee-joint forces during walking (21). In other obese adults, however, excessive biomechanical joint stress represents one possible pathway for the pathogenesis and progression of knee OA. We hypothesized a significant and direct relationship between weight loss and attenuation of knee-joint forces and moments during walking in overweight and obese older adults with knee OA after an 18-month clinical trial of diet and exercise. Since obesity is a known risk factor for the development and progression of knee OA, determining the relationship between weight loss and knee-joint loads will clarify the pathophysiologic role of obesity in knee OA.

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**PATIENTS AND METHODS**

**Design.** The Arthritis, Diet, and Activity Promotion Trial (ADAPT) was a single-blind, randomized, controlled clinical trial of overweight and obese, sedentary older adults with symptomatic knee OA (20,22). The study was designed to compare the effects of assignment to 1 of 4 distinct 18-month interventions, as follows: 1) exercise only, 2) dietary weight loss only (diet), 3) dietary weight loss plus exercise (diet plus exercise), and 4) healthy lifestyle (control). ADAPT was conducted at the Claude D. Pepper Older Americans Independence Center of Wake Forest University, with approval provided by the university’s institutional review board.

**Participants.** Sedentary, overweight and obese older adults with radiographic evidence of knee OA were recruited in 6 waves over an 18-month period. Detailed inclusion and exclusion criteria are described elsewhere (20,22). A total of 316 individuals were randomized to 1 of the 4 treatment groups. A subset of this population (n = 142), equally represented in the 4 intervention groups, was randomized to undergo biomechanics testing. The descriptive characteristics of the biomechanics subset were similar to those of the entire ADAPT cohort (Table 1).

**Interventions.** For the purpose of this study, the data from the 4 groups were combined to determine the relationship between weight loss and knee-joint kinetics, independent of group assignment. Prior to intervention, the experimental procedures were explained to each participant and an informed consent document was signed.

**Gait analysis.** Prior to testing at baseline and at the 6-month and 18-month followup visits, participants’ freely chosen walking speeds were assessed using a Lafayette Model 63501 photoelectric control system (Lafayette, IN) interfaced with a digital timer. The photocells were positioned 7.3 meters apart on an elevated walkway. Participants traversed the 7.3-meter course 6 times. Freely chosen walking speed was expressed as the mean value of the 6 trials. This speed (±3.5%) was used in all subsequent gait evaluations for the particular test period.

To control for the effects of footwear, each participant wore an identical make and model of athletic shoe during testing. Three-dimensional (3-D) high-speed (60-Hz) videography was performed using a 4-camera motion analysis system (Motion Analysis Corporation, Santa Rosa, CA) and a set of 25 passive reflective markers arranged in the Cleveland Clinic configuration. Raw coordinate data from the 3-D system were smoothed using a Butterworth low-pass digital filter with a cutoff frequency of 6 Hz. Temporal and lower extremity kinematic and kinetic variables were computed using Orthotrac software and an AMTI model SGA6-4 force platform (AMTI, Watertown, MA) which was set to sample data at 1,000 Hz. Kinematic and kinetic data were synchronized, which allowed calculation of knee-joint forces using an inverse dynamics model (23). The primary outcomes were the peak values of 9 knee-joint kinetic variables, comprising compressive, anteroposterior shear, and resultant forces, and peak values of 6 internal knee moments, comprising flexion, extension, abduction, adduction, medial rotation, and lateral rotation. These joint moments represent the internal moments produced by the muscles and other tissues crossing the joints. Figures 1 and 2 show the average baselines curves, and these results, along with the kinematic description of the participants’ lower extremity and related anatomic and physiologic characteristics, were used to

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**Table 1.** Comparison of the descriptive characteristics of the ADAPT biomechanics subset with the entire ADAPT cohort*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Biomechanics subset (n = 142)</th>
<th>ADAPT cohort (n = 316)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Value</td>
<td>Range</td>
</tr>
<tr>
<td>Age, years</td>
<td>68.5 ± 0.52</td>
<td>60–89</td>
</tr>
<tr>
<td>Height, cm</td>
<td>165.5 ± 0.75</td>
<td>144.8–188.0</td>
</tr>
<tr>
<td>Baseline body mass, kg</td>
<td>93.2 ± 1.31</td>
<td>66.6–148.2</td>
</tr>
<tr>
<td>Baseline BMI, kg/m²</td>
<td>34.0 ± 0.42</td>
<td>27.0–49.8</td>
</tr>
<tr>
<td>Baseline WOMAC function score (scale 0–68)</td>
<td>25.1 ± 0.99</td>
<td>2–68</td>
</tr>
<tr>
<td>Baseline WOMAC pain score (scale 0–20)</td>
<td>7.2 ± 0.30</td>
<td>0–20</td>
</tr>
<tr>
<td>Sex, no. (%)</td>
<td>105 (73.9)</td>
<td>230 (72.8)</td>
</tr>
<tr>
<td>Female</td>
<td>105 (73.9)</td>
<td>230 (72.8)</td>
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<tr>
<td>Race, no. (%)</td>
<td>107 (75.4)</td>
<td>238 (75.4)</td>
</tr>
<tr>
<td>White</td>
<td>107 (75.4)</td>
<td>238 (75.4)</td>
</tr>
<tr>
<td>African American</td>
<td>34 (23.9)</td>
<td>71 (22.4)</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean ± SEM. ADAPT = Arthritis, Diet, and Activity Promotion Trial; BMI = body mass index; WOMAC = Western Ontario and McMaster Universities Osteoarthritis Index.
calculate muscle, lateral collateral ligament, and joint forces of the knee during the stance phase of walking. We have previously applied this model to healthy individuals and OA patients (23), which resulted in values and ranges for muscle and joint forces (2.8–6.0 body weight) similar to those reported in previous studies (25,26). A more detailed description of the model can be found elsewhere (23,24).

**Statistical analysis.** For each of the 9 kinetic outcome variables, a mixed model with random effect for subject was created using SAS software (version 8.02; SAS Institute, Cary, NC) to explain the change in the kinetic variable from baseline to followup. Mixed models were chosen so that all available followup information (at 6 months and 18 months) could be included. Each mixed model produced a regression equation to predict followup values of the kinetic outcome variable, with followup body mass serving as the primary explanatory variable. Since baseline body mass was also included in each mixed model, followup body mass represents the change in body mass, which is directly related to weight loss. The use of followup values rather than change values in our statistical analysis permits us to observe an estimate for change while allowing our model to account for variability in both baseline and followup measures separately.

Age, sex, race, group assignment, baseline values of the

**Figure 1.** Baseline mean knee-joint forces in 134 subjects with knee osteoarthritis. The biomechanical analyses measured A, anteroposterior force, B, compressive force, and C, resultant force.

**Figure 2.** Baseline mean knee-joint moments in 134 subjects with knee osteoarthritis. Gait mechanics involved A, flexion/extension moments, B, abduction/adduction moments, and C, medial/lateral rotation moments.
kinetic outcome variable, walking speed, and scores on the pain and function subscales of the Western Ontario and McMaster Universities Osteoarthritis Index (27) were included in the mixed model as potential covariates. Height was included only in models of knee-joint moments to account for differences in subjects’ limb lengths. In addition, each model included an adjustment factor for followup visit (6 months or 18 months).

The mixed model for each kinetic outcome variable produced a best-fit regression equation. This equation used the change in body mass and all potential covariates to model a followup value for the kinetic outcome variable. The regression equation can be viewed as a prediction equation in which a given predictor variable is judged statistically significant when its corresponding beta coefficient is significantly different from 0. Significance was determined by computing a T score (beta coefficient divided by standard error) and using the Student’s 2-tailed t-test, with the significance level set at a P value of less than or equal to 0.05.

**RESULTS**

**Retention.** Of the 142 participants randomized to undergo biomechanics testing, complete data on biomechanics were obtained at baseline from 134 subjects (94%). A total of 116 of the 142 participants (82%) had complete data from at least 1 biomechanics followup visit (at 6 and/or 18 months). Of the 116 participants who completed at least 1 followup visit, 94 completed the 6-month followup, 84 completed the 18-month followup, and 62 completed both visits. There were no significant visit effects (6 or 18 months) for any of the outcome measures; therefore, the average intervention effects over the followup period were estimated and used in all subsequent analyses. The proportion of subjects who completed followup biomechanics testing (116 of 142; 82%) was similar to the completion rate for the primary outcome in the entire ADAPT cohort (252 of 316; 80%).

**Body mass and body mass index (BMI).** The mean ± SEM body mass and BMI of the cohort at baseline were 93.2 ± 1.3 kg and 34.0 ± 0.4 kg/m², respectively, and the mean ± SEM values at followup were 90.8 ± 1.4 kg and 33.0 ± 0.4 kg/m², respectively. Thus, participants lost 2.6% of their body mass and lowered their BMI by 3.0%.

**Gait mechanics.** The unadjusted peak values for gait mechanics at baseline and followup are presented in Table 2. The baseline values, along with the covariates, were used as input data for our statistical model.

Our primary hypothesis was that change in body mass from baseline to followup (i.e., weight loss) was directly related to changes in knee-joint forces and moments. Baseline and followup body masses were included in the regression models that were used to predict followup values of kinetic outcomes. The beta coefficient for followup body mass ($\beta_{\text{followup mass}}$) in the regression models takes into account the baseline value for body mass; it is therefore a surrogate measure for the change in body mass, or weight loss. The $P$ value for the $\beta_{\text{followup mass}}$ coefficient is the criterion for judging the accuracy of the primary hypothesis. The sign accompanying each beta coefficient indicates whether the relationship between a kinetic outcome variable and followup body mass is positive (direct) or negative (indirect) after adjusting for covariates. The beta coefficients, T scores, and $P$ values for followup body mass in relation to all 9 kinetic outcome variables are shown in Table 3.

**Knee forces.** After adjusting for baseline body mass, baseline knee-joint force, and the 9 covariates, significant associations were found between followup body mass and followup values for peak compressive ($P = 0.001$) and peak resultant ($P = 0.002$) knee force. No association was found between followup body mass and followup anteroposterior shear force ($P = 0.91$). The positive beta coefficient and significant $P$ values for the association between followup body mass and followup compressive and resultant forces indicate that a decrease in body mass was associated with a significant reduction in compressive and resultant knee forces. Specifically, a 9.8N reduction in body weight, equivalent to a 1 kg reduction in body mass, was associated with a 40.6N reduction in compressive force and a 38.7N reduction in resultant force. Thus, each unit of weight loss was associated with an ~4-unit reduction in knee forces (e.g., 40.6N divided by 9.8N = 4.1, and 38.7N divided by 9.8N = 3.9). In clinical terms, each pound lost

| Table 2. Unadjusted peak knee forces and moments during gait at baseline and followup* |
|---------------------------------|-----------------|-----------------|
|                                 | Baseline (n = 134) | Followup (n = 116) |
| **Forces, Newtons**             |                 |                 |
| AP shear force                  | 475.9 ± 16.2    | 499.2 ± 19.0    |
| Compressive force               | 2,892.2 ± 73.0  | 2,968.2 ± 73.0  |
| Resultant force                 | 2,926.2 ± 73.5  | 3,007.0 ± 73.7  |
| **Moments, Nm**                 |                 |                 |
| Flexion moment                  | 29.55 ± 0.92    | 26.06 ± 0.78    |
| Extension moment                | 29.34 ± 1.54    | 29.05 ± 1.61    |
| Medial rotation moment          | 9.61 ± 0.57     | 9.05 ± 0.43     |
| Lateral rotation moment         | 18.45 ± 0.70    | 17.65 ± 0.71    |
| Abduction moment                | 33.52 ± 1.31    | 32.78 ± 1.36    |
| Adduction moment                | 10.35 ± 0.63    | 10.55 ± 0.67    |
| **Resultant force**             |                 |                 |
| AP shear force                  | 73.5 ± 3,007.0  | 73.0 ± 3,007.0  |
| Compressive force               | 73.7 ± 3,007.0  | 73.0 ± 3,007.0  |
| Resultant force                 | 73.7 ± 3,007.0  | 73.0 ± 3,007.0  |

*Values are the mean ± SEM. Mean body weights at baseline and followup were 913.9 Newtons and 890.4 Newtons, respectively. AP = anteroposterior.
would result in a 4-pound reduction in a knee-joint load that peaks at ~650 pounds for each step.

Knee moments. The significant \( P = 0.03 \) positive beta coefficient for knee abduction moment indicates that a reduction in body mass was associated with a decreased peak knee abduction moment. The beta coefficient for followup mass indicates that for every 1 kg reduction in body mass from baseline to followup, the followup peak abduction moment was reduced 0.496 Nm (Table 3). With an average peak baseline abduction moment of 33.52 Nm, a 9.8N (1 kg) reduction in body weight would result in a 1.4% reduction in knee abduction moment.

Likewise, the significant \( P = 0.03 \) positive beta coefficient for knee medial rotation moment indicates that for every 1 kg reduction in followup mass, the followup medial rotation moment was reduced 0.31 Nm. The average baseline value for peak medial rotation moment was 18.45 Nm; therefore, the ratio of reduction in medial rotation moment for a 9.8N (1 kg) reduction in body weight would result in a 1.6% reduction in knee medial rotation moment.

DISCUSSION

Given the strong evidence linking obesity and knee OA (1,11–16), our approach involving measurement of the association between change in body mass (i.e., weight loss) and changes in knee-joint forces and knee-joint moments clarifies the pathophysiologic process. By adjusting statistically for age, sex, and baseline covariates including walking velocity, this analysis isolated the association between weight loss and changes in knee-joint kinetics. To our knowledge, this is the first study to link these variables in overweight and obese adults with knee OA.

The significant relationship between weight loss and reduction in compressive knee-joint loads indicates that the force reduction was larger than the actual weight reduction. The 1:4 ratio of weight loss to load reduction indicates that, for every 1 pound of weight loss, there is a 4-pound reduction in knee-joint load per step. The accumulated reduction in knee load for a 1-pound loss in weight would be more than 4,800 pounds per 1 mile walked (assuming 1,200 strides/mile). For people losing 10 pounds, each knee would be subjected to 48,000 pounds less in compressive load per mile walked. Although there are no longitudinal studies indicating that weight loss in humans slows the progression of knee OA, a reduction of this magnitude would appear to be clinically relevant. For example, Felson et al (13) revealed that a weight loss of 11.2 pounds over a 10-year period decreased the likelihood of developing knee OA by \( >50\% \).

Knee-joint moments contribute to the stress placed on the knee during walking. Specifically, higher external adduction moments (or internal abduction moment) are related to increased compressive loads transmitted to the medial compartment of the knee (28). Schipplein and Andriacchi proposed that increased compressive forces at the knee represent an adaptive gait strategy to increase dynamic stability in the presence of a high external adduction moment (26). Our analysis showed a significant direct association \( P = 0.03 \) between followup peak internal knee abduction moment (external adductor moment) and followup body mass after adjusting for baseline covariates. Therefore, the decrease in knee abduction moments with increased weight loss contributed to the attenuated joint loads.

Schipplein and Andriacchi (26) also found higher peak external extension and flexion moments in OA patients compared with normal controls after adjusting for walking speed. Higher flexion/extension moments may improve stability by increasing knee compressive forces, especially in the presence of higher external adduction moments. The additional stability afforded by

**Table 3. Change in body mass as a predictor for knee-joint kinetic variables at followup**

<table>
<thead>
<tr>
<th>Outcome</th>
<th>( \beta_{\text{followup mass}} )</th>
<th>Units</th>
<th>T score</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak AP shear force*</td>
<td>-0.38</td>
<td>N/kg</td>
<td>-0.11</td>
<td>0.91</td>
</tr>
<tr>
<td>Peak compressive force</td>
<td>40.59</td>
<td>N/kg</td>
<td>3.45</td>
<td>0.001</td>
</tr>
<tr>
<td>Peak resultant force</td>
<td>38.71</td>
<td>N/kg</td>
<td>3.28</td>
<td>0.002</td>
</tr>
<tr>
<td>Peak lateral rotation moment</td>
<td>0.15</td>
<td>Nm/kg</td>
<td>1.71</td>
<td>0.09</td>
</tr>
<tr>
<td>Peak medial rotation moment</td>
<td>0.31</td>
<td>Nm/kg</td>
<td>2.32</td>
<td>0.03</td>
</tr>
<tr>
<td>Peak abduction moment</td>
<td>0.50</td>
<td>Nm/kg</td>
<td>2.24</td>
<td>0.03</td>
</tr>
<tr>
<td>Peak adduction moment</td>
<td>-0.07</td>
<td>Nm/kg</td>
<td>-0.70</td>
<td>0.49</td>
</tr>
<tr>
<td>Peak extension moment</td>
<td>0.08</td>
<td>Nm/kg</td>
<td>0.52</td>
<td>0.61</td>
</tr>
<tr>
<td>Peak flexion moment</td>
<td>0.10</td>
<td>Nm/kg</td>
<td>0.29</td>
<td>0.77</td>
</tr>
</tbody>
</table>

* AP = anteroposterior.
greater compressive forces may exacerbate disease progression. However, we did not observe a significant association between weight loss and knee flexion/extension internal moments.

Knee stability is also challenged during the stance phase by subtalar pronation, which initiates medial tibial and knee rotation (29). Excessive subtalar pronation, common in obese adults (30), is counteracted by higher internal lateral rotation knee moments (29). Weight loss in this obese population, then, should result in a reduction in the lateral rotation moment. Although this trend was evident in the present study, the correlation did not reach statistical significance \((P = 0.09)\). There was, however, a significant association \((P = 0.03)\) between weight loss and internal medial rotation moment. Resupination (inversion) in late stance locks the intertarsal joints and provides a firm base on which to toe-off. In obese adults with excessive rearfoot motion, however, this resupination is delayed and the stability at toe-off may be provided, in part, by the higher internal medial rotation moment at the knee. We suggest that weight loss may be related to improved subtalar motion and less demand on proximal muscles to provide stability.

Shear forces, both anteroposterior and mediolateral, are thought to increase the wear and tear on the articular cartilage. We found no relationship between anteroposterior shear force and weight loss, and our model did not estimate the mediolateral shear forces. The importance of these forces in disease progression remains unknown and should be investigated in future studies.

A common perception is that weight gain increases the load on the knee, causing joint pain and the avoidance of daily activities. This investigation sought to determine the relationship between change in body weight and change in knee-joint loads during gait. Our results indicate that each pound of weight lost will result in a 4-fold reduction in the load exerted on the knee per step during daily activities. Accumulated over thousands of steps per day, a reduction of this magnitude would appear to be clinically meaningful. A critical question that remains is whether this relationship holds true in an experimental longitudinal study, and whether such an approach results in a slowing of disease progression.

ACKNOWLEDGMENT

We would like to acknowledge the contributions of Jovita Jolla, research technician, who assisted in data collection.

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Association of Cartilage Defects With Loss of Knee Cartilage in Healthy, Middle-Age Adults

A Prospective Study

Flavia Cicuttini,1 Changhai Ding,2 Anita Wluka,1 Susan Davis,2 Peter R. Ebeling,3 and Graeme Jones2

Objective. The significance of asymptomatic knee cartilage defects in healthy individuals is not known. The aim of this study was to examine the association between cartilage defects in the knee and cartilage volume both cross-sectionally and longitudinally in healthy, middle-age adults.

Methods. Eighty-six healthy men and women (mean ± SD age 53.8 ± 8.8 years) underwent T1-weighted fat-suppressed magnetic resonance imaging of their dominant knees at baseline and at the 2-year followup visit. Knee cartilage volume was measured. Cartilage defects were scored according to a grading system (0–4) and as present (a defect score of ≥2) or absent in the medial and lateral tibiofemoral compartments.

Results. Cartilage defects in the medial and lateral tibiofemoral compartments were very common (in 61% and 43% of subjects, respectively). Those with cartilage defects had a 25% reduction in medial tibial cartilage volume, a 15% reduction in lateral tibial cartilage volume, and a 19% reduction in total femoral cartilage volume relative to those with no cartilage defects in cross-sectional analyses (all P < 0.05). In the medial tibiofemoral compartment, the annual loss of tibial cartilage in those with cartilage defects was 2.5% (95% confidence interval [95% CI] 2.2%, 3.1%) compared with an annual loss of tibial cartilage of 1.3% (95% CI 0.5%, 2.0%) in those with no defects (P = 0.028), independent of other known risk factors for osteoarthritis (OA).

Conclusion. These data suggest that the presence of asymptomatic, non–full-thickness medial tibiofemoral cartilage defects identifies healthy individuals most likely to lose knee cartilage in the absence of radiographic knee OA. Thus, interventions aimed at reducing or reversing cartilage defects may reduce the risk of subsequent knee OA.

Full-thickness defects of articular cartilage in the knee are well recognized as a clinical entity often presenting with symptoms (1,2). It has been thought that such defects may progress to osteoarthritis (OA) and to the eventual requirement for total knee joint replacement (3). Treatments such as subchondral drilling, which may be effective in producing fibrocartilage to replace full- and partial-thickness cartilage defects, as well as autologous chondrocyte transplantation have been used to treat cartilage defects (4,5). There is some evidence that this improves symptoms attributable to the defect, but it is not known whether any of the numerous, costly treatments that have been recommended for cartilage defects alters the natural history of the untreated lesions (4). Knee cartilage defects have been shown to be common in those with radiographic knee OA (6,7) and to be variably associated with pain (7,8). However, it has not been demonstrated whether asymptomatic cartilage defects are a risk factor for OA or cartilage loss at the knee, although these relationships are often assumed (9).
Knee cartilage defects can be identified noninvasively using magnetic resonance imaging (MRI), and MRI findings have been shown to correlate well with arthroscopic (10–12) and histologic (13) findings. Knee cartilage volume can be measured using MRI (14–17). It has been shown to be a valid and reproducible measure of knee cartilage (14–17), to correlate with radiographic grade of knee OA (18), and to be sensitive to change in longitudinal studies of both people with OA and healthy subjects (19,20). Loss of knee cartilage volume has been shown to be clinically relevant (21). Loss of knee cartilage volume is associated with worsening of knee symptoms (21), and those with knee OA who are in the highest versus the lowest tertile of knee cartilage loss have a 7-fold increased risk of requiring knee joint replacement within 4 years (22). The aim of the present study was to examine the association between cartilage defects in the knee and cartilage volume both cross-sectionally and longitudinally in healthy adults.

SUBJECTS AND METHODS

Subjects. Healthy, normal subjects were recruited through advertising in newspapers, through sporting clubs, and through the hospital staff association. Subjects were excluded if any form of arthritis other than OA was present, including evidence of chondrocalcinosis on plain radiographs. Subjects were excluded if they had a contraindication to MRI, hemiparesis of either lower limb, or planned total knee replacement. The study was approved by the Alfred Hospital and Human Research Ethics Committees, and all participants gave written informed consent.

Subjects completed a questionnaire that included demographic data, medical and surgical history, and current physical activity (23). Weight was measured to the nearest 0.1 kg using a single pair of electronic scales, with the subject’s shoes, socks, and bulky clothing removed. Height was measured to the nearest 0.1 cm using a stadiometer, with the subject’s shoes and socks removed. Body mass index (BMI; weight [kg]/height [m²]) was calculated.

Each subject underwent MRI of his/her dominant knee, defined as the lower limb from which he/she stepped off when walking, at baseline and ~2 years later. Knee cartilage volume was determined by image processing on an independent work station using Osiris software (University of Zurich, Zurich, Switzerland) as previously described (18,19). Knees were imaged in the sagittal plane on a 1.5T whole-body MR unit (Signa Advantage HiSpeed; GE Medical Systems, Milwaukee, WI) using a commercial transmit–receive extremity coil. The following sequence and parameters were used: a T1-weighted fat-suppressed 3-dimensional (3-D) gradient recall acquisition in the steady state, flip angle 5°, repetition time 58 msec, echo time 12 msec, field of view 16 cm, 60 partitions, 513 × 196–pixel matrix, acquisition time 11 minutes 56 seconds, and 1 acquisition. Sagittal images were obtained at a partition thickness of 1.5 mm and an in-plane resolution of 0.31 × 0.83 mm (512 × 196 pixels).

The image data were transferred to a work station. The volumes of the individual cartilage plates (medial and lateral tibial) were isolated from the total volume by manually drawing disarticulation contours around the cartilage boundaries on each section. These data were resampled by bilinear and cubic interpolation (area of 312 μm × 312 μm and 1.5-mm thickness, continuous sections) for the final 3-D rendering. The volume of the particular cartilage plate was determined by summing the pertinent voxels within the resultant binary volume. A trained observer read each MRI. Each subject’s baseline and followup MRI scans were scored within a 2-week period, unpaired and with the reader blinded to subject identification and timing of MRI. Medial and lateral tibial cartilage volume and femoral cartilage volume were measured as previously described (15,19). The coefficients of variation (CVs) for medial and lateral tibial cartilage volumes and femoral cartilage volume were 2.6%, 3.3%, and 2.0%, respectively (15,19). Medial and lateral tibial plateau areas were measured from images obtained by creating an isotropic volume from the 3 input images closest to the knee joint which were reformatted in the axial plane (24). The CVs for the medial and lateral tibial plateau areas were 2.2% and 2.3%, respectively (24).

The cartilage defects were graded on the MR images with a modification of a previous classification system (10–12) at medial tibial, medial femoral, lateral tibial, and lateral femoral sites as follows: grade 0 = normal cartilage; grade 1 = focal blistering and intracartilaginous low–signal intensity area with an intact surface and bottom; grade 2 = irregularities on the surface or bottom and loss of thickness of <50%; grade 3 = deep ulceration with loss of thickness of >50%; grade 4 = full-thickness cartilage wear with exposure of subchondral bone (Figure 1). We found that the cartilage surface in some images was still regular but that cartilage adjacent to subchondral bone became irregular, so we included this in the classification system. A cartilage defect also had to be present in at least 2 consecutive slices. The cartilage was considered to be normal if the band of intermediate signal intensity had a uniform thickness. The cartilage defects were regraded 1 month later, and the average scores of cartilage defects at medial tibiofemoral sites (0–8) and lateral tibiofemoral sites (0–8) were used in the study. A prevalent cartilage defect was defined as a cartilage defect score of ≥2 at any site of that compartment. Intraobserver reliability (expressed as intraclass correlation coefficient [ICC]) was 0.90 for the medial tibiofemoral compartment and 0.89 for the lateral tibiofemoral compartment. Interobserver reliability was assessed in 50 MR images and yielded ICCs of 0.90 for the medial tibiofemoral compartment and 0.85 for the lateral tibiofemoral compartment.

Statistical analysis. Descriptive statistics for characteristics of the subjects were tabulated. Medial and lateral tibial cartilage volume were initially assessed for normality. Multivariate regression models were then constructed adjusting for known demographic predictors, including age, sex, BMI, bone size (either medial or lateral tibial bone area), and current level of physical activity, as well as the presence and absence of cartilage defects to determine the effect of these on cartilage volume. The annual difference in medial and lateral tibial cartilage volume was calculated as follows: (initial tibial carti-
Multivariate regression models were then constructed adjusting for age, sex, BMI, bone size (either medial or lateral tibial bone area), initial tibial cartilage volume, and the presence and absence of cartilage defects to determine the effect of these on change in tibial cartilage volume. P values less than 0.05 were considered significant. All analyses were performed using the SPSS statistical package, version 10.0.5 (SPSS, Chicago, IL).

**RESULTS**

Baseline characteristics of the study population are presented in Table 1. Six subjects (4 women and 2 men) had OA according to the American College of Rheumatology criteria (25). The women tended to be older than the men and had a higher proportion of cartilage defects, which were also of higher grade than...
those in the men. The men were more physically active than the women, and they had significantly more medial and lateral tibial cartilage volume. Although a high proportion of subjects had tibiofemoral cartilage defects (61% with medial tibiofemoral cartilage defects and 43% with lateral tibiofemoral cartilage defects), the mean ± SD defect scores were low (2.0 ± 1.0 for medial tibiofemoral cartilage defects; 1.6 ± 1.1 for lateral tibiofemoral cartilage defects). There was a moderate, but significant, correlation between femoral and tibial cartilage defects in both the medial and lateral tibiofemoral compartments (R = 0.41, P < 0.001 and R = 0.42, P < 0.001, respectively).

In the cross-sectional analyses, medial and lateral tibiofemoral cartilage defects were associated with a consistent decrease in the respective tibial cartilage volume both in univariate analyses and following adjustment for age, sex, BMI, physical activity, and respective tibial plateau area, all known risk factors for OA (Table 2). The mean ± SD adjusted medial tibial cartilage volume in those with cartilage defects was 1.62 ± 0.38 ml compared with 2.17 ± 0.67 ml in those with no defects (P < 0.001), a 25% reduction. The mean ± SD adjusted lateral tibial cartilage volume in those with cartilage defects was 2.17 ± 0.54 ml compared with 2.56 ± 0.78 ml in those with no defects (P = 0.002), a 15% reduction. The mean ± SD adjusted total femoral cartilage volume in those with defects was 7.4 ± 1.8 ml compared with 9.1 ± 2.2 ml in those with no defects (P < 0.001), a 19% reduction.

After 2 years, those with cartilage defects demonstrated an annual loss of medial tibial cartilage of 1.2% (95% confidence interval [95% CI] 0.1%, 2.3%), which was greater than that seen in those with no defects, independent of age, sex, BMI, medial tibial bone area, and baseline medial tibial cartilage volume (Table 3). This corresponded to an actual 2.5% (95% CI 2.2%, 3.1%) annual loss of tibial cartilage in those with cartilage defects compared with an actual 1.3% (95% CI 0.5%, 2.0%) annual loss of tibial cartilage in those with no defects (P = 0.028 for difference), a 1.9-fold increase. There was a significant increase in the rate of medial tibial cartilage loss as the grade of cartilage lesions increased. For every increase in grade of cartilage

### Table 1. Characteristics of participants*

<table>
<thead>
<tr>
<th></th>
<th>Women (n = 43)</th>
<th>Men (n = 43)</th>
<th>P†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>55.1 ± 10.4 (49.9 to 76.2)</td>
<td>52.5 ± 13.2 (35.5 to 75.0)</td>
<td>0.02</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>26.3 ± 5.1 (17.6 to 45.5)</td>
<td>25.6 ± 3.5 (19.4 to 36.7)</td>
<td>0.40</td>
</tr>
<tr>
<td>Physical activity score, 0–12</td>
<td>6.4 ± 1.9 (3 to 11)</td>
<td>7.2 ± 1.7 (3 to 11)</td>
<td>0.04</td>
</tr>
<tr>
<td>Medial tibial cartilage defect, %</td>
<td>71</td>
<td>51</td>
<td>&lt;0.000‡</td>
</tr>
<tr>
<td>Lateral tibial cartilage defect, %</td>
<td>48</td>
<td>38</td>
<td>0.01‡</td>
</tr>
<tr>
<td>Medial tibiofemoral cartilage defect score, 0–8</td>
<td>2.2 ± 0.9 (0 to 6)</td>
<td>1.4 ± 1.0 (0 to 5)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lateral tibiofemoral cartilage defect score, 0–8</td>
<td>1.8 ± 1.1 (0 to 8)</td>
<td>1.4 ± 1.0 (0 to 7)</td>
<td>0.09</td>
</tr>
<tr>
<td>Knee OA, no. (%)</td>
<td>4.7 (7.0)</td>
<td>2 (4.7)</td>
<td>0.08‡</td>
</tr>
<tr>
<td>Medial tibial cartilage volume, ml</td>
<td>1.5 ± 0.3 (1.0 to 2.5)</td>
<td>2.5 ± 0.5 (1.2 to 4.1)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Lateral tibial cartilage volume, ml</td>
<td>2.0 ± 0.4 (1.2 to 2.5)</td>
<td>3.2 ± 0.6 (1.9 to 4.8)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Total femoral cartilage volume, ml</td>
<td>7.1 ± 1.4 (4.2 to 10.2)</td>
<td>10.1 ± 1.8 (6.6 to 14.3)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Annual % loss in medial tibial cartilage volume</td>
<td>2.3 ± 2.1 (−1.1 to 12.2)</td>
<td>1.9 ± 2.3 (−5 to 15.2)</td>
<td>0.08</td>
</tr>
<tr>
<td>Annual % loss in lateral tibial cartilage volume</td>
<td>1.8 ± 2.2 (−2.3 to 10.2)</td>
<td>1.6 ± 2.1 (−2.1 to 9.2)</td>
<td>0.10</td>
</tr>
<tr>
<td>Annual % loss in femoral cartilage volume</td>
<td>8.6 ± 9.4 (−7.5 to 17.5)</td>
<td>11.9 ± 9.2 (−7.5 to 17.5)</td>
<td>0.10</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean ± SD (range). BMI = body mass index; OA = osteoarthritis.
† By t-test, except where indicated otherwise.
‡ By Mann-Whitney U test.

### Table 2. Relationship between presence of cartilage defects and cartilage volume

<table>
<thead>
<tr>
<th></th>
<th>Univariate analysis regression coefficient (95% CI)*</th>
<th>Multivariate analysis regression coefficient (95% CI)†</th>
<th>P‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medial tibial cartilage volume, ml</td>
<td>−0.49 (−0.73, −0.25)</td>
<td>−0.17 (−0.32, −0.02)</td>
<td>0.02</td>
</tr>
<tr>
<td>Lateral tibial cartilage volume, ml</td>
<td>−0.39 (−0.50, −0.18)</td>
<td>−0.20 (−0.37, −0.02)</td>
<td>0.03</td>
</tr>
<tr>
<td>Total femoral cartilage volume, ml</td>
<td>−4.35 (−6.95, −1.75)</td>
<td>−2.67 (−5.22, −0.12)</td>
<td>0.04</td>
</tr>
</tbody>
</table>

* Change in tibial cartilage volume per unit increase in cartilage defect score. 95% CI = 95% confidence interval.
† Adjusted for age, sex, body mass index, physical activity, and tibial bone size.
‡ For multivariate analysis.
lesions, the annual loss of medial tibial cartilage increased by 0.5% (95% CI 0.1%, 1.0%) ($P_{/H11021}/H110210.019$). Similar findings were observed when the 6 subjects with radiographic knee OA were excluded from the analyses (data not shown). Such a relationship was not seen in the lateral tibiofemoral compartment or when total femoral cartilage was examined.

**DISCUSSION**

In this study we demonstrated a high prevalence of tibiofemoral cartilage defects in normal, asymptomatic middle-age people. However, although the defects were of mild severity, they were associated with large reductions in medial and lateral tibial cartilage volume and femoral cartilage volume compared with subjects with no defects. After 2 years, those with cartilage defects in the medial tibiofemoral compartment demonstrated a greater loss of medial tibial cartilage compared with those with no defects, but this was not observed for lateral tibial cartilage or the femoral compartments.

No previous study has examined whether asymptomatic cartilage defects are a risk factor for OA or cartilage loss in the knee joint in normal subjects. We have shown that the presence of tibiofemoral cartilage defects is associated with less tibial cartilage in the respective compartment. Previous studies of subjects with radiographic knee OA have shown a significant association between cartilage defects and the Kellgren/Lawrence grade of radiographic OA (7) and between cartilage defects and patellofemoral and tibiofemoral osteophytes (1). Findings of those studies in subjects with chronic knee pain or knee OA are consistent with our results. Although articular cartilage volume correlates with radiographic grade of knee OA, most of our subjects had normal radiographs. However, by the time radiographic changes of OA are present, $\sim$10–15% of articular cartilage is already lost (26).

Cross-sectional data cannot be used to determine causal directions; thus, the reduction in cartilage volume may be due to the presence of cartilage defects or subjects with less cartilage may be more prone to defect formation. However, in this study we found that cartilage defects were prospectively associated with loss of joint cartilage in the medial compartment when subjects were followed up prospectively over 2 years. The annual loss of medial tibial cartilage was 1.9-fold greater in those with cartilage defects than in those with no defects, independent of other known OA risk factors.

We also demonstrated a dose-response relationship between increasing severity of cartilage lesions and an increase in the rate of medial tibial cartilage loss. Although we found that lateral tibiofemoral cartilage and femoral cartilage were reduced by 15% and 19%, respectively, in those with cartilage defects, we did not demonstrate that cartilage defects were associated with loss of joint cartilage at these sites over the 2 years of this study. It may be that we did not have the power to show this, since the prevalence of cartilage defects was lower in the lateral than in the medial tibiofemoral compartment, and the lesions were of milder severity. The femoral cartilage as measured in this study was total femoral cartilage, which included both the medial and lateral tibiofemoral compartments. Our results are consistent with those of a previous study that found that cartilage defects were far more common in the medial tibiofemoral compartment than in the lateral tibiofemoral compartment in subjects presenting with knee pain (1). It may be, as those investigators speculated, that the medial compartment is more susceptible to articular cartilage degeneration, which may in part explain the 4-fold higher prevalence of OA in the medial compartment compared with the lateral compartment (27).

Our study has a number of potential limitations. Our subjects were generally healthy, with few having clinically apparent knee OA. Repeating our analyses excluding those who had OA did not change the magni-

<table>
<thead>
<tr>
<th>Table 3. Relationship between presence of tibiofemoral cartilage defects and annual percentage loss in cartilage volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate analysis</td>
</tr>
<tr>
<td>regression coefficient</td>
</tr>
<tr>
<td>(95% CI)*</td>
</tr>
<tr>
<td>Annual % loss in medial tibial cartilage volume</td>
</tr>
<tr>
<td>Annual % loss in lateral tibial cartilage volume</td>
</tr>
<tr>
<td>Annual % loss in total femoral cartilage volume</td>
</tr>
</tbody>
</table>

* Annual percentage change in tibial cartilage (in each respective compartment) in those with cartilage defects compared with those with no cartilage defects. 95% CI = 95% confidence interval.
† Adjusted for age, sex, body mass index, tibial bone size, and baseline tibial cartilage volume.
‡ For multivariate analysis.
tude or direction of our findings. Thus, it is unlikely that our findings are due to significant, preexisting OA. Furthermore, we adjusted our longitudinal analyses to take into account the baseline amount of knee cartilage. Our measure of cartilage volume directly visualized the articular cartilage and is more accurate than radiography for assessing articular cartilage volume (28). We have recently shown that tibial cartilage volume correlates with radiographic OA (18), and that those who have grade 1 joint space narrowing have already lost up to 10–15% of their tibial cartilage (26). Thus, adjusting for baseline knee cartilage volume allowed us to further take into account possible early, subclinical cartilage loss.

In this study we have used tibial cartilage loss rather than femoral cartilage loss as the measure of joint cartilage loss at the tibiofemoral joint. We have previously shown a strong correlation between tibial and femoral cartilage loss in the medial and lateral tibiofemoral joint compartments both in cross-sectional analysis (24) and when longitudinal change was examined (29). Since the femoral cartilage articulates with 3 joints (the medial and lateral tibiofemoral joints and the patellofemoral joints), it is more difficult to clearly identify the relevant component of the femoral joint when assessing the medial and lateral tibiofemoral joints, since this requires arbitrary definitions. In contrast, each of the tibial cartilage plates, which we examined in this study, only forms part of 1 joint (either the medial or the lateral tibiofemoral joint). However, when we examined the effect of femoral cartilage defects on total femoral cartilage, we found similar results. Although our sample size was large enough to measure cartilage loss, it may not have been large enough to identify a weaker association between lateral tibiofemoral cartilage defects and cartilage loss in that compartment or in the femoral compartment. It was also not large enough to allow us to examine sex differences in loss. Larger studies will be needed for this.

The annual rate of cartilage loss of 2.5% in those with cartilage defects is likely to be clinically significant, since we are examining a population of healthy, middle-age persons whose average residual life expectancy is 30 years. It has been shown that there is ~60% cartilage loss in end-stage knees (22,30). Once OA develops, the mean ± SD rate of cartilage loss is more rapid (4.7 ± 6.5% per year) (19), and we have recently shown that 16% of subjects required a knee replacement within 4 years, independent of other risk factors (22). This suggests that knee cartilage loss, from normal to requiring knee replacement, may vary according to the different stages of the disease process.

Our findings suggest that it may be possible to affect the risk of knee OA in two ways. Since the presence of medial tibiofemoral cartilage defects identifies healthy individuals most likely to lose knee cartilage, this may be a group that should be targeted for early risk factor modification before the development of clinical knee OA. Furthermore, interventions aimed at reducing or reversing cartilage defects may reduce the risk of subsequent knee OA in the population. Larger studies may be able to identify patterns of physical activity, drugs, or dietary factors that increase or decrease the risk of cartilage defects, and thus identify novel preventive or management strategies in knee OA.

REFERENCES


Lack of Support for the Presence of an Osteoarthritis Susceptibility Locus on Chromosome 6p

Gary K. Meenagh,1 David McGibbon,2 James Nixon,1 Gary D. Wright,1 Michael Doherty,3 and Anne E. Hughes2

Objective. To replicate, in a Northern Irish population, the previously reported association between a locus on chromosome 6 and hip osteoarthritis (OA).

Methods. Patients with hip OA were identified from a registry of patients who had undergone total hip replacement surgery over an 8-year period at a single large orthopedic unit in Northern Ireland. Patients identified as index cases were contacted by mail and asked to reply only if another family member also had undergone total hip replacement surgery. Using this approach, we identified 288 sibling pairs concordant for primary hip OA. DNA was extracted from peripheral blood, and microsatellite markers were amplified by polymerase chain reaction and subsequently genotyped.

Results. No evidence of linkage to this region was demonstrated by either 2-point analysis or multipoint analysis of 17 microsatellites.

Conclusion. The reported association between a locus on chromosome 6 and hip OA could not be confirmed in this population. Different methods of ascertainment and phenotyping of OA may contribute to the current inability to replicate genetic associations for hip OA.

Osteoarthritis (OA) is a common, complex disorder that shows heterogeneity with respect to the pattern of joint involvement, number of joints affected, age at onset, and clinical outcome. Several extrinsic and intrinsic risk factors that may differ between joint sites have been identified (1). A strong genetic component for development of hip OA is evident from the increased concordance for radiographic hip OA seen in monozygotic twins compared with dizygotic twins (2) and from the increased risk of radiographic hip OA in siblings of patients who have undergone total hip replacement (THR) surgery for primary hip OA (3). The high estimated heritability observed in such studies (>50%) helps justify the search for site-specific genes that predispose to OA (4).

Chapman and colleagues (the Loughlin group) studied sibling pairs with large-joint (hip, knee) OA requiring joint replacement; subjects were identified from several centers within the UK (5). Their genome-wide linkage analysis in 194 pedigrees revealed a region suggestive of linkage on chromosome 6p, with a maximum multipoint logarithm of odds (LOD) score of 2.9. In a subsequent analysis, these investigators genotyped chromosome 6 to a higher density in an expanded cohort of 378 THR pedigrees. Finer linkage analysis of this 11.4-cM region with stratification for sex and site resulted in a maximum multipoint LOD score of 4.6 at marker D6S1573 in 166 female-only pedigrees (6). This finding represents the strongest evidence to date for a region that may harbor an OA susceptibility gene. Two attractive candidate genes that reside in this region are COL9A1, which encodes a minor cartilage collagen that may act to stabilize cartilage, and BMP5, which encodes a protease belonging to the transforming growth factor β family. However, single-nucleotide polymorphism analysis of these genes failed to show an association (7).

The aim of the present study was to perform linkage analysis on this region of chromosome 6 in a separate cohort of Northern Irish pedigrees with primary hip OA.

PATIENTS AND METHODS

Subjects. This study was approved by the local research ethics committee. Subjects in the Northern Irish sibling pair...
Table 1. Novel primer sequences used in multipoint analysis of chromosome 6p

<table>
<thead>
<tr>
<th>Marker</th>
<th>Forward primer (5’→3’)</th>
<th>Reverse primer (5’→3’)</th>
<th>Heterozygosity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL17</td>
<td>GACCTACCCCAACTGGAATGTCC</td>
<td>AGTGCGCAGAAGTGCATTTGC</td>
<td>66</td>
</tr>
<tr>
<td>IL17F</td>
<td>TCACAGTGGTGGATCTTTGATGG</td>
<td>TTGCTACATTGTATTTGTATGG</td>
<td>63</td>
</tr>
<tr>
<td>FBOX9</td>
<td>GGTTAAATCTAGAAGGAGTGG</td>
<td>CTCTGAGAATTTACATTCAGG</td>
<td>78</td>
</tr>
<tr>
<td>BMP5</td>
<td>GATGAGAATTTCCTAAGCAGC</td>
<td>TGTTACACGAGCAAGACCTGC</td>
<td>80</td>
</tr>
<tr>
<td>2326k (ca)n</td>
<td>CCACTCAGGTCTTTTAGAGG</td>
<td>GTTCACATTCCACTTACAGG</td>
<td>76</td>
</tr>
<tr>
<td>2524k (gt)n</td>
<td>CAACTCAGGTCTTTTAGAGG</td>
<td>GTTGAATCCTGCTGGAACTCC</td>
<td>80</td>
</tr>
<tr>
<td>COL9A1</td>
<td>AGGGTGCCCTATTCTTCTTCC</td>
<td>AAGGGTTCCGTTTGTGGGC</td>
<td>71</td>
</tr>
</tbody>
</table>


dip OA cohort were identified from the records of Musgrave Park Hospital and included patients who had undergone THR surgery for primary hip arthritis between 1996 and 2003. This hospital has the largest orthopedic unit in Western Europe. Index patients were contacted by mail and were asked to reply only if they had a first-degree relative who had also undergone THR surgery. Families containing at least 1 affected sibling pair concordant for hip OA were thus identified. Patients who had previously donated DNA samples for the Loughlin group (Oxford cohort) study were excluded, along with any patient with a history of inflammatory arthritis, hip trauma, or congenital hip abnormality. The method of exclusion according to results of radiography is described below. A control group of 10 Northern Irish adults was chosen at random and was used to estimate allele frequencies of the markers studied. Based on standard formulae, we estimated that if we assumed a genotype relative risk (γ) of 4.0, our hip OA cohort had 80% power at a 5% significance level.

### Phenotypic characterization.

All THR patients underwent clinical assessment to enable accurate site-specific OA phenotyping according to American College of Rheumatology (ACR) criteria and to enable further exclusion on a clinical basis (8). Patients with secondary causes of hip OA were excluded based on the assessment of preoperative radiographs, including measurement of the acetabular depth (9) and the center edge angle (10). Hip dysplasia was defined as an acetabular depth including measurement of the acetabular roof, was measured by a single observer (GKM) using Vernier calipers that were accurate to 0.02 mm. The degree of reproducibility for radiographic scoring was assessed using the weighted kappa test with 50 random pelvic radiographs that were graded 4 weeks apart.

### Genetic analysis.

Peripheral blood was obtained from participants, and genomic DNA was extracted using the Puregene DNA Isolation Kit (Flowgen, Nottingham, UK). Nine microsatellite markers for chromosome 6 from the ABI LMS-MD10 v2.5 kit (Applied Biosystems, Warrington, UK) were amplified using multiplex polymerase chain reaction (PCR) (Qiagen, Crawley, UK). The primer sequences for 8 additional markers that were developed in-house and were used in the analysis are shown in Table 1. Hardy-Weinberg equilibrium was established for all of the markers studied, PCR cycling conditions were as follows: 15 minutes at 95°C, then 30 cycles at 94°C for 30 seconds, 60°C for 90 seconds, and 72°C for 60 seconds, followed by 60°C for 30 minutes.

Allelic genotyping was performed using GeneScan and Genotyper software (Applied Biosystems). Multipoint linkage analysis was subsequently performed with the GeneHunter-Plus program (http://hgmp.mrc.ac.uk/Registered/Option/genehunter-plus.html) (12), using allele frequencies found in our control population. The allele frequencies were compared between the control group and a random sample of 40 affected siblings (the first member of each sibling pair in the first 40 pedigrees), using Wilcoxon’s signed rank test. P values less than 0.05 were considered significant.

### Results

A total of 3,505 patients who had undergone THR surgery were contacted by mail. Four hundred eighty-two replies were obtained, representing a positive response rate of 13.7% for self-reported THR in family members. Thirty-four index patients were excluded due to concomitant inflammatory arthritis (n = 17), congenital hip abnormality (n = 15), and hip trauma (n = 2). The remaining group of 288 sibling pairs comprised 180 women and 112 men from 109 THR pedigrees. The mean acetabular depth in men was 12.4 mm (95% confidence interval [95% CI] 11.9–12.7) and in women was 12.6 mm (95% CI 12.2–12.9). The mean center edge angle in men was 35.6° (95% CI 35.1–36.0) and in women was 34.7° (95% CI 34.4–34.9). The mean minimum joint space width in men was 2.44 mm (95% CI 2.16–2.88) and in women was 2.34 mm (95% CI 2.02–2.48). The kappa values for intraobserver reproducibility of measurements of the center edge angle, acetabular depth, and minimum joint space width were 0.85, 0.90, and 0.87, respectively. The specific OA phenotypes of the cohort are shown in Table 2.

Table 2. Subjects fulfilling ACR criteria for hip OA, according to phenotype

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>No. in group</th>
<th>% fulfilling criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hip only</td>
<td>162</td>
<td>56</td>
</tr>
<tr>
<td>Hip and knee</td>
<td>63</td>
<td>21.5</td>
</tr>
<tr>
<td>Hip and hand</td>
<td>22</td>
<td>7.5</td>
</tr>
<tr>
<td>Hip, knee, and hand</td>
<td>45</td>
<td>15</td>
</tr>
</tbody>
</table>

* ACR = American College of Rheumatology; OA = osteoarthritis.
Both 2-point (data not shown) and multipoint LOD scores at each marker locus remained negative throughout all intervals between markers, indicating that pathogenetic loci for OA are very unlikely to exist between the markers. These scores remained negative after substratification for female-only sibling pairs. There was no significant difference between the distribution of alleles in affected sibling pairs and controls (Table 3). Thus, there was no evidence to support linkage of an OA susceptibility locus to this region on chromosome 6p.

**DISCUSSION**

This study failed to demonstrate evidence for an OA susceptibility locus on chromosome 6p in Northern Irish families concordant for primary hip OA requiring THR surgery. Our data showed neither linkage nor a trend toward it before or after stratification for female-only families.

This study is the first to attempt to confirm the presence of an OA susceptibility locus that was previously identified by genome-wide screening. Replication of promising data is an important step toward identification of genes that predispose to complex disorders and avoids the need for rigorous statistical correction due to multiple testing. We typed many of the most influential markers from chromosome 6p used by Loughlin et al and undertook a comprehensive investigation of the region. We added several new markers in the vicinity of OA candidate genes, including \(IL17\), which has been shown to stimulate collagenase 3 activity in OA (13), and \(FBOX9\), which is a member of the ubiquitin ligase family that is thought to influence synovial proliferation in animal models of arthritis (14). Our data also provide further evidence against \(COL9A1\) and \(BMP5\) as major OA susceptibility genes (7).

Several factors may have contributed to the discordant results between our study and that of Loughlin et al (6). First, our cohort was assembled from sequential patients undergoing THR surgery for defined clinical and radiographic OA in a single large orthopedic center serving a defined population. All subjects satisfied the ACR criteria for site-specific OA, and we were careful to exclude patients with dysplasia or other arthropathy. In contrast, the Oxford cohort was gathered in a less systematic manner from several centers throughout the UK, and radiographic details of the cohort have not been published. Therefore, the generalizability of the findings from each study may differ. The inclusion of subjects with hip dysplasia is unlikely to explain the promising multipoint LOD score obtained by Loughlin’s group. Those investigators initially studied 297 OA families and obtained a modest maximum multipoint LOD score of 1.0, which was increased to 2.9 only after a subanalysis of 194 families containing sibling pairs concordant for THR. Increasing the number of THR families to 478 led to a slight reduction in significance, but further subanalysis of female-only THR families would be required to confirm the preliminary results.

### Table 3. Distribution of alleles in affected sibling pairs and controls

<table>
<thead>
<tr>
<th>Microsatellite</th>
<th>Chromosome 6p map position, Mb</th>
<th>Multipoint LOD score*</th>
<th>Allele frequency, (P)†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All pedigrees</td>
<td>Females only</td>
<td></td>
</tr>
<tr>
<td>D6S276</td>
<td>38.0</td>
<td>−2.16</td>
<td>−1.57</td>
</tr>
<tr>
<td>D6S1549</td>
<td>46.5</td>
<td>−2.11</td>
<td>−1.55</td>
</tr>
<tr>
<td>D6S282</td>
<td>48.2</td>
<td>−1.68</td>
<td>−1.40</td>
</tr>
<tr>
<td>IL17</td>
<td>52.0</td>
<td>−1.50</td>
<td>−1.38</td>
</tr>
<tr>
<td>IL17F</td>
<td>52.1</td>
<td>−1.48</td>
<td>−1.31</td>
</tr>
<tr>
<td>FBOX9</td>
<td>52.8</td>
<td>−1.42</td>
<td>−1.23</td>
</tr>
<tr>
<td>D6S1573</td>
<td>53.7</td>
<td>−1.34</td>
<td>−1.19</td>
</tr>
<tr>
<td>D6S294</td>
<td>55.1</td>
<td>−1.16</td>
<td>−1.02</td>
</tr>
<tr>
<td>BMP5</td>
<td>55.7</td>
<td>−1.12</td>
<td>−0.94</td>
</tr>
<tr>
<td>D6S1276</td>
<td>55.8</td>
<td>−0.91</td>
<td>−0.63</td>
</tr>
<tr>
<td>D6S257</td>
<td>56.0</td>
<td>−0.60</td>
<td>−0.35</td>
</tr>
<tr>
<td>D6S223</td>
<td>56.2</td>
<td>−0.75</td>
<td>−0.48</td>
</tr>
<tr>
<td>232k(ca)(_n)</td>
<td>56.4</td>
<td>−0.92</td>
<td>−0.54</td>
</tr>
<tr>
<td>252k(gt)(_n)</td>
<td>56.6</td>
<td>−1.01</td>
<td>−0.87</td>
</tr>
<tr>
<td>276k(ca)(_n)</td>
<td>56.8</td>
<td>−1.25</td>
<td>−1.01</td>
</tr>
<tr>
<td>D6S1557</td>
<td>70.8</td>
<td>−2.34</td>
<td>−1.92</td>
</tr>
<tr>
<td>COL9A1</td>
<td>70.9</td>
<td>−2.87</td>
<td>−2.06</td>
</tr>
</tbody>
</table>

* LOD = logarithm of odds.
† By Wilcoxon’s signed rank test.
that comprise a joint and the multiple systemic and extrinsic factors that may interact to influence phenotypic expression. This makes the hunt for genetic factors all the more challenging. Use of genome-wide screens of higher marker density with rigorous followup of candidate loci may provide the best strategy. The important starting point, however, is agreement with respect to the characterization of the phenotype that is recognized as common OA.

REFERENCES

Prevalence of and Risk Factors for Low Bone Mineral Density and Vertebral Fractures in Patients With Systemic Lupus Erythematosus

Irene E. M. Bultink, Willem F. Lems, Piet J. Kostense, Ben A. C. Dijkmans, and Alexandre E. Voskuyl

Objective. To examine the prevalence of and risk factors for low bone mineral density (BMD) and vertebral fractures in patients with systemic lupus erythematosus (SLE).

Methods. We studied 107 SLE patients. Demographic and clinical data were collected, and radiographs of the thoracic and lumbar spine and BMD measurements by dual x-ray absorptiometry were performed. Vertebral deformities were scored according to the method of Genant et al: fractures were defined as a reduction of ≥20% of the vertebral body height. Osteoporosis was defined as a T score less than −2.5 SD and osteopenia as a T score less than −1.0 SD in at least 1 region of measurement.

Results. Osteopenia was present in 39% of the patients and osteoporosis in 4% (93% female; mean age 41.1 years). In multiple regression analysis, low BMD in the spine was associated with a low body mass index (BMI), postmenopausal status, and 25-hydroxyvitamin D deficiency. Low BMD in the hip was associated with low BMI and postmenopausal status. At least 1 vertebral fracture was detected in 20% of the patients. Vertebral fractures were associated with ever use of intravenous methylprednisolone and male sex.

Conclusion. Risk factors for low BMD in SLE patients are low BMI, postmenopausal status, and vitamin D deficiency. While osteoporosis defined as a low T score was found in only 4% of the patients, osteoporotic vertebral fractures were detected in 20%. The high prevalence of low BMD and vertebral fractures implies that more attention must be paid to the prevention and treatment of osteoporosis and fractures in SLE.

Over the last few decades, the survival of patients with systemic lupus erythematosus (SLE) has improved dramatically (1), and the morbidity pattern has shown a shift toward long-term complications, including osteoporosis. Several studies have demonstrated a high prevalence of low bone mineral density (BMD) in patients with SLE, especially female patients. For example, osteopenia is reported in 25–46% of SLE patients (2–4) and osteoporosis defined as a T score less than −2.5 SD, is reported in 1–23% (5–7).

In contrast, little attention is paid to osteoporotic fractures, one of the items of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology (SLICC/ACR) damage index for SLE (8). Studies on fractures in SLE have focused on incident cases of symptomatic vertebral and nonvertebral fractures (2,3,9,10) or on prevalent vertebral deformities, i.e., fractures (11–17). However, the method used to assess vertebral fractures in 6 of these studies (11.13–17) is not clear, and vertebral fractures were scored using dual x-ray absorptiometry (DXA) images in 1 study (12). Moreover, in the majority of these studies, only a limited number of patients were evaluated (11,12,14–17).

The importance of identifying prevalent vertebral fractures in SLE patients is illustrated by the observed association between prevalent vertebral deformities and reduced quality of life in postmenopausal women with osteoporosis (18) as well as increased mortality rates and increased risk of future vertebral and nonvertebral frac-
tures in the general population (19,20). The aim of the present study was to investigate the prevalence of low BMD and vertebral fractures, as determined by a standardized assessment, and to identify risk factors associated with low BMD and prevalent vertebral fractures in a large population of SLE patients.

PATIENTS AND METHODS

**Patients.** One hundred seven consecutive patients with a diagnosis of SLE were included in the study. All patients regularly attended the outpatient rheumatology clinic of either the VU University Medical Center, the Jan van Breemen Institute, or the Slotervaart Hospital. These institutes provide primary, secondary, and tertiary care for SLE patients. All patients fulfilled the ACR revised criteria for the classification of SLE (21) and provided informed consent for their participation. The local ethics committee approved the study.

**Data collection and clinical measures.** All measurements were performed systematically between August 2001 and February 2003. Demographic, patient, and disease characteristics were recorded by interview, self-reported questionnaire, chart review, and a clinical examination that was performed by 1 rheumatologist (IEMB). Data collected at the time of study inclusion were age, disease duration, race, menstrual status, age at menopause, periods of amenorrhea, family history of osteoporosis, ultraviolet (UV) light intolerance, sunshine avoidance, use of sunscreens in the previous year, calculated mean daily dietary calcium intake in the last 3 months, history of (non)vertebral fractures after the age of 25 years, comorbidity, alcohol and tobacco intake, and exercise status. Exercise was determined as the weekly frequency of a minimum of 40 minutes of aerobic exercise performed.

History of corticosteroid use, including intravenous (IV) methylprednisolone use (past and current) and oral corticosteroid use (past use, duration of use in months, maximum dosage ever taken, current use, and actual dosage), was documented. The cumulative corticosteroid dose was not calculated since assessment of the patients in the outpatient clinic takes place every 3 months and some patients had, in the past, been allowed to gradually lower their dosage of oral corticosteroids during the time between 2 visits. As a result, the exact dosage of oral corticosteroids used at every point in time was not available for some patients. For this reason, we preferred to use the available exact data on corticosteroid use only. Past and current use of antirheumatic drugs, calcium supplements, vitamin D supplements, multivitamin supplements, hormone-replacement therapy (HRT), oral contraceptives, antiosteoporosis medications, antiepileptic agents, and anticoagulants were also documented.

Body weight, height, and body mass index (BMI) were assessed. Disease activity was scored using the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) (22) and the European Consensus Lupus Activity Measure (ECLAM) (23). Accumulated organ damage was assessed with the SLICC/ACR damage index (DI) (8). A modified DI score was derived as the DI score excluding osteoporotic fractures as a damage item.

Laboratory investigations at the time of study inclusion included routine clinical biochemistry profile, immunologic measures (anti–double-stranded DNA antibodies, complement components, antiphospholipid antibodies), and biochemical and hormonal variables related to mineral metabolism (serum levels of calcium, phosphate, and alkaline phosphatase), thyroid function (thyroid-stimulating hormone), and serum levels of 25-hydroxyvitamin D (25(OH)D). Deficiency of 25(OH)D was defined as a serum level <25 nmoles/liter, based on the laboratory reference value.

**BMD measurements.** BMD measurements of the hip (total hip and femoral neck) and the lumbar spine (L1–L4) were performed in the same radiology department by a trained operator and according to a standardized protocol. All radiographs were of good quality, with good visibility and reliable identification of all vertebrae. Spine radiographs were scored by 2 experienced observers (WFL and BACD) using a standardized semiquantitative method described by Genant et al (24). This method grades vertebrae on a scale of 0–3, where grade 0 = normal, grade 1 = 20–25% reduction in height, grade 2 = >25–40% reduction in height, and grade 3 >40% reduction in height. For the anterior and middle heights, the posterior height of the same vertebra was used as a reference. A vertebral fracture was defined as a reduction of at least 20% of the vertebral body height.

For quality assurance, blinded scoring was done after 7 months, involving 30 SLE patients, 60% of whom had at least 1 vertebral fracture, and 10 controls, 50% of whom had at least 1 vertebral fracture. The kappa value for whether an SLE patient was classified as having any vertebral fracture was 0.62.

**Statistical analysis.** Variables possibly associated with a decreased BMD or the presence of vertebral fractures were examined first by univariate tests and subsequently by multiple regression analysis. The following variables were examined in relationship to BMD by univariate analyses: age, sex, race, menopause status, BMI, disease duration, disease activity, modified DI score, exercise, use of sunscreens, UV light intolerance, dietary calcium intake, previous nonvertebral fractures, creatinine clearance, 25(OH)D deficiency, ever use of corticosteroids and IV methylprednisolone, duration of corticosteroid use, current use of corticosteroids and IV methylprednisolone, and past and current use of anticoagulants. Univariate analyses of variables possibly associated with vertebral fractures included BMD of the lumbar spine and hip as a variable.

To determine which factors were significantly associated with low BMD or with vertebral fractures, the demographic, clinical, and treatment variables showing $P < 0.2$ in the univariate analyses and variables with supposed clinical relevance were entered into the respective multiple regression analyses. The multiple regression models were refined by
RESULTS

Clinical, demographic, and treatment variables. The clinical and demographic characteristics of the 107 SLE patients included in the study are shown in Table 1. The majority of the patients were premenopausal, female, and Caucasian. At the time of study inclusion, most patients had mild disease activity and little organ damage. Decreased renal function was found in only a small percentage of the patients. 25(OH)D deficiency was detected in 8% of the patients. A history of at least 1 nonvertebral fracture following the diagnosis of lupus was present in 11% of the patients. The majority of patients had taken corticosteroids and hydroxychloroquine. Bisphosphonates and HRT were taken by a small percentage of the patients. 25(OH)D deficiency and low BMD at the spine (B < 0.001), but not at the hip. Moreover, every use of phenprocoumon (a slow-acting coumarin derivative) was significantly associated with low BMD at the hip (B = -0.19, P = 0.01), but not at the spine. BMD at the spine and at the total hip were not associated with age, race, disease duration, disease activity, or measures of corticosteroid exposure (past and current IV methylprednisolone use, past use of oral corticosteroids, duration of oral corticosteroid use, maximum dosage of oral corticosteroid ever taken, current use of oral corticosteroids, and actual oral corticosteroid dosage).

Multiple regression analyses. In a multiple regression analysis of the relationship between menstrual status, BMI, age, and serum 25(OH)D deficiency as independent variables and BMD at the spine as the dependent variable, postmenopausal status (P = 0.001),

Table 1. Demographic, clinical, and treatment variables in the study patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>All study patients (n = 107)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Demographic variables</td>
<td></td>
</tr>
<tr>
<td>Female sex, %</td>
<td>93</td>
</tr>
<tr>
<td>Premenopausal, %</td>
<td>72</td>
</tr>
<tr>
<td>Caucasian race, %</td>
<td>79</td>
</tr>
<tr>
<td>Age, mean ± SD years</td>
<td>41 ± 13</td>
</tr>
<tr>
<td>Body mass index, mean ± SD kg/m²</td>
<td>25 ± 6</td>
</tr>
<tr>
<td>Current smoker, %</td>
<td>22</td>
</tr>
<tr>
<td>Exercise ≥3 times weekly, %</td>
<td>29</td>
</tr>
<tr>
<td>Daily dietary calcium intake, mean ± SD mg</td>
<td>775 ± 317</td>
</tr>
<tr>
<td>Clinical variables</td>
<td></td>
</tr>
<tr>
<td>Disease duration, mean ± SD years</td>
<td>6.9 ± 6.7</td>
</tr>
<tr>
<td>SLEDAI, mean ± SD</td>
<td>4.9 ± 4.0</td>
</tr>
<tr>
<td>ECLAM score, mean ± SD</td>
<td>3.1 ± 1.6</td>
</tr>
<tr>
<td>SLICC/ACR damage index, mean ± SD</td>
<td>1.4 ± 1.9</td>
</tr>
<tr>
<td>SLICC/ACR damage index modified, mean ± SD</td>
<td>1.3 ± 1.9</td>
</tr>
<tr>
<td>Erythrocyte sedimentation rate, mean ± SD mm/hour</td>
<td>26 ± 25</td>
</tr>
<tr>
<td>C-reactive protein, mean ± SD mg/liter</td>
<td>11 ± 17</td>
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<tr>
<td>Creatinine clearance &lt; 70 ml/minute, %</td>
<td>18</td>
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<tr>
<td>Ever had lupus nephritis, %</td>
<td>21</td>
</tr>
<tr>
<td>25-hydroxyvitamin D deficiency, %</td>
<td>8</td>
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<tr>
<td>Ultraviolet light intolerance, %</td>
<td>59</td>
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<tr>
<td>Use of sunscreen, %</td>
<td>61</td>
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<tr>
<td>Previous nonvertebral fracture, %</td>
<td>11</td>
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<tr>
<td>Previous symptomatic vertebral fracture, %</td>
<td>2</td>
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<td>Treatment variables</td>
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<td>Oral corticosteroids</td>
<td>81</td>
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<tr>
<td>Ever use, %</td>
<td>54</td>
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<tr>
<td>Treatment duration in ever users, mean ± SD months</td>
<td>62 ± 69</td>
</tr>
<tr>
<td>Actual prednisone dosage, mean ± SD mg/day</td>
<td></td>
</tr>
<tr>
<td>All patients</td>
<td>7 ± 11</td>
</tr>
<tr>
<td>Current users only</td>
<td>13 ± 12</td>
</tr>
<tr>
<td>Ever use of other medications, %</td>
<td></td>
</tr>
<tr>
<td>Methylnprednisolone</td>
<td>17</td>
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<tr>
<td>Hydroxychloroquine</td>
<td>87</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>11</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>13</td>
</tr>
<tr>
<td>Phenprocoumon (slow-acting coumarin derivative)</td>
<td>4</td>
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<tr>
<td>Hormone-replacement therapy</td>
<td>11</td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td>22</td>
</tr>
<tr>
<td>Current use of other medications, %</td>
<td></td>
</tr>
<tr>
<td>Methotrexate</td>
<td>3</td>
</tr>
<tr>
<td>Hormone-replacement therapy</td>
<td>5</td>
</tr>
<tr>
<td>Bisphosphonates</td>
<td>16</td>
</tr>
<tr>
<td>Calcium supplements</td>
<td>51</td>
</tr>
<tr>
<td>Vitamin D supplements</td>
<td>33</td>
</tr>
</tbody>
</table>

* SLEDAI = Systemic Lupus Erythematosus Disease Activity Index (range 0–105); ECLAM = European Consensus Lupus Activity Measure (range 0–10); SLICC/ACR = Systemic Lupus International Collaborating Clinics/American College of Rheumatology (modified damage index excludes osteoporotic fractures as a damage item).
Table 2. BMD variables and assessment of vertebral deformities*

<table>
<thead>
<tr>
<th>Variable</th>
<th>All study patients (n = 107)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD, mean ± SD gm/cm²</td>
<td></td>
</tr>
<tr>
<td>Spine L1-L4</td>
<td>1.02 ± 0.15</td>
</tr>
<tr>
<td>Total hip</td>
<td>0.91 ± 0.13</td>
</tr>
<tr>
<td>T score, mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Spine L1-L4</td>
<td>−0.23 ± 1.32</td>
</tr>
<tr>
<td>Total hip</td>
<td>−0.27 ± 1.06</td>
</tr>
<tr>
<td>Z score, mean ± SD</td>
<td></td>
</tr>
<tr>
<td>Spine L1-L4</td>
<td>0.24 ± 1.35</td>
</tr>
<tr>
<td>Total hip</td>
<td>0.49 ± 1.11</td>
</tr>
<tr>
<td>Osteopenia, %</td>
<td></td>
</tr>
<tr>
<td>Lumbar spine and/or total hip</td>
<td>39</td>
</tr>
<tr>
<td>Lumbar spine</td>
<td>35</td>
</tr>
<tr>
<td>Total hip</td>
<td>29</td>
</tr>
<tr>
<td>Osteoporosis, %</td>
<td></td>
</tr>
<tr>
<td>Lumbar spine and/or total hip</td>
<td>4</td>
</tr>
<tr>
<td>Lumbar spine</td>
<td>3</td>
</tr>
<tr>
<td>Total hip</td>
<td>2</td>
</tr>
<tr>
<td>Vertebral deformities in 90 SLE patients, %</td>
<td></td>
</tr>
<tr>
<td>At least 1 vertebral deformity</td>
<td>20</td>
</tr>
<tr>
<td>At least 2 vertebral deformities</td>
<td>7</td>
</tr>
<tr>
<td>Severity of 26 vertebral deformities in 90 SLE patients, %</td>
<td></td>
</tr>
<tr>
<td>Grade 1 (20–25% reduction of height)</td>
<td>73</td>
</tr>
<tr>
<td>Grade 2 (25–40% reduction of height)</td>
<td>23</td>
</tr>
<tr>
<td>Grade 3 (&gt;40% reduction of height)</td>
<td>4</td>
</tr>
</tbody>
</table>

* Osteopenia is defined as a T score less than −1.0 SD. Osteoporosis is defined as a T score less than −2.5 SD in at least 1 region of measurement. Vertebral deformities are defined as at least a grade 1 deformity (≥20% reduction of vertebral body height) according to the method of Genant et al (24). BMD = bone mineral density; SLE = systemic lupus erythematosus.

low BMI (P = 0.025), and serum 25(OH)D deficiency (P = 0.047) were significantly associated with low BMD at the spine. In a multiple regression analysis of the relationship between menstrual status, sex, and BMI as independent variables and BMD at the hip as the dependent variable, low BMI (P = 0.0001) and postmenopausal status (P = 0.037) were significantly associated with low BMD at the hip (Table 3). None of the other variables investigated demonstrated a significant contribution to the model for spine or hip BMD. The final models explained 40% of the variation for spine BMD and 43% of the variation for hip BMD.

Vertebral deformities. Lateral spine radiographs were available in 90 patients. Osteopenia was present in 39% of these patients and osteoporosis in 3%. The results of the assessment of vertebral deformities are shown in Table 2. The total number of vertebral fractures, defined according to Genant et al as a reduction of the vertebral body height by at least 20%, was 26. Of all vertebral fractures, 89% were located in the thoracic spine and 11% in the lumbar region. At least 1 vertebral fracture was observed in 18 patients (20%; 95% confidence interval 13–29) and at least 2 vertebral fractures in 6 patients (7%; 95% confidence interval 3–14). Twenty-seven percent of vertebral fractures were grade 2 (at least 25% reduction of vertebral body height) or higher.

Variables associated with vertebral deformities. Univariate analyses. There was a significant association between male sex (B = 0.47, P = 0.001) and age (B = 0.0075, P = 0.024) and fractures in the thoracic and/or lumbar spine. Furthermore, there was a trend toward previous nonvertebral fractures (B = 0.27, P = 0.054), ever use of IV methylprednisolone (B = 0.21, P = 0.054), and non-Caucasian race (B = −0.19, P = 0.058).

Multivariate analyses. In a multivariate analysis of the relationship between vertebral fractures as the dependent variable and sex, age, race, modified DI, previous nonvertebral fractures, and ever use of IV methylprednisolone as the independent variables, ever use of IV methylprednisolone (P = 0.01) and male sex (P = 0.002) were significantly associated with fractures in the thoracic and/or lumbar spine (Table 3).

DISCUSSION

This is the first study on the estimation of prevalent vertebral fractures in a large group of SLE patients, using a standardized semiquantitative method of scoring vertebral deformities. Associations between clinical data and low BMD and vertebral fractures were also evaluated in this large group of SLE patients. The main conclusion from our study is that low BMD and vertebral fractures are observed frequently in SLE patients, which emphasizes that osteoporosis is a common feature in SLE. In addition, we found a significant association between the prevalence of vertebral fractures and ever use of methylprednisolone and male sex.

The frequency of osteopenia found in our population (39%) is consistent with previous studies showing osteopenia in 25–46% of SLE patients (2–4). The prevalence of osteoporosis in our patients (4%) was in the lower range seen in previous studies of patients with SLE (1.4–23%) (5–7). The association between postmenopausal status and low BMD found in 2 previous studies in female patients with SLE (5,25) and the association between low BMI and low BMD demonstrated in other studies in SLE patients (5,6,26–28) were confirmed in the present study.

Deficiency of serum 25(OH)D was significantly associated with low BMD in the lumbar spine in our patients. Low serum levels of 25(OH)D in SLE patients have been previously described and are usually ascribed...
to conscious avoidance of exposure to the sun and/or the use of sunscreens by these patients (29–32).

Surprisingly, a relationship between corticosteroid use and low BMD could not be demonstrated in our study. This observation is supported by various studies in SLE patients (12–15,28–30,33–35) but is in conflict with other studies in SLE patients in which an association between corticosteroid use and low BMD in the lumbar spine and/or the hip was demonstrated (2,3,5–7,11,16,17,25,26). The reasons for this discrepancy are unclear but may be related to differences between patient populations in, for example, size, mean age, disease duration, and menstrual status, as well as differences between centers in treatment strategies for osteoporosis, use of corticosteroids, and differences in assessments of corticosteroid use.

Subanalyses of variables associated with low BMD at the lumbar spine and at the hip in patients who had never been treated with bisphosphonates and/or HRT (n = 77) confirmed the importance of low BMI and postmenopausal status as major risk factors for low BMD at lumbar spine and at the hip, both in univariate and multiple regression analyses (data not shown). In these subanalyses, 25(OH)D deficiency was not significantly associated with low BMD, a finding that might be explained by the small number of patients with 25(OH)D deficiency in the subgroup.

The most striking finding of the present study was the high prevalence of vertebral fractures in our patients (20%), who had a mean age of 41 years, compared with a prevalence of 12% in the general population of Europe ages 65–69 years (36). One would expect a lower prevalence of vertebral fractures in a younger population (36).

Only a few studies on fractures in SLE have been published, and these were focused on incident symptomatic vertebral and nonvertebral fractures. In 4 studies, symptomatic vertebral and nonvertebral fractures occurring since the onset of lupus were documented in 9–16.5% of patients (2,3,5,9). However, studies focusing on symptomatic fractures have a disadvantage in that only one-third of all vertebral fractures come to clinical attention (37). In the present study, only 2 of 18 patients with 1 or more prevalent vertebral fractures had a documented previous symptomatic fracture, which illustrates the possibility of underestimating vertebral fractures in patients with SLE if only symptomatic fractures are considered in the scoring.

The association between ever use of IV methylprednisolone and the prevalence of vertebral fractures in this study is consistent with 2 studies documenting an association between corticosteroid use and symptomatic fractures in SLE (9,10). The association between male sex and prevalent vertebral fractures in our study is not surprising, since in the general population, the prevalence of vertebral fractures at ages younger than 65 years is higher in men than in women (36).

A subanalysis of factors associated with grade 1 vertebral fractures (according to the method of Genant et al) demonstrated the same significant association.

| Table 3. Multiple regression analyses of BMD at the lumbar spine, BMD at the total hip, and vertebral deformities (dependent variables) and demographic, clinical, and treatment variables (independent variables)* |
| Variable | B | SE | P |
| BMD at the lumbar spine in 107 SLE patients | | | |
| Postmenopause | −0.14 | 0.04 | <0.01 |
| Body mass index | 0.0058 | 0.0030 | 0.025 |
| Age | 0.0021 | 0.001 | 0.140 |
| 25-hydroxyvitamin D deficiency | −0.10 | 0.051 | 0.047 |
| BMD at the total hip in 107 SLE patients | | | |
| Postmenopause | −0.060 | 0.029 | 0.037 |
| Male sex | 0.088 | 0.048 | 0.07 |
| Body mass index | 0.0092 | 0.0020 | <0.0001 |
| Vertebral deformities in 90 SLE patients | | | |
| Ever use of IV methylprednisolone | 0.28 | 0.105 | 0.01 |
| Nonvertebral fractures | 0.19 | 0.141 | 0.18 |
| SLICC/ACR damage index modified | −0.013 | 0.024 | 0.61 |
| Male sex | 0.47 | 0.143 | 0.002 |
| Age | 0.0039 | 0.0040 | 0.29 |
| Noncaucasian | −0.13 | 0.099 | 0.21 |

*BMD = bone mineral density; SLE = systemic lupus erythematosus; IV = intravenous; SLICC/ACR = Systemic Lupus International Collaborating Clinics/American College of Rheumatology; B = regression coefficient (when considered in the multiple regression model); SE = standard error of B.
between ever use of IV methylprednisolone and prevalent vertebral fractures, both in univariate and multiple regression analyses (data not shown). These results demonstrate that ever use of IV methylprednisolone is also a strong risk factor for prevalent vertebral fractures after the 26% more severe fractures were excluded from the analyses. The subanalysis of factors associated with Genant grade 1 vertebral fractures did not show an association with male sex (data not shown). This finding can be explained by the small number of male patients in the study and the fact that 3 of the 5 male patients with at least 1 vertebral fracture had a fracture that was a Genant grade 2 or more.

Limitations of the present study are the racial background of the study population and the method used to assess corticosteroid use. As a consequence of the rather high percentage of Caucasians in the study population (79%), the associations found in the present study may not be generalized to lupus cohorts with a significantly different racial background. Second, since the cumulative oral corticosteroid dose was not calculated, associations between the cumulative corticosteroid dose and BMD and prevalent vertebral fractures could not be assessed. However, all other measures of oral corticosteroid use we assessed were not associated with BMD or vertebral fractures.

The results of this study suggest that attention must be paid to the prevention and treatment of osteoporosis and fractures as an important disease complication. Prevention strategies directed toward SLE patients who are at risk of osteoporosis include advice for maintaining a normal body weight and performing weight-bearing physical activity, calcium and vitamin D supplementation in cases of deficiency, and treatment with appropriate antiosteoporosis medication in cases of osteoporosis and/or a vertebral fracture. When considering osteoporosis, osteopenia in combination with corticosteroid use and/or the prevalence of 1 or more vertebral fractures as a reason for treatment with antiosteoporosis drugs, 40% of the patients in our study should have been treated with antiosteoatosis drugs. At the time of the study, only 16% of the patients were taking bisphosphonates and 5% were taking HRT.

The high prevalence of vertebral fractures in our study indicates that the assessment of fracture risk in SLE patients should include assessment of vertebral fractures, since these are often asymptomatic and are clinically important in terms of morbidity, mortality, and future fracture risk. Therefore, we recommend that in the assessment of osteoporosis and future fracture risk in SLE patients, spine radiographs (analyzed using a standardized method for scoring vertebral deformities) and measurements of spine and hip BMD be performed.

**REFERENCES**

18. Oleksik A, Lips P, Dawson A, Minshall ME, Shen W, Cooper C,
Frequency of Osteopenia in Children and Young Adults With Childhood-Onset Systemic Lupus Erythematosus

Vibke Lilleby,1 Gunhild Lien,1 Kathrine Frey Frøslie,1 Margaretha Haugen,2 Berit Flatø,1 and Øystein Førre1

Objective. To determine the frequency of osteopenia in children with childhood-onset systemic lupus erythematosus (SLE) compared with that in healthy matched controls, and to evaluate the relationship between disease-related variables and bone mineral mass.

Methods. Bone mineral density (BMD) and bone mineral content (BMC) were measured in a cohort of 70 patients with childhood-onset SLE (mean ± SD disease duration 10.8 ± 8.3 years, mean ± SD age 26.4 ± 9.9 years) and 70 age- and sex-matched healthy controls. BMD and BMC of the femoral neck, lumbar spine, total body, and distal one-third of the radius were measured by dual x-ray absorptiometry. We investigated the relationship between BMC and the following disease variables: cumulative dose of corticosteroids, organ damage, current use of corticosteroids, use of cyclophosphamide, age at disease onset, and disease activity at the time of diagnosis. Biochemical markers of bone metabolism were also measured.

Results. BMD values for the lumbar spine and femoral neck were significantly lower in patients than in healthy controls. The reduction in BMD of the lumbar spine was significantly greater than that of the total body. In multiple linear regression analyses, a higher cumulative corticosteroid dose was significantly associated with lower BMC of the lumbar spine and femoral neck. Decreased lumbar spine BMC was also related to male sex.

Conclusion. The frequency of osteopenia was higher in patients with childhood-onset SLE than in matched controls. The lumbar spine was the most seriously affected skeletal site, followed by the femoral neck. The cumulative dose of corticosteroids was shown to be an important explanatory variable for BMC values in the lumbar spine and femoral neck.

Systemic lupus erythematosus (SLE) is a chronic, inflammatory, multisystem disease for which long-term corticosteroid therapy often is required. Use of corticosteroids was a breakthrough in the treatment of SLE and has led to increased survival; however, the longer survival time has meant that these patients now experience a range of complications, some of which are attributable to the disease itself and some of which are medication side effects (1). These complications include reduced bone mass and osteoporosis, which are recognized as major health problems in patients with SLE (2).

Several studies of osteoporosis have involved adult patients with SLE (3–11), but only a few such studies have addressed childhood-onset SLE (12,13). Bone mass increases throughout childhood and adolescence, reaching a peak in the late teens or early adulthood (14). Peak bone mass is partly genetically determined but is also influenced by hormonal factors, physical activity, and nutritional factors (15,16). The presence of lower-than-expected peak bone mass during the period of skeletal growth increases the risk of osteoporosis and fractures later in life (17,18).

Several other factors are associated with the risk of osteopenia in patients with SLE, including inflammation, renal failure, ovarian dysfunction, lack of sun exposure (due to conscious avoidance), and intake of corticosteroids (19,20). Endocrinologically, corticosteroids are known to adversely affect bone mass.
through decreased formation and increased resorption of bone (21,22), but the impact of corticosteroids on bone loss in the setting of SLE is unclear (6,9,10,19).

We evaluated the frequency of osteopenia in a cohort of Norwegian patients with childhood-onset SLE and compared it with that in healthy age- and sex-matched controls. We also investigated the relationship between disease-related variables and bone mass in the lumbar spine, femoral neck, total body, and forearm.

PATIENTS AND METHODS

Patients and healthy controls. The current cross-sectional study was performed at the Department of Rheumatology, Rikshospitalet University Hospital, which serves the majority of the population of southern Norway (~2.5 million people). The study population comprised 70 children, adolescents, and young adults with childhood-onset SLE. These subjects represent 91% of 77 patients with childhood-onset SLE who were identified by the search strategy described below. The inclusion criteria were disease onset before the age of 16 years, a minimum disease duration of 12 months, and the presence of at least 4 of the American College of Rheumatology (ACR) criteria for classification of systemic lupus erythematosus (23).

Sixty-four patients who had been admitted to Rikshospitalet University Hospital between January 1980 and June 2003 were identified from the patient register. Fifty-eight of those patients were included in the study; the other 6 patients were not enrolled (1 had a history of drug-induced lupus, 4 had died, and 1 could not participate due to severely impaired health status). Thirteen patients who had been admitted to other hospitals were identified as a result of a request to the National Register of Autoimmune Disease and questionnaires about new and previously treated patients with childhood-onset SLE that were mailed to all pediatric and rheumatology clinics in Norway. Of these patients, 12 were enrolled and 1 chose not to participate. No significant differences with respect to age, sex, disease duration, disease activity, and medication requirements were observed between patients recruited from Rikshospitalet and those recruited from other hospitals.

Healthy controls (matched for age and sex with each SLE patient) were selected randomly from the population registry of Oslo and the neighboring county of Akershus. An invitation to participate was mailed to 255 individuals, 85 of whom (33.3%) accepted. For some SLE patients, we identified 2 or more matched controls due to the different numbers of responders and nonresponders. In such cases, the first control to be examined was selected as a matched control for the patient in question.

Informed consent was obtained from the patients, controls, and the parents of patients younger than age 16 years. This study was approved by the Regional Ethics Committee for Medical Research.

Data collection and clinical measures. All of the patients and controls were examined in accordance with a 1-day program at Rikshospitalet University Hospital, which included a clinical examination by a single physician (VL), laboratory measures, self-reported health status questionnaires, and bone mass measurements. In patients with SLE, disease activity and cumulative organ damage were measured by the SLE Disease Activity Index (SLEDAI) (24,25) and the Systemic Lupus International Collaborating Clinics (SLICC)/ACR Damage Index (SDI) (26,27). In addition, SLEDAI scores for the time of diagnosis were calculated retrospectively. Information on the cumulative corticosteroid dose, including pulses of methylprednisolone (expressed as prednisolone equivalents), and other reported disease variables were obtained from patients' medical records. Disease onset was defined as the time at which the initial clinical symptoms clearly attributable to SLE appeared. Disease duration was defined as the period of time from the diagnosis of SLE until the time of the study.

The erythrocyte sedimentation rate (ESR) and serum levels of albumin, ionized calcium, and parathyroid hormone (PTH) were measured by routine laboratory methods. Bone formation was assessed by measuring serum levels of osteocalcin and bone-specific alkaline phosphatase. Bone resorption was evaluated by measuring serum levels of C-terminal type I telopeptide and the urinary concentration of deoxypyridinoline.

Physical activity was quantified on a 6-point scale, where daily physical activity for 20 minutes or longer = 6, and no or very rare physical activity = 1. Physical activity in this context was defined as activity causing the subject to perspire and experience shortness of breath. Food and nutrient intake were estimated by a standardized quantitative food frequency questionnaire (28), and calculations were performed with use of the Norwegian Food Composition Table (29). Physical disability was measured by the Childhood Health Assessment Questionnaire (C-HAQ) and, for patients older than age 18 years, by the HAQ (30,31).

Bone mass measurements. Bone mineral mass in the femoral neck, the second through fourth lumbar vertebrae, the total body, and the distal one-third of the radius was measured with dual x-ray absorptiometry (DXA) equipment (Lunar Expert-XL; GE Lunar, Madison, WI). All analyses were performed by a single investigator using Expert-XL software version 1.91 (GE Lunar). The scanner was calibrated daily with an aluminum spine phantom, and the in vitro coefficient of variation (CV) was 0.5%. The in vivo CV (patients and healthy subjects) was 1.6% for the spine (second through fourth lumbar vertebrae) and 2% for the femoral neck. Four of our patients underwent bone mineral measurements with other DXA equipment (Lunar DPX-L; GE Lunar), which was located at the Endocrinology Department of the Rikshospitalet University Hospital. The Lunar Expert-XL (fan-beam) produces results that correlate highly with the Lunar DPX series (pencil-beam) (32,33). Due to the presence of prosthetic joints, measurements of the total body were not obtained in 2 patients. Measurements of the distal one-third of the radius were missing in 1 patient and 1 control.

Bone mass was expressed as bone mineral density (BMD), bone mineral content (BMC), or as a Z score in terms of the number of SDs above or below the age-specific mean for healthy individuals. At present, Norwegian reference values for bone mass measurement in children and adults are not available. We therefore chose to use age- and sex-specific numerical data provided by the manufacturer (GE Lunar) to calcu-
late Z scores for BMD using the following formula: Z score = (subject’s measurement – mean measurement of the reference population)/SD of the reference population. BMD reference values for the forearm were available only for individuals ages 20 years and older. Reduced bone mass (osteopenia) was defined as a Z score less than -1 SD (34).

Bone mass was also expressed as the T score (the number of SDs above or below the mean value for young adults at the time of peak bone mass) (35), but only for patients ages 20 years and older (n = 45). This score is not applicable to children or adolescents, because they have not yet achieved peak bone mass. For the same reason, the World Health Organization definition of osteopenia (a T score between 1 and 2.5 SD below the mean value for young adults) and osteoporosis (a T score less than -2.5 SD) cannot be applied to children and adolescents (36).

**Statistical analysis.** Differences between patients and matched controls were tested by the paired samples t-test, Wilcoxon’s test, and McNemar’s test. Differences between patient groups (e.g., patients younger than age 20 years versus patients age 20 years and older) were tested by the independent sample t-test, the Mann-Whitney test, and the chi-square test. Reductions in BMD (BMD in controls - BMD in patients) at the various skeletal sites were compared using Friedman’s test, with paired samples t-tests (with Bonferroni correction) as post hoc tests. Univariate and multiple linear regression analyses were used to investigate the impact of disease variables on bone mass measurements. To avoid the possibility of size-related artifacts, bone area, weight, and height were included in the multiple regression models, as proposed by Prentice et al (37). Additional variables that are considered to be possible predictors of BMC are listed in Table 4. The variable “disease duration” was not included in regression analyses, due to high correlation with the variable “age.” The variables were entered in multiple analyses by forward variable selection methods. We thoroughly checked for possible violations from the model assumptions during analyses. P values less than or equal to 0.05 were considered significant. All statistical analyses were performed using SPSS version 12.01 software (Chicago, IL).

**RESULTS**

**Characteristics of the study subjects.** The study population comprised 70 patients with childhood-onset SLE and 70 healthy controls, who were individually matched for age and sex. The mean ± SD disease

<table>
<thead>
<tr>
<th>Table 1. Characteristics of SLE patients and healthy controls*</th>
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<tbody>
<tr>
<td>Characteristic</td>
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<tr>
<td>Females, no. (%)</td>
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<tr>
<td>Age, years (range)</td>
</tr>
<tr>
<td>Caucasian, no. (%)</td>
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<tr>
<td>Body mass index, kg/m²</td>
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<tr>
<td>Weight, kg</td>
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<tr>
<td>Height, cm</td>
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<tr>
<td>Smoker, current or previous, no. (%)</td>
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<tr>
<td>Physical activity of ≥20 minutes’ duration</td>
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<td>Calcium intake, mg/day</td>
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<tr>
<td>Vitamin D intake, μg/day</td>
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<tr>
<td>Vitamin D supplementation, μg/day‡</td>
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<tr>
<td>Disease duration, years</td>
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<td>Age at disease onset, years (range)</td>
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<td>Osteoporotic fractures, no. (%)</td>
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<td>HAQ/C-HAQ score (range 0–3)</td>
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<td>SLEDAI at time of diagnosis</td>
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<tr>
<td>SLICC/ACR Damage Index</td>
</tr>
<tr>
<td>Nephritis, no. (%)</td>
</tr>
<tr>
<td>Corticosteroids</td>
</tr>
<tr>
<td>Ever user, no. (%)</td>
</tr>
<tr>
<td>Current user, no. (%)</td>
</tr>
<tr>
<td>Prednisolone, current dosage, mg/day</td>
</tr>
<tr>
<td>Cumulative dose, gm</td>
</tr>
<tr>
<td>Duration of use, months</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean ± SD. SLE = systemic lupus erythematosus; NA = not applicable; HAQ = Health Assessment Questionnaire; C-HAQ = Childhood HAQ; SLEDAI = SLE Disease Activity Index; SLICC/ACR = Systemic Lupus International Collaborative Clinics/American College of Rheumatology.
† P < 0.05 versus controls.
‡ For patients with childhood-onset SLE, n = 32; for controls, n = 24.
§ P < 0.001 versus controls.
duration in the patients was 10.8 ± 8.3 years, and their mean ± SD age was 26.4 ± 9.9 years (range 9.8–49.3 years). Characteristics of the study groups are presented in Table 1. The patients and healthy controls were comparable with respect to body mass index, weight, daily calcium and vitamin D intake, physical activity, and smoking habits, but the mean height of the patients was significantly lower than that of controls. The patient group was more disabled physically than the healthy group, as reflected by significantly higher C-HAQ/HAQ scores in the patients.

A comparison of female and male patients showed a similar age distribution (mean ± SD age 26.5 ± 10.0 years and 26.4 ± 9.9 years, respectively) between sexes and revealed no significant differences with regard to clinical manifestations, disease duration, age at disease onset, the SLEDAI, the SDI, or the cumulative corticosteroid dose (data not shown).

### BMD findings

BMD in the lumbar spine and femoral neck was significantly lower in patients with childhood-onset SLE than in healthy controls; this difference was independent of the age group (age <20 years or ≥20 years) (Table 2 and Figure 1). The difference between patients and controls was also significant for total body BMD and for BMD of the distal one-third of the radius, when patients and controls were not divided into age groups (data not shown). The reduction in lumbar spine BMD (BMD in matched control – BMD in patient) was significantly greater than the reduction in total body BMD and BMD of the distal one-third of the radius, but after Bonferroni correction, this difference for the radius did not reach significance ($P = 0.051$).

Male patients had significantly lower mean Z scores for the lumbar spine and total body compared with female patients ($P = 0.034$ and $P = 0.023$, respectively). In

<table>
<thead>
<tr>
<th>Variable</th>
<th>Patients with childhood-onset SLE (n = 70)</th>
<th>Healthy controls (n = 70)</th>
<th>$P$†</th>
<th>Mean reduction in BMD, %‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>BMD, gm/cm²</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spine, L2-L4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;20 years</td>
<td>1.03 ± 0.20</td>
<td>1.16 ± 0.19</td>
<td>0.003</td>
<td>10.1§</td>
</tr>
<tr>
<td>Age ≥20 years</td>
<td>1.13 ± 0.18</td>
<td>1.28 ± 0.17</td>
<td>&lt;0.001</td>
<td>11.1§</td>
</tr>
<tr>
<td>Femoral neck</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;20 years</td>
<td>0.95 ± 0.18</td>
<td>1.05 ± 0.16</td>
<td>0.045</td>
<td>7.4</td>
</tr>
<tr>
<td>Age ≥20 years</td>
<td>0.94 ± 0.17</td>
<td>1.06 ± 0.13</td>
<td>&lt;0.001</td>
<td>10.1</td>
</tr>
<tr>
<td>Distal one-third of the radius</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;20 years</td>
<td>0.56 ± 0.10</td>
<td>0.61 ± 0.09</td>
<td>0.046</td>
<td>3.4</td>
</tr>
<tr>
<td>Age ≥20 years</td>
<td>0.70 ± 0.09</td>
<td>0.72 ± 0.13</td>
<td>0.305</td>
<td>4.2</td>
</tr>
<tr>
<td>Total body</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;20 years</td>
<td>1.07 ± 0.11</td>
<td>1.12 ± 0.11</td>
<td>0.107</td>
<td>3.9</td>
</tr>
<tr>
<td>Age ≥20 years</td>
<td>1.16 ± 0.09</td>
<td>1.21 ± 0.08</td>
<td>0.001</td>
<td>4.1</td>
</tr>
<tr>
<td>Z score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Spine, L2-L4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;20 years</td>
<td>−0.72 ± 1.43</td>
<td>0.60 ± 1.21</td>
<td>0.002</td>
<td>–</td>
</tr>
<tr>
<td>Age ≥20 years</td>
<td>−0.68 ± 1.03</td>
<td>0.41 ± 0.98</td>
<td>&lt;0.001</td>
<td>–</td>
</tr>
<tr>
<td>Femoral neck</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;20 years</td>
<td>−0.45 ± 1.52</td>
<td>0.45 ± 1.05</td>
<td>0.041</td>
<td>–</td>
</tr>
<tr>
<td>Age ≥20 years</td>
<td>−0.64 ± 1.42</td>
<td>0.41 ± 0.98</td>
<td>&lt;0.001</td>
<td>–</td>
</tr>
<tr>
<td>Distal one-third of the radius</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;20 years</td>
<td>NA</td>
<td>NA</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Age ≥20 years</td>
<td>−0.39 ± 0.95</td>
<td>−0.16 ± 0.88</td>
<td>0.305</td>
<td>–</td>
</tr>
<tr>
<td>Total body</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &lt;20 years</td>
<td>0.02 ± 1.07</td>
<td>0.47 ± 0.90</td>
<td>0.087</td>
<td>–</td>
</tr>
<tr>
<td>Age ≥20 years</td>
<td>0.09 ± 1.12</td>
<td>0.51 ± 0.88</td>
<td>0.057</td>
<td>–</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean ± SD. Twenty-five patients were younger than age 20 years, and 45 patients were age 20 years or older. BMD = bone mineral density; SLE = systemic lupus erythematosus; NA = not available.
† By paired samples t-test.
‡ BMD in matched control – BMD in patient with childhood-onset SLE.
§ $P < 0.01$, lumbar spine versus total body, by Friedman’s test, with Bonferroni correction.
addition, Z scores for the femoral neck tended to be nonsignificantly lower in male patients (data not shown).

**Frequency of reduced bone mass.** The frequency of osteopenia (Z score less than $-1$ SD) was significantly higher in patients with childhood-onset SLE than in matched healthy controls for all 4 sites at which BMD was measured (Figure 2). The sites affected most frequently were the femoral neck (40% of patients versus 6% of controls) and the lumbar spine (41% of patients versus 7% of controls). When the patients were divided into 2 groups on the basis of age, a comparison between the groups showed that the frequency of reduced bone mass in patients younger than age 20 years was almost the same as that in patients ages 20 years and older (Figure 2).

Osteoporosis (T score less than $-2.5$ SD) was detected only in patients with childhood-onset SLE and not in any healthy controls. Furthermore, osteoporosis was observed only in the femoral neck and the lumbar spine, where the frequencies were 7% and 9%, respectively.

The level of deoxypyridinoline was significantly higher in young adult patients with SLE than in controls (Table 3). There were no statistically significant differences for other markers of bone metabolism, ionized calcium, or PTH.

**Relationship between disease variables and BMC.** Multiple linear regression analyses showed that the impact of the cumulative corticosteroid dose on the BMC was significant for the femoral neck and the lumbar spine (Table 4). In the femoral neck and lumbar spine, an increasing cumulative corticosteroid dose was associated with a decreasing BMC. In addition to the cumulative dose of corticosteroids, sex was also found to be a significant predictor of BMC in the lumbar spine, where the BMC was lower in men than in women. Although univariate analysis demonstrated that age at disease onset was highly associated with lumbar spine BMC ($P < 0.001$), the effect was only borderline significant ($P = 0.054$) after adjustment for sex and corticosteroids. No disease-related variables were identified as independent predictors of BMC in the distal one-third of the radius and the total body.

**DISCUSSION**

In the present cohort of 70 patients with childhood-onset SLE with a mean disease duration of $10.8 \pm 8.3$ years, BMD in the lumbar spine and femoral neck was significantly lower than that in matched healthy controls. We also found that in our patients with childhood-onset SLE, a higher cumulative corticosteroid dose was significantly associated with a lower bone mass in the lumbar spine and femoral neck. To our knowledge, this study is the first to describe the frequency of reduced bone mass and associated factors in patients with childhood-onset SLE of long-term duration.

The lower bone mass observed in our patients with childhood-onset SLE is consistent with results reported in several studies of adult patients with SLE.
Two previous studies of childhood-onset SLE (12,13) also found lower BMD in the spine and femoral neck of patients compared with controls. The frequency of osteoporosis in the femoral neck (7%) and lumbar spine (9%) observed in our study was in the lower range as compared with the frequency reported in other studies (range 8–22%) (10,39,40). This might partly be explained by the younger mean age of our patients, and may also be attributable to less selection bias in favor of severely diseased patients. In southern Norway, our department represents the only pediatric rheumatology clinic, and the patients who were recruited from Rikshospitalet University Hospital presumably represent the vast majority of patients in whom childhood-onset SLE was diagnosed during the given time period. Alternatively, in 2 North American studies (26, 41), higher SDI scores were reported, and based on this, a milder disease in our patient population may be another explanation. However, our patients demonstrated a relatively high frequency of osteopenia in the femoral neck (40%) and lumbar spine (41%) during the early stages of life, which is associated with a potentially higher risk of osteoporosis developing later in life.

The cumulative dose of corticosteroids was found to be an important variable in explaining decreased bone mass in the femoral neck and lumbar spine. These findings are consistent with those of several studies in adult-onset SLE (6,8–10) and contradict the results of other studies (4,38). Trapani et al also reported an association between the cumulative steroid dose and BMD in patients with childhood-onset SLE (13), whereas Castro et al did not observe such an association (12). The impact of corticosteroids have been a subject of controversy (19,42,43), but some of the differences between studies may be attributable to patient selection, study design, and different uses of corticosteroids (i.e., high-dose, low-dose, oral, intravenous). Interestingly, in our patients, corticosteroids were not identified as predictors of bone loss in the distal one-third of the radius and the total body. This probably indicates that corticosteroids have a greater impact on bone loss in areas containing greater proportions of trabecular bone (e.g., the lumbar spine), as was noted in 2 other studies (22,43).

Male patients had lower Z scores for the lumbar spine and the total body than did female patients; additionally, in multiple regression analysis, male sex was associated with decreased bone mass. We found no differences in clinical manifestations or disease activity, severity, or duration between male and female patients, which may explain the more severe bone loss in male patients. Only a few studies have investigated bone mass in male patients with SLE, and the findings of those studies contradict ours (44,45). However, a comparison of the results of those studies and our findings may have some limitations, in that those patients presumably did not have lower-than-expected peak bone mass during adolescence and early adulthood. Another reason that could be considered for the more severe bone loss in

<table>
<thead>
<tr>
<th>Table 3. Markers of bone metabolism in patients with childhood-onset SLE and age- and sex-matched healthy controls*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Marker</td>
</tr>
<tr>
<td>Serum osteocalcin, nmoles/liter</td>
</tr>
<tr>
<td>Age &lt; 20 years</td>
</tr>
<tr>
<td>Age ≥ 20 years</td>
</tr>
<tr>
<td>Serum bone-specific alkaline phosphatase, units/liter</td>
</tr>
<tr>
<td>Age &lt; 20 years</td>
</tr>
<tr>
<td>Age ≥ 20 years</td>
</tr>
<tr>
<td>Serum C-telopeptide type I, µg/liter</td>
</tr>
<tr>
<td>Age &lt; 20 years</td>
</tr>
<tr>
<td>Age ≥ 20 years</td>
</tr>
<tr>
<td>Urinary deoxypyridinoline, nM/mM creatinine</td>
</tr>
<tr>
<td>Age &lt; 20 years</td>
</tr>
<tr>
<td>Age ≥ 20 years</td>
</tr>
</tbody>
</table>

* Except where indicated otherwise, values are the mean ± SD. Twenty-five patients were younger than age 20 years, and 45 patients were age 20 years or older. SLE = systemic lupus erythematosus.
† By paired samples t-test.
Table 4. Relationship between disease variables and BMC in 70 patients with childhood-onset SLE*

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lumbar spine</th>
<th>Femoral neck</th>
<th>Distal one-third of the radius</th>
<th>Total body, P, univariate analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>B</td>
<td>95% CI</td>
<td>P</td>
<td>B</td>
</tr>
<tr>
<td>Age, years</td>
<td>&lt;0.001</td>
<td>0.662</td>
<td>0.001</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sex (female = 1, male = 2)</td>
<td>0.313</td>
<td>0.066</td>
<td>0.006</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Age at disease onset</td>
<td>&lt;0.001</td>
<td>0.531</td>
<td>0.079</td>
<td>0.023</td>
</tr>
<tr>
<td>Cumulative CS dose</td>
<td>0.050</td>
<td>0.022</td>
<td>0.089</td>
<td>0.952</td>
</tr>
<tr>
<td>Current prednisolone user (yes = 1, no = 2)</td>
<td>0.002</td>
<td>0.332</td>
<td>0.259</td>
<td>0.073</td>
</tr>
<tr>
<td>Cyclophosphamide ever used (yes = 1, no = 2)</td>
<td>0.068</td>
<td>0.538</td>
<td>0.724</td>
<td>0.409</td>
</tr>
<tr>
<td>SDI</td>
<td>0.120</td>
<td>0.202</td>
<td>0.997</td>
<td>0.334</td>
</tr>
<tr>
<td>SLEDAI at diagnosis</td>
<td>0.338</td>
<td>0.289</td>
<td>0.247</td>
<td>0.391</td>
</tr>
<tr>
<td>ESR at diagnosis</td>
<td>0.754</td>
<td>0.820</td>
<td>0.970</td>
<td>0.809</td>
</tr>
</tbody>
</table>

* All multiple linear regression analyses were adjusted for bone area, weight, and height. R² values (the total explained variance of the model) were as follows: for the lumbar spine, R² = 79%; for the femoral neck, R² = 52%; for the distal one-third of the radius, R² = 81%; and for total body, R² = 90%. Beta values are nonstandardized coefficients. BMC = bone mineral content; SLE = systemic lupus erythematosus; 95% CI = 95% confidence interval; CS = corticosteroids; SDI = Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index; SLEDAI = SLE Disease Activity Index; ESR = erythrocyte sedimentation rate.
male patients with SLE is sex hormone abnormalities, which we did not analyze in our patients. Elevated estrogen-to-androgen ratios and hyperprolactinemia, which are associated with a potential risk of osteoporosis, have been described in male patients with SLE (45).

In randomly selected matched healthy controls, the frequency of osteopenia was low (3–7%). Based on a Gaussian distribution, we would have expected up to 16% of controls to have a Z score less than -1 SD. Despite the fact that our controls were randomly selected from the population registry, there may have been a selection bias in favor of healthier individuals, because such persons were probably more willing to participate in the BMD measurements. The mean height of controls was higher than that of patients, a finding that corroborates previous reports of growth retardation in patients with childhood-onset SLE (46). The higher mean height of the controls may have influenced and aggravated the differences between patients and controls to a certain degree, because shorter height is associated with decreased bone mass.

The higher deoxypyridinoline levels observed in young adults with SLE compared with those in controls are consistent with results reported by Teichmann et al (11). Osteocalcin levels tended to be lower in pediatric-age SLE patients than in controls, a finding reported in previous studies of children with rheumatic diseases (47) and in adults with SLE (11,40). Reduced osteoblast function and increased bone resorption are also known to be effects of corticosteroids (21,48,49). However, the alteration of bone resorption and markers of bone formation has been found to be independent of corticosteroid treatment in patients with SLE (11).

A limitation of the study might be the small sample size. The ages of the SLE patients in the present study were highly variable, and for this reason, we used controls that were individually matched for age and sex, and we adjusted for age, bone area, weight, and height in all multiple linear regression analyses. The successful matching of the study population may be a strength of our study. The relatively high male-to-female ratio in our cohort was similar to that in many previous reports of childhood-onset SLE (41,50). The reason for the higher proportion of male patients with childhood-onset SLE compared with male patients with adult-onset SLE is unknown but may be partly influenced by hormonal factors (41). The high proportion of Caucasian participants in our study may limit the generalizability of our findings to nonwhite patients with lupus.

In conclusion, we observed lower bone mass in the lumbar spine and femoral neck in patients with childhood-onset SLE compared with healthy controls, and the frequency of osteopenia was higher in patients with SLE. Decreasing BMC in the lumbar spine and femoral neck was also associated with a higher cumulative steroid dose. These results should alert clinicians to the potentially higher risk for the development of osteoporosis later in life in patients with childhood-onset SLE. For the prevention of osteoporosis, this would mean administering the lowest possible dose of corticosteroids and using corticosteroid-sparing drugs in clinical practice.

ACKNOWLEDGMENTS

We thank Gunn J. Hovland, Berit Brenden, and Gunhild A. Isaksen for conducting the DXA scans.

REFERENCES

OSTEOPENIA IN PATIENTS WITH CHILDHOOD-ONSET SLE


Systemic Lupus Erythematosus in a Multiethnic US Cohort (LUMINA)

XXV. Smoking, Older Age, Disease Activity, Lupus Anticoagulant, and Glucocorticoid Dose as Risk Factors for the Occurrence of Venous Thrombosis in Lupus Patients

Jaime Calvo-Alén,1 Sergio M. A. Toloza,1 Mónica Fernández,1 Holly M. Bastian,1 Barri J. Fessler,1 Jeffrey M. Roseman,1 Gerald McGwin, Jr.,1 Luis M. Vilá,2 John D. Reveille,3 and Graciela S. Alarcón,1 for the LUMINA Study Group

Objective. Venous thrombosis is a relatively frequent and serious complication in systemic lupus erythematosus (SLE) that has been associated with the presence of antiphospholipid antibodies (aPL). However, venous thrombotic events can also be seen in patients without aPL, and only a few patients with aPL develop venous thrombosis. This study was carried out to ascertain other factors contributing to the development of venous thrombosis in SLE.

Methods. Patients with SLE, ages ≥16 years with ≤5 years disease duration and of Hispanic, African American, or Caucasian ethnicity, from LUMINA (LUpus in MInorities, NAture versus nurture), a multiethnic, longitudinal study of outcome, were studied. Selected socioeconomic/demographic, clinical, laboratory, and treatment-exposure variables were compared between patients who developed and those who did not develop venous thrombotic events. Significant and clinically relevant variables were then entered into different multivariable models (Cox proportional hazards and unconditional stepwise logistic regression) to identify independent risk factors associated with the primary outcome. In another model, only patients who developed an event after enrollment (time 0) in the cohort were included.

Results. Of 570 LUMINA patients, 51 developed at least 1 venous thrombotic event after SLE diagnosis. In univariable analyses, smoking (P = 0.020), shorter disease duration at time 0 (P = 0.017), serum levels of total cholesterol, low-density lipoprotein, and triglycerides (all P < 0.0001), and presence of lupus anticoagulant (LAC) (P = 0.045) were associated with venous thrombotic events. Survival analyses showed a time-dependent significant association of the primary outcome with smoking (P = 0.008) and a borderline significant association with the presence of LAC (P = 0.070). Multivariable models showed an independent association with smoking, age at time 0, disease activity over time, LAC, mean dose of glucocorticoids, and shorter disease duration at time 0.

Conclusion. Venous thrombotic events occur early in the course of SLE. Our data confirm the association between LAC and venous thrombotic events. Smoking, shorter disease duration, older age, disease activity over
time, and higher mean daily glucocorticoid dose were identified as additional risk factors for the development of this vascular complication. These findings may have implications for the management of patients with SLE.

Venous thrombosis is a relatively common manifestation and/or complication occurring in patients with systemic lupus erythematosus (SLE), being reported in ~10% of patients (1,2). Venous thrombosis may lead to life-threatening complications such as pulmonary thromboembolism and pulmonary hypertension or to residual damage such as venous stasis with chronic edema and ulcers of the lower limbs. Furthermore, proportionate mortality in SLE resulting from thrombotic events (arterial and venous together) has been estimated to be ~27% (1). The association of venous thrombosis with antiphospholipid antibodies (aPL) (IgG and IgM antiphospholipid antibodies and lupus anticoagulant [LAC]) has been well established; this has been observed in patients without an underlying autoimmune disease (3), but has particularly been found in patients with SLE (4). The association has been primarily identified in retrospective studies; more recently, a systematic review, a meta-analysis, and a prospective study have confirmed this association (2,4,5).

In SLE, not all patients with aPL develop venous thrombosis and not all patients with venous thrombosis have aPL. For example, the frequency of antiphospholipid antibodies in these patients is reported to be ~34%, and the frequency of LAC is up to 44% (5,6), yet only ~10% of SLE patients develop venous thrombotic events. These data suggest that there may be other factors predisposing lupus patients to develop venous thrombosis. We thus decided to identify these other factors in a multiethnic SLE cohort. The identification of such factors may be important for the management of these patients.

PATIENTS AND METHODS

Patients. As previously described (7,8), LUMINA (LUpus in MInorities, NAture versus nurture) is a longitudinal study of outcome in SLE and includes patients from 3 ethnic groups living in the US: Hispanics from Texas and Puerto Rico, African Americans, and Caucasians. The study is being conducted in 3 geographic areas (Alabama, Texas, and Puerto Rico) and at 3 institutions (The University of Alabama at Birmingham, The University of Texas Health Science Center at Houston, and The University of Puerto Rico Medical Sciences Campus). Patients with SLE in accordance with the American College of Rheumatology (ACR) criteria (9,10), with a disease duration of ≤5 years, with defined ethnicity (all 4 grandparents of the same ethnicity as the patient), and living within the geographic recruitment areas of the participating institutions are eligible to be enrolled in LUMINA.

Every patient participated in a baseline visit after enrollment (time 0); followup visits were conducted every 6 months for the first year (time 0.5 and time 1, respectively) and yearly thereafter (consecutively numbered up to the last available visit). At each visit, an interview, a physical examination, and laboratory tests were performed; a review of all previously available medical records was also done to obtain pertinent clinical information for the interval. Data for missed study visits were obtained by review of all available medical records.

Duration of disease was defined as the time elapsing from the date that the patient met 4 of the ACR criteria for SLE (diagnosis date) to time 0. Duration of followup (or followup time) in the cohort was defined as the period between time 0 and last visit.

Variables. As previously reported (8,11), the LUMINA database includes variables from the following domains: socioeconomic/demographic, clinical, immunologic, immunogenetic, and behavioral/psychological. These variables are measured at time 0 and at every subsequent visit. Only the variables included in the present analyses will be described. From the socioeconomic/demographic domain, the variables included were ethnicity, age, sex, and unhealthy behaviors (smoking, drinking, and sedentary lifestyle). From the clinical domain, the variables included were disease duration, followup time, body mass index, disease activity and damage, the presence of renal involvement, concomitant or immediately preceding pregnancy or malignancy, comorbidities, ancillary laboratory test results, and medication use.

Disease activity was assessed at each study visit using the Systemic Lupus Activity Measure (SLAM) (12). For these analyses, we computed the average SLAM value (mean of all study visits from diagnosis date to last visit) as a measure related to the degree of disease activity experienced by our patients over time. Disease damage was assessed using the Systemic Lupus International Collaborating Clinics/ACR Damage Index (SDI) (13). For patients with disease duration of <6 months at time 0, the first SDI score obtained (at time 0.5) was used in the analyses.

Comorbidities considered were diabetes mellitus (self-reported and/or physician-based diagnosis and/or a requirement for pharmacologic treatment, regardless of glucocorticoid intake) and hypertension, regardless of the cause (defined as a systolic blood pressure ≥140 mm Hg and/or a diastolic blood pressure ≥90 mm Hg on at least 2 occasions and/or patient’s self-reported intake of antihypertensive medications [unless being taken for Raynaud’s phenomenon and/or proteinuria]). Laboratory variables measured were nonfasting serum levels of lipoproteins (total cholesterol, high-density lipoprotein cholesterol, triglycerides, and low-density lipoprotein [LDL] cholesterol, calculated using the Friedewald formula), and serum C-reactive protein (CRP) (measured as high-sensitivity CRP on immunometric assay; Immulite 2000 Diagnostic Products, Los Angeles, CA).

Autoantibodies were measured at time 0 and included IgG and IgM aPL (abnormal levels defined as >13 IgG and/or >13 IgM phospholipid units/ml) by enzyme-linked immunosorbent assay (ELISA) technique (14), LAC (Staclot test; Diagnostica Stago 92000, Asnières-Sur-Seine, France) (15,16), and IgG and/or IgM anti–oxidized LDL antibodies (for the
purpose of this study, abnormal values were defined as higher than the mean ± SD value in 50 unselected healthy individuals by ELISA (Specialty Laboratories, Santa Monica, CA) (17). Patients were considered to be aPL positive if they had IgM and/or IgG aPL and/or LAC, as described above; in addition, patients in whom these antibodies had been recorded as positive prior to time 0, as obtained by medical record review, were also considered to be aPL positive.

We also took into account current and past use of medications, including nonsteroidal antiinflammatory drugs (NSAIDs) (traditional and cyclooxygenase 2 [COX-2]), statins, oral contraceptives, and hydroxychloroquine. The dose of prednisone equivalent at each study visit and the exposure time were used to calculate the mean daily dose of prednisone.

**Outcome variable.** Venous thrombotic events (peripheral and/or visceral) were noted as the primary outcome, as ascertained by physician assessment during LUMINA study visits and/or documented in the medical records reviewed for these visits. All patients with at least 1 venous thrombotic event documented after the diagnosis date were included in these analyses; thus, the observation time was the period from diagnosis date to last visit, or disease duration plus followup time. We identified a total of 51 cases of venous thrombosis in our cohort; 19 (37%) of these were incident cases, since they were observed during the followup time (time 0 to last visit), whereas 32 (63%) were prevalent cases, since they occurred between the diagnosis date and time 0.

**Statistical analysis.** To maximize the information obtained from these data, different analytic strategies were followed. The dependent variable in all analyses was venous thrombosis. Univariable analyses were conducted by including all 51 patients with venous thrombotic events and the variables from the main domains ascertained either at diagnosis date or at time 0 (particularly the laboratory variables). Categorical and continuous variables were examined by chi-square and Student's t-test, respectively; the Fisher's exact test was used when appropriate. For those categorical variables found to be significant in these analyses, survival analyses were performed using Kaplan-Meier and log-rank tests.

Two multivariable Cox proportional hazards models were generated. The first model included all 51 cases of venous thrombotic events as well as those variables found to be significant at \( P \leq 0.10 \) in the univariable analyses and those considered to be clinically relevant; the starting time point in this model was the diagnosis date. The second model included only the incident cases (starting time point, time 0); in this model, in addition to all variables entered in the diagnosis date model, the medication variables were also included (medications could not have been entered into the first model because they had not been accurately recorded prior to time 0).

Finally, as an alternative model to the time-dependent analyses, a multivariable unconditional stepwise logistic regression analysis that included all 51 patients with venous thrombotic events was performed. In this later model, disease duration (defined as the diagnosis date to time 0) was entered as a variable.

Age, sex, and ethnicity were entered in all regression models, regardless of statistical significance in the univariable analyses. In all cases, the unit of analysis was the patient and not the event. All analyses were done using SPSS software, version 10.0 (Chicago, IL).

**RESULTS**

**Descriptive analyses.** Five hundred seventy LUMINA patients (90% women) were included in the analyses. One hundred ten patients (19%) were Hispanics from Texas, 90 (16%) were Hispanics from Puerto Rico, 208 (37%) were African Americans, and 162 (28%) were Caucasians. The patients’ age at enrollment was 37 ± 12.6 years (mean ± SD) and the total observation time (diagnosis date to last visit) was 53.0 ± 40.6 months, whereas the disease duration (diagnosis date to time 0) was 17.3 ± 16.1 months. Fifty-one patients developed venous thrombotic events after the SLE diagnosis. Thirty-nine percent of these patients developed this complication within the first year after diagnosis, 57% within the first 2 years after diagnosis, and 81% within the first 5 years after diagnosis.

**Univariable analyses.** Univariable comparisons between patients who developed venous thrombotic events and those who did not develop this complication are shown in Tables 1 and 2. Generally, all socioeconomic/demographic variables were comparable between the 2 patient groups, with the exception of smoking, which was twice as frequent among those patients who developed venous thrombotic events (26%) than among those who did not develop them (13%) (\( P = 0.020 \)).

With regard to the clinical features, patients who developed venous thrombotic events had a shorter observation time (diagnosis date to last visit) (mean ± SD

<table>
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<th>No (n = 519)</th>
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<tr>
<td>Hispanic Puerto Rico</td>
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<td>Caucasian</td>
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<td>Age, mean ± SD years</td>
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<tr>
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<tr>
<td>Smoking, %</td>
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<td>Drinking alcohol, %</td>
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</tbody>
</table>

* As defined by the US federal government, adjusted for the number of household inhabitants.
† \( P = 0.020 \) versus patients without venous thrombotic events.
32.4 ± 35.0 months versus 55.0 ± 40.6 months; \( P = 0.071 \) and shorter disease duration at time 0 (diagnosis date to time 0) (13.5 ± 13.4 months versus 17.7 ± 16.3 months; \( P = 0.017 \)) than did patients without this complication. The frequency of renal involvement was numerically higher among those with venous thrombotic events (11% versus 7%), but the difference did not reach statistical significance (1.2; \( P = 0.068 \)). No pregnancies occurred during the months preceding the visit in which the venous thrombotic event was recorded or at the time of the visit. Likewise, no malignancies were recorded during the visit at which the venous thrombotic event was recorded. The frequencies of diabetes mellitus and hypertension were comparable between the 2 patient groups. Furthermore, the mean SLAM score was comparable in both groups. The SDI, however, was higher at time 0 in those patients who developed venous thrombotic events than in those who did not develop them (1.2 ± 1.2 versus 0.7 ± 1.2; \( P = 0.005 \)), even though disease duration among patients with this complication was shorter.

Higher levels of total cholesterol, LDL cholesterol, and triglycerides at time 0 were observed in those patients who developed venous thrombotic events versus those who did not develop them (14.7 ± 9.7 mg) compared with those who did not (12.5 ± 10.4 mg), but the difference was not statistically significant. No pregnancies were recorded. The frequencies of diabetes mellitus and hypertension were comparable in both groups. No malignancies were recorded during the visit at which the venous thrombotic event was recorded. The SDI was higher at the time of the visit in those patients who developed venous thrombotic events than in those who did not develop them (1.2 ± 1.2 versus 0.7 ± 1.2; \( P = 0.005 \)), even though disease duration among patients with this complication was shorter.

Higher levels of total cholesterol, LDL cholesterol, and triglycerides at time 0 were observed in those patients who developed venous thrombotic events versus those who did not develop them (14.7 ± 9.7 mg) compared with those who did not (12.5 ± 10.4 mg), but the difference was not statistically significant.

Finally, survival analyses were performed with those categorical variables (smoking and LAC) found to be significant. No pregnancies were recorded. The frequencies of diabetes mellitus and hypertension were comparable in both groups. No malignancies were recorded during the visit at which the venous thrombotic event was recorded. The SDI was higher at the time of the visit in those patients who developed venous thrombotic events than in those who did not develop them (1.2 ± 1.2 versus 0.7 ± 1.2; \( P = 0.005 \)), even though disease duration among patients with this complication was shorter.
be associated with the development of venous thrombosis in the previous analyses. The probability of developing a venous thrombotic event was higher among those patients with LAC (Figure 1) and among smokers (Figure 2) than among patients without these characteristics; however, the differences were statistically significant only for smoking ($P = 0.008$) and not for LAC ($P = 0.070$).

**Multivariable analyses.** Tables 3 and 4 show the results of the Cox models. In the first model (Table 3), which includes all patients who developed venous thrombotic events after the diagnosis date ($n = 51$), smoking (hazard ratio [HR] 2.487, 95% confidence interval [95% CI] 1.223–5.056, $P = 0.012$), LAC (HR 2.288, 95% CI 1.022–5.120, $P = 0.044$), and disease activity over time (HR 1.106, 95% CI 1.008–1.213, $P = 0.032$) were independently associated with the occurrence of venous thrombosis, whereas age at diagnosis was only of borderline significance (HR 1.023, 95% CI 0.997–1.049, $P = 0.082$). Sensitive analyses using the unconditional stepwise logistic regression approach yielded results consistent with those of the Cox model (for smoking, $P = 0.028$; for LAC, $P = 0.018$; for disease activity over time, $P = 0.028$; and for age at diagnosis, $P = 0.050$ (data not shown). Of note, these variables were representative of the patients’ status either at the diagnosis date (smoking), at time 0 (LAC), or over time (disease activity).

In the second Cox model (Table 4), which included only the incident cases ($n = 19$) and also

<table>
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<th>Feature</th>
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<th>95% CI</th>
<th>$P$</th>
</tr>
</thead>
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<tr>
<td>Age at diagnosis</td>
<td>1.023</td>
<td>0.997–1.049</td>
<td>0.082</td>
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<td>Smoking</td>
<td>2.487</td>
<td>1.223–5.056</td>
<td>0.012</td>
</tr>
<tr>
<td>Disease activity (by SLAM)</td>
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<td>1.008–1.213</td>
<td>0.032</td>
</tr>
<tr>
<td>Lupus anticoagulant</td>
<td>2.288</td>
<td>1.022–5.120</td>
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</table>

* Starting time was the diagnosis date. HR = hazard ratio; 95% CI = 95% confidence interval; SLAM = Systemic Lupus Activity Measure.

<table>
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<th>Feature</th>
<th>HR</th>
<th>95% CI</th>
<th>$P$</th>
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</thead>
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<td>Age at diagnosis</td>
<td>1.055</td>
<td>1.012–1.099</td>
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<tr>
<td>Disease duration at T0</td>
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<td>0.918–0.997</td>
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<tr>
<td>Disease activity (by SLAM)</td>
<td>1.162</td>
<td>0.972–1.389</td>
<td>0.099</td>
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<tr>
<td>Mean dose of glucocorticoids</td>
<td>1.076</td>
<td>1.023–1.389</td>
<td>0.005</td>
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</table>

* Starting time was baseline (time 0 [T0]). See Table 3 for other definitions.
incorporated the medication variables, an independent association between venous thrombosis and the mean dose of glucocorticoids (HR 1.076, 95% CI 1.023–1.389, \( P = 0.005 \)), age at diagnosis (HR 1.055, 95% CI 1.012–1.099, \( P = 0.009 \)), and disease duration at time 0 (HR 0.957, 95% CI 0.918–0.997, \( P = 0.035 \)) was found. In this model, disease activity over time was only of borderline significance (HR 1.162, 95% CI 0.972–1.389, \( P = 0.099 \)). The impact of smoking and of the presence of LAC could not be ascertained in this analysis, since the number of subjects exposed to these risk factors was relatively small and the results were unstable.

**DISCUSSION**

The present study is the first to identify risk factors other than aPL for the occurrence of venous thrombosis in SLE. The risk factors identified were smoking, shorter disease duration, older age, disease activity over time, and the average dose of glucocorticoids used. Our data also confirm the association between venous thrombotic events and aPL (specifically, LAC). Although an overrepresentation of patients of African American and Hispanic (from Texas) ethnicities was seen among those patients who developed venous thrombotic events, ethnicity does not seem to constitute a risk factor that predisposes to the occurrence of these events, according to the results of our univariable and multivariable analyses.

Smoking has been associated with the development of SLE (18–21) as well as with a worse outcome (22–25). It has been proposed that cigarette consumption may interfere with the therapeutic effect of hydroxychloroquine (26). However, smoking has not been previously reported to be associated with the development of venous thrombosis in SLE patients, although such an association has been variably reported in healthy individuals (27,28). In fact, there are earlier reports about this possible association (29,30). However, these studies may have been methodologically flawed, since thrombotic events were not properly defined and adjustment for potential confounders such as age was not performed (31). More recently, 2 large prospective studies have demonstrated an association between smoking and pulmonary embolism (in women only) (32) and deep venous thrombosis (in both men and women) (33). Moreover, it has been demonstrated that cigarette smoking may activate the coagulation pathway (34); it is possible that smoking-induced clotting abnormalities may be more harmful in lupus patients than in healthy individuals, since lupus patients may harbor a more prothrombogenic environment than that in patients without lupus (35).

We also found an association between glucocorticoid use (mean daily dose) and the development of venous thrombotic events. Given the widespread utilization of glucocorticoids among lupus patients, this finding is of concern. However, we were only able to examine this variable in the incident cases; thus, some caution in the interpretation of these data is in order. Glucocorticoids have been considered to contribute to accelerated atherosclerosis in SLE (36–39). Although venous and arterial thrombosis may share some clotting abnormalities, atherogenic factors, which are very important in the arterial system, are less important in the venous compartment. Nevertheless, thromboembolic complications have also been reported during the initial phase of glucocorticoid use for the treatment of giant cell arteritis (40). Moreover, Cushing’s syndrome is often regarded as a prothrombotic condition (41), since high levels of adrenal steroids have been shown to be associated with several clotting abnormalities, including increased levels of factor VIII, von Willebrand factor, and plasminogen activator inhibitor activity, as well as with decreased fibrinolysis (41–43). Similar clotting abnormalities have been reported in patients treated with exogenous glucocorticoids (44), indicating that glucocorticoids may, in fact, increase the risk of thrombotic events in individuals with a predisposition to develop them.

When occurrence of venous thrombotic events in conjunction with any of the aPL was examined, no association between these 2 variables was found; however, when only the presence of LAC was examined, a significant and independent association with the outcome of interest was observed. These results are consistent with data from the Hopkins Lupus Cohort, in which, as in our study, the association was only with LAC, suggesting that this is a more specific marker for venous thrombosis than are the other aPL (2). These antibodies encompass a heterogeneous group that has been broadly associated with thrombotic events (45–47), although, in the majority of studies, arterial and venous thrombotic events have been examined together. Our group has found an association between vascular arterial events and the presence of any of the aPL (48), but not when arterial and venous thrombotic events were examined together (49). In addition, it is important to note that our patients were considered positive or negative for IgM and IgG aPL regardless of the antibody titer, whereas the association between these autoantibodies and thrombotic events has been, primarily, with medium and high antibody titers (50). By including patients with
low titers among the aPL-positive patients, we may have been unable to find some level of association. Finally, these autoantibodies were not always examined immediately prior to the event; thus, it is possible that we may have missed a true association.

The association between older age and venous thrombotic events was clearly significant in the incident model, but only of borderline significance in the overall (Cox and logistic) models, which is not surprising given that venous thrombotic events are seen more frequently in elderly than in young patients from the general population. A recent study demonstrated the association between older age and thrombosis in patients with anticardiolipin antibodies in the setting of different rheumatic and nonrheumatic disorders (51), but the association between venous thrombotic events and age in SLE has not been reported previously. Although menopause and hormone replacement therapy were not included in the analyses presented, we do not believe age is a proxy, since neither of these 2 variables has been found to be associated with the occurrence of venous thrombotic events in analyses performed separately (52,53).

In the Cox model of incident cases, an independent association between the occurrence of venous thrombotic events and shorter disease duration at time 0 was also found. This finding suggests that these events occur early in the course of the disease, in contrast to vascular arterial events, which are more commonly seen later in its course, as has been noted by other authors (2). Finally, the association between disease activity over time and venous thrombosis, which was evident in the overall models, has not been previously reported and it may have important implications in the care of the lupus patient.

Dyslipidemia is another traditional risk factor for vascular events, but it has been related preferentially to arterial events rather than to venous thrombotic events. However, an association between hypercholesterolemia and venous thrombosis has been observed in the Hopkins Lupus Cohort (2). We could not validate such an association in our multivariable analyses, although we did observe an association between venous thrombosis and higher levels of total cholesterol, LDL cholesterol, and triglycerides in the univariable analyses.

Some limitations of the present study should be addressed. First, our study did not include other potential prothrombotic factors such as hyperhomocysteinemia or genetic and acquired clotting abnormalities of factor V Leiden, protein S or C, or activated protein C, which can also play a role in some patients (54). Second, we could not enter the medication variables in the analyses that included both prevalent and incident cases; therefore, we could not properly examine whether glucocorticoid use may have predisposed our patients to the occurrence of venous thrombotic events from disease onset. Third, the association between disease activity over time and venous thrombosis found in the multivariable models that included all patients was not found in the incident Cox model. Lack of power is a definite possibility; an alternative explanation, however, is that glucocorticoid use, which is associated with disease activity, is a proxy for this variable. Fourth, we could not properly examine the role of LAC or smoking in the prospective part of our analyses. Despite these limitations, we think our data are unique, given the multi-ethnic composition of our cohort, the duration of follow-up time for our patients, and the fact that this report describes the largest number of venous thrombotic events of any lupus study to date.

In summary, venous thrombotic events represent an early, rather than late, manifestation of SLE. In addition to the presence of LAC, older age, higher disease activity, and smoking were identified as risk factors for the development of such events in patients with SLE. Our data also suggest that a higher dose of glucocorticoids predisposes lupus patients to the occurrence of venous thrombotic events. Other clinical factors that have been associated with venous thrombosis were either not present in our patients around the time of the event (malignancy and pregnancy, for example) or were comparable in both patient groups. Our data have direct applicability to the management of patients with SLE.

ACKNOWLEDGMENTS

We acknowledge all of the LUMINA patients, without whom this study would not have been possible, our supporting staff (Martha L. Sanchez, MD, MPH and Ellen Sowell at The University of Alabama at Birmingham, Carmine Pinilla, MT at The University of Puerto Rico Medical Sciences Campus, and Robert Sandoval, BA and Li-Lu Wang, MS, BS at The University of Texas Health Science Center at Houston) for their efforts in securing our patients’ followup and performing other LUMINA-related tasks, Drs. Ruihua Wu and Yehuda Schonfeld for determining anti–oxidized LDL antibodies in all patients, and Ms Ella Henderson for her expert assistance in the preparation of this manuscript.

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thematous during a 5-year period: a multicenter prospective study of 1,000 patients. Medicine (Baltimore) 1999;78:167–75.


Increased Levels of Serum Protein Oxidation and Correlation With Disease Activity in Systemic Lupus Erythematosus

Philip E. Morgan,1 Allan D. Sturgess,2 and Michael J. Davies1

**Objective.** To examine protein oxidation in systemic lupus erythematosus (SLE) and to correlate levels of protein oxidation products with disease activity.

**Methods.** Serum was collected from SLE patients and healthy control subjects. Protein-bound carbonyls and the pro-oxidant enzyme myeloperoxidase (MPO) were quantified by enzyme-linked immunosorbent assay. Protein thiols were quantified using 5,5′-dithionitrobenzoic acid. Protein-bound amino acids and methionine, tyrosine, and phenylalanine oxidation products were quantified by acid hydrolysis and high-performance liquid chromatography. Disease activity was assessed by the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI). Levels of anti-double-stranded DNA (anti-dsDNA) antibodies were measured by radioimmunoassay.

**Results.** Compared with control subjects, SLE patients exhibited elevated levels of protein carbonyls (0.108 ± 0.078 versus 0.064 ± 0.028 nmoles/mg of protein; \( P = 0.046 \)), decreased levels of protein thiols (3.9 ± 1.1 versus 4.9 ± 0.7 nmoles/mg of protein; \( P = 0.003 \)), decreased levels of protein-bound methionine (\( P = 0.0007 \)), and increased levels of protein-bound methionine sulfoxide (\( P = 0.0043 \)) and 3-nitrotyrosine (\( P = 0.0477 \)). SLE patients with high SLEDAI scores or elevated anti-dsDNA antibody levels exhibited increased oxidation compared with patients with low SLEDAI scores or low antibody levels. Serum MPO levels were decreased in SLE patients (\( P = 0.03 \)), suggesting that this enzyme is not responsible for the enhanced protein oxidation.

**Conclusion.** We found elevated levels of multiple markers of protein oxidation in sera from SLE patients compared with controls, and these levels correlated with disease activity. The findings suggest that protein oxidation may play a role in the pathogenesis of chronic organ damage in SLE.

Systemic lupus erythematosus (SLE) is the prototypical systemic chronic autoimmune disease, characterized by diverse clinical manifestations and production of multiple autoantibodies. Common long-term complications of SLE include damage to the musculoskeletal, neuropsychiatric, renal, and cardiovascular systems (for review, see ref. 1). In recent years, it has been widely appreciated that premature atherosclerosis is a particularly striking feature of the disease (2), especially in women (3); furthermore, traditional risk factors are not believed to fully account for the increased atherosclerosis (2).

Although the cause of SLE is unknown, evidence of a complex genetic contribution has been reported, with an increased incidence in families in which one or more members already has the disease or another autoimmune disease (for review, see ref. 4). Other factors implicated in the development or progression of SLE include altered cytokine levels (5), modulated sex hormone metabolism (6), increased apoptosis (7), and elevated levels of oxidative stress. With respect to oxidative stress, evidence of increased levels of phospholipid oxidation products, particularly in patients with antiphospholipid antibodies (8) has been reported, as well as elevated plasma DNA oxidation products, such as 8-hydroxydeoxyguanosine (8-oxodG), a product that also appears to be processed abnormally (9). Many of the autoantibodies produced in SLE exhibit a preference...
for oxidized substrates, including oxidized double-stranded DNA (dsDNA) (10) and phospholipids (11). Oxidative stress and antiphospholipid antibodies have also been implicated in the increased atherosclerosis seen in patients with SLE (8). Elevated levels of the oxy radical–producing enzyme xanthine oxidase (12) and decreased levels of the protective enzyme superoxide dismutase and endogenous antioxidants (13) have also been reported; the latter have been suggested to be predictive of SLE onset (14).

Despite this evidence for a role for oxidative stress in SLE, there are few data on the occurrence of protein oxidation. In addition, there is no information on whether the extent of oxidation correlates with disease activity or cumulative organ damage. Such data are required for an assessment of the importance of oxidative damage as a causal agent in disease development, since enhanced oxidative stress may merely be a secondary consequence of chronic inflammation. Proteins would be expected to be major targets for oxidative damage since they are major components of most tissues, cells, and plasma (15) and exhibit rapid rates of reaction with many oxidants (15). Oxidized proteins are known to cause major physiologic perturbations, including loss of structure or function (for review, see ref. 16).

The long-lived nature and slow rates of removal of many oxidized proteins (see, for example, refs. 17 and 18) may make these materials valuable quantitative markers of oxidative stress. Previous studies have shown elevated levels of protein oxidation products in a number of pathologic conditions in humans, including atherosclerosis, lens cataracts, diabetes mellitus, and neurodegenerative syndromes (for review, see refs. 19 and 20).

Protein oxidation has been implicated as a cause of the pathology in at least some of these conditions.

In this study, the oxidation of serum proteins was quantified in SLE patients and healthy control subjects. Loss of parent, protein-bound amino acids was examined, as well as generic markers of oxidation (protein carbonyls) and specific side-chain oxidation products (methionine sulfoxide [MetSO], 3,4-dihydroxyphenylala-nine [DOPA], dityrosine [di-Tyr], 3-chlorotyrosine [3ClTyr], 3-nitrotyrosine [3NO2-Tyr], and o-tyrosine [o-Tyr]). Levels of the pro-oxidant heme enzyme myeloperoxidase (MPO), which generates the potent oxidant hypochlorous acid among others (21), were also examined, since recent studies found elevated levels in patients with atherosclerosis (22), an important complication of SLE and a major cause of death in people with this disease. We also examined whether the extent of protein oxidation, assessed by this battery of assays and the levels of MPO, correlate with the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and with elevated levels of antibodies to dsDNA, an SLE-specific autoantibody.

MATERIALS AND METHODS

Materials. Chemicals were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia) unless noted otherwise. High-performance liquid chromatography (HPLC) solvents were purchased from EMD Chemicals (Merck, Kil-syth, Victoria, Australia) and were filtered before use through VacuCap 90 filter units with 0.2-μm Supor membranes (Pall, Cheltenham, Victoria, Australia). Water used in all experiments was passed through a 4-stage Milli Q system. Phosphate buffer solutions were pretreated with Chelex 100 resin (Bio-Rad, Hercules, CA) to remove trace metal ions.

Patients and controls. This study was approved by the Human Ethics Committee of The St. George Hospital. All SLE patients were outpatients of the hospital Rheumatology Department, and all were classified as having SLE, as defined by the American College of Rheumatology 1997 revised criteria (23). Controls were Rheumatology Department outpatients with miscellaneous (nonautoimmune) complaints and staff of the Rheumatology Department or the Heart Research Institute. The demographics of the controls and SLE patients, including renal status and drug therapy in the SLE patients, are given in Table 1.

Patient and control groups were matched as closely as possible with regard to age, sex, and race. There were no statistically significant differences in the ages of the subjects, either between or within the SLE patient and control groups, by two-way analysis of variance (ANOVA), or in the disease activity markers in the various SLE patient groups (categorized by SLEDAI score and anti-dsDNA antibodies) by one-way ANOVA.

Blood collection and serum preparation. Venous blood was collected into plain clot tubes, and after ~30 minutes, serum was prepared by centrifugation at 3,500 revolutions per minute for 10–15 minutes. Since only small volumes of serum were available, not all experiments were performed on all samples/subjects.

Measurement of disease activity. Disease activity was assessed, at the time of blood sampling, using the SLEDAI. This instrument, which has been validated extensively (24), examines 9 organ systems, and weighted scores are assigned according to disease severity. All patients were assessed by the same clinician (ADS), who has extensive experience in the use of this scale. A SLEDAI score of ≥6 was taken as an indicator of high levels of disease activity (25).

Measurement of anti-dsDNA antibody. Anti-dsDNA autoantibodies were determined as part of the routine assessment of all patients with SLE. A commercially available anti-dsDNA kit in which serum antibodies to dsDNA are bound to 125I-labeled recombinant DNA (Diagnostic Products, Los Angeles, CA) was used according to the manufacturer’s instructions. The upper limit of the 95th percentile of anti-dsDNA antibody levels in a reference population of healthy adults is 4.2 IU ml⁻¹. Patients positive for anti-dsDNA auto-
<table>
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<th>Study group, marker of interest</th>
<th>No. of women/men</th>
<th>Age, mean ± SD</th>
<th>Race/ethnicity, %</th>
<th>SLEDAI score</th>
<th>Anti-dsDNA, IU ml⁻¹</th>
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<th>Corticosteroids</th>
<th>Anti-malarials</th>
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<td>7.7</td>
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<td>40.2 ± 100.8</td>
<td>26.9</td>
<td>42.3</td>
<td>34.6</td>
</tr>
<tr>
<td>Amino acid analysis</td>
<td>25/1</td>
<td>42.8 ± 15.0</td>
<td>53.8</td>
<td>34.6</td>
<td>3.8</td>
<td>7.7</td>
<td>3.7 ± 2.8</td>
<td>28.3 ± 42.7</td>
<td>27.1</td>
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<td>Tyr oxidation products</td>
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<td>45.1 ± 15.9</td>
<td>52.6</td>
<td>42.1</td>
<td>5.3</td>
<td>0</td>
<td>5.1 ± 4.9</td>
<td>46.0 ± 121.3</td>
<td>21.1</td>
<td>36.8</td>
<td>21.1</td>
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<tr>
<td>Myeloperoxidase</td>
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<td>42.5 ± 15.0</td>
<td>52.6</td>
<td>36.8</td>
<td>5.3</td>
<td>5.3</td>
<td>3.6 ± 2.9</td>
<td>27.5 ± 41.7</td>
<td>21.1</td>
<td>36.8</td>
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<td><strong>Control patients</strong></td>
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<td></td>
<td></td>
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<tr>
<td>Protein thiols</td>
<td>12/0</td>
<td>43.1 ± 10.8</td>
<td>83.3</td>
<td>8.3</td>
<td>8.3</td>
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</tr>
<tr>
<td>Protein carbonyls</td>
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<td>40.8 ± 7.9</td>
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<td>10.0</td>
<td>10.0</td>
<td>0</td>
<td></td>
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<tr>
<td>Amino acid analysis</td>
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<td>75.0</td>
<td>12.5</td>
<td>12.5</td>
<td>0</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Tyr oxidation products</td>
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<td>40.3 ± 9.6</td>
<td>86.7</td>
<td>6.7</td>
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<td>0</td>
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<tr>
<td>Myeloperoxidase</td>
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<td>72.7</td>
<td>9.1</td>
<td>18.2</td>
<td>0</td>
<td></td>
<td></td>
<td></td>
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</tbody>
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* SLE = systemic lupus erythematosus; SLEDAI = Systemic Lupus Erythematosus Disease Activity Index; anti-dsDNA = anti-double-stranded DNA; COX-2 = cyclooxygenase 2.
antibodies (>4.2 IU ml⁻¹) were compared with those who were negative for these antibodies (≤4.2 IU ml⁻¹).

Determination of protein concentrations. Protein concentrations were determined by the bicinchoninic acid assay (Pierce, Rockford, IL) using 96-well plates, with incubation at 60°C for 30 minutes. Absorbance was measured at 562 nm using a 96-well plate reader (Tecan, Grödig, Austria) and was converted to absolute values using bovine serum albumin (BSA) standards (0–1 mg ml⁻¹).

Determination of serum protein thiol levels. Protein thiols were quantified spectrophotometrically using 5,5'-dithiobisnitrobenzoic acid (DTNB) (26). Briefly, using 96-well plates, 1:2 and 1:4 dilutions of freshly thawed serum were prepared to a final volume of 10 μl. To these preparations was added either 200 μl of freshly prepared 500 μM DTNB in 100 mM phosphate buffer, pH 7.4, or 200 μl of buffer alone. Following incubation in the dark for 30 minutes at 21°C, release of 5-thiobenzonic acid was quantified by absorbance at 412 nm and converted to absolute values using reduced glutathione standards (0–0.5 mM). The absorbance of samples without added DTNB was subtracted to account for background absorbance at 412 nm. Samples were analyzed in triplicate.

Determination of serum protein carbonyl levels. Carbonyl concentrations were determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (Zenith Bonyl, Hills, Victoria, Australia), lysozyme, and human plasma. Hydrolysis at 130°C is slightly underestimated.

Preparation of samples for protein hydrolysis. Protein samples (free of free amino acids) were prepared for hydrolysis as described previously (for review, see ref. 20). Two methods using methanesulfonic acid (MSA) and HCl were employed that used different amounts of protein, which are shown here as MSA hydrolysis volume/HCl hydrolysis volume. Briefly, 5/60-μl serum samples were aliquotted into 8 × 40-mm clear vials (Alltech, Baulkham Hills, New South Wales, Australia) in triplicate. These were diluted with 100/560 μl of 100 mM phosphate buffer, pH 7.4, and 5/10 μl of freshly prepared 10 mg ml⁻¹ sodium borohydride (Merck) was added to remove peroxides. MetSO is not affected by this treatment (Hawkins CL: unpublished observations). After incubation for 5–10 minutes at 21°C, samples were delipidated by adding 5/50 μl of 0.3% (weight/volume) sodium deoxycholate, and proteins were precipitated with 10/100 μl of 10% trichloroacetic acid, followed by centrifugation at 4,300g for 2 minutes. The protein pellets were then washed twice with 500 μl of ice-cold acetone, repelleted by centrifugation at 7,000g for 2 minutes, and dried by vacuum centrifugation for 10–15 minutes.

Protein hydrolysis with MSA and amino acid analysis. Protein pellets were hydrolyzed using 150 μl of 4M MSA (27) containing 0.2% (w/v) trypstatine per vial. Eighty nanomoles of l-homoarginine (Fluka, Buchs, Switzerland) was added as an internal standard. The vials were placed in Pico-Tag reaction vessels (Alltech) and subjected to 3 cycles of purging with nitrogen gas and evacuation. The samples were then incubated for 17 hours at 110°C, neutralized with 150 μl of 4M sodium hydroxide (ICN Biomedicals, Aurora, OH), and filtered through 0.45-μm Nanosep MF GHF centrifugal devices (500-μl sample capacity; Pall). Samples were then diluted 50-fold in water, stored at 4°C, and analyzed by HPLC within ~24 hours.

Analysis was performed by reverse-phase HPLC using a Zorbax ODS 5-μm, 4.6 × 250–mm column (Agilent, Forest Hill, Victoria, Australia) and a Supelco Pellicular LC-18 2-cm guard cartridge (Sigma-Aldrich). Samples were derivatized using an autoinjector system (Shimadzu Oceania, Rydalmere, New South Wales, Australia), which added 20 μl of an o-phthaldialdehyde (1 ml)/β-mercaptoethanol (5 μl) solution to 40 μl of sample. Fifteen microliters of the derivatized sample was injected into the column after 1 minute.

Samples were separated (flow rate 1 ml minute⁻¹) using a gradient consisting of buffer A with 5% buffer B for 7 minutes, 5–25% buffer B over 10 minutes, 25–45% buffer B over 2 minutes, 45–50% buffer B over 8 minutes, 50–58% buffer B over 8 minutes, 58–100% buffer B over 5 minutes, 100% buffer B for 5 minutes, 100–5% buffer B over 1 minute, and reequilibration at 5% buffer B for 9 minutes. Buffer A consisted of 20% methanol and 2.5% tetrahydrofuran (THF) in 20 mM sodium acetate (BDH-Merck, Sydney, New South Wales, Australia), pH 5.4. Buffer B consisted of 80% methanol and 2.5% THF in 20 mM sodium acetate, pH 5.4. Buffers were degassed by sonication under vacuum and sparged with helium during chromatography. Derivatized amino acids were detected by fluorescence (excitation and emission spectra λex 340 nm and λem 440 nm) and quantified using standards containing added l-homoarginine and l-methionine sulfoxide.

Validation of the methanesulfonic acid protein hydrolysis method. Validation experiments were conducted with BSA (owing to its sequence homology with human serum albumin), lysozyme, and human plasma. Hydrolysis at 130°C for 16 hours resulted in significant loss of Met and Trp residues (~33% and 20% respectively), whereas hydrolysis at 110°C for 17 hours gave good recovery (>75% for Met and >85% for all other amino acids except Cys and cystine). Spiking experiments with added MetSO revealed slightly greater Met concentrations than expected, which is consistent with a low extent of conversion of MetSO to Met. Spiking also demonstrated a low extent of artifactual oxidation of Met to MetSO; this was quantified as 8–13% when Met was hydrolyzed alone, with a further 2–10% unaccounted for. Approximately 20% of MetSO was converted back to Met when MetSO was hydrolyzed alone, with a further 10% unaccounted for (data not shown). These data suggest that under the conditions used, the proportion of total Met plus MetSO that is present as MetSO is slightly underestimated.

Protein hydrolysis with hydrochloric acid and product analysis. Hydrolysis using HCl was performed essentially as described previously (20); this method does not produce significant artifactual chlorination in our laboratory (28). Vials containing pretreated serum were placed in Pico-Tag reaction vessels containing 1 ml of 6M HCl (BDH-Merck) and 50 μl of mercaptoacetic acid, evacuated, then incubated at 110°C for 18 hours. The samples were then dried by vacuum centrifugation.
for 1 hour, redissolved in 200 μl of water, and filtered through 0.45-μm Nanosep MF GHP centrifugal devices. Samples were maintained at 4°C prior to HPLC analysis, which was performed within ~24 hours.

Parent amino acids and Tyr and Phe oxidation products were separated by reverse-phase HPLC as described above. Samples (20 μl) were injected and eluted using a gradient consisting of buffer A with 2% buffer B for 20 minutes, 2–50% buffer B over 30 minutes, 50% buffer B for 5 minutes, 50–2% buffer B over 1 minute, and reequilibration at 2% buffer B for 4 minutes, where buffer A was 100 mM sodium perchlorate in 10 mM orthophosphoric acid and buffer B was 80% methanol in water. Buffers were degassed before and during use.

Compounds were identified by serial ultraviolet (280 nm) and fluorescence (λex 280 nm and λem 320 nm for 0–31 minutes; λex 280 nm and λem 410 nm for the remainder) detection. DOPA, o-Tyr, and di-Tyr were quantified by fluorescence; p-Tyr, 3Cl-Tyr, and 3NO2-Tyr were quantified by ultraviolet absorption. Data are expressed as micromoles of product per mole of p-Tyr.

Statistical analysis. Statistical analysis was performed using GraphPad Prism version 4.0b for Macintosh software (GraphPad Software, San Diego, CA). Student’s t-test was used to compare one condition with its corresponding control. For multiple conditions, one-way ANOVA was used, with Newman-Keuls post hoc test. Where multiple conditions in different groups were compared, a two-way ANOVA was used, with Bonferroni posttests. In all cases, P values less than 0.05 were considered significant.

RESULTS

Serum levels of protein thiols. Protein thiol levels were significantly decreased in SLE patients compared with controls (3.9 ± 1.1 versus 4.9 ± 0.7 nmoles/mg protein; P = 0.003) (Figure 1A). Thiol concentrations correlated with the activity of disease, as measured the SLEDAI (Figure 1B). Lower levels of thiols were found in patients with high levels of disease activity (SLEDAI score ≥6) as compared with patients with low levels of disease activity (SLEDAI score <6). No statistically significant difference was detected between thiol levels in patients who were negative (≤4.2 IU ml−1) and those who were positive (>4.2 IU ml−1) for anti-dsDNA antibodies (Figure 1C).

Serum levels of protein carbonyls. Carbonyl levels were significantly increased in SLE patients compared with controls (0.108 ± 0.078 nmoles/mg of protein versus 0.064 ± 0.028 nmoles/mg of protein; P = 0.046) (Figure 2A). When these data were stratified according to low and high levels of disease activity as measured by the SLEDAI, the statistical significance was lost because of the small sample sizes (Figure 2B). Nevertheless, a trend toward increasing protein carbonyl concentrations with increasing disease activity was evident. We found no difference for SLE patients who were positive or negative for anti-dsDNA antibodies (data not shown).

Serum levels of MPO. MPO levels were significantly decreased in SLE patients compared with controls (86 ± 46 ng/ml of serum versus 140 ± 84 ng/ml of serum;
A trend toward decreased MPO levels with increasing disease activity, as indicated by low versus high SLEDAI scores and by negative versus positive anti-dsDNA antibody levels, was observed, but these differences were not statistically significant (data not shown).

**Analysis of protein-bound amino acids and specific side-chain oxidation products.** Chromatograms from the protein-bound amino acid analysis experiments, showing a typical SLE patient and a typical healthy control subject, are presented in Figure 3. As can be seen, some of the amino acid peaks were off the scale and, therefore, not all amino acids could be analyzed. This was done in order to maximize the size of the smaller peaks of interest, notably, Met, MetSO, and Trp.

Statistically significant decreases in levels of Met, increases in MetSO and 3NO₂-Tyr, and decreases in total Met plus MetSO were detected in SLE patients compared with controls (Table 2). Correlation of the Met oxidation data with disease activity (SLEDAI score) and anti-dsDNA antibody levels revealed increasing oxidation with increasing disease activity and anti-dsDNA positivity (Figure 4). With the exception of 3NO₂-Tyr, no statistically significant changes were observed for any of the Tyr (DOPA, di-Tyr, 3Cl-Tyr) or Phe (o-Tyr) oxidation products quantified. A small increase in o-Tyr levels in SLE patients failed to achieve significance, although a significant loss of the parent amino acid (Phe) was detected (Table 2). In addition, statistically significant increases in Gly levels and significant decreases Arg levels were observed in SLE patients compared with controls. The changes in Arg and Phe levels also correlated with disease activity and anti-dsDNA antibody levels.
Many enzymes (31). Unlike other protein oxidation residues, Met may be of particular significance, since these residues play an important role in the catalytic activity of many enzymes (31). Unlike other protein oxidation products, the oxidation of Cys and Met residues can be at least partly reversed. The formation of cystine from Cys can be readily reversed by reductase and isomerase enzymes (15, 30), although there is no evidence for the repair of oxyacids, such as RSO₂H and RSO₃H, which are formed in competition with cystine (15, 30). MetSO can be reduced to Met by methionine sulfoxide reductase enzymes (15, 32), although such repair does not occur in serum or plasma. Further oxidation of MetSO to the sulfone MetSO₂ is irreversible (15, 32), with the formation of this species probably accounting for the observed decrease in the total Met plus MetSO.

It has been hypothesized that the oxidation of Met to MetSO and its subsequent reduction by methionine sulfoxide reductases may be important in the regulation of the biologic activity of proteins and an important antioxidant defense mechanism (32, 33). Oxidation of Met residues in proteins can bring about changes in hydrophobicity and protein unfolding, with resulting loss of function (15, 34). Although free methionine can spontaneously oxidize to MetSO (35), this is unlikely to be a significant problem in the current study.

### Table 2. Amino acid analysis of serum proteins from SLE and control patients by HPLC*

<table>
<thead>
<tr>
<th>Amino acid/oxidation product</th>
<th>SLE</th>
<th>Control</th>
<th>Increase or decrease in SLE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>2.169 ± 0.118</td>
<td>2.286 ± 0.074</td>
<td>Decrease</td>
<td>0.0133</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.433 ± 0.109</td>
<td>2.297 ± 0.128</td>
<td>Increase</td>
<td>0.0056</td>
</tr>
<tr>
<td>Histidine</td>
<td>1.456 ± 0.073</td>
<td>1.514 ± 0.069</td>
<td>No change</td>
<td>0.0550</td>
</tr>
<tr>
<td>Lysine</td>
<td>4.608 ± 0.335</td>
<td>4.795 ± 0.353</td>
<td>No change</td>
<td>0.1816</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.348 ± 0.061</td>
<td>0.431 ± 0.048</td>
<td>Decrease</td>
<td>0.0007†</td>
</tr>
<tr>
<td>Methionine sulfoxide</td>
<td>0.321 ± 0.048</td>
<td>0.262 ± 0.062</td>
<td>Increase</td>
<td>0.0043†</td>
</tr>
<tr>
<td>Methionine + methionine sulfoxide</td>
<td>0.668 ± 0.031</td>
<td>0.693 ± 0.023</td>
<td>Decrease</td>
<td>0.0234†</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>2.373 ± 0.137</td>
<td>2.513 ± 0.112</td>
<td>Decrease</td>
<td>0.0135</td>
</tr>
<tr>
<td>o-Tyrosine‡</td>
<td>65.9 ± 29.5</td>
<td>60.1 ± 18.0</td>
<td>No change</td>
<td>0.5152</td>
</tr>
<tr>
<td>Threonine</td>
<td>3.651 ± 0.141</td>
<td>3.597 ± 0.101</td>
<td>No change</td>
<td>0.3239</td>
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<tr>
<td>Tryptophan</td>
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<td>0.384 ± 0.025</td>
<td>No change</td>
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</tr>
<tr>
<td>Tyrosine</td>
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<td>1.913 ± 0.054</td>
<td>No change</td>
<td>0.7222</td>
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<tr>
<td>3-Chlorotyrosine‡</td>
<td>49.1 ± 17.7</td>
<td>43.3 ± 13.8</td>
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<td>0.3083</td>
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<tr>
<td>Dityrosine§</td>
<td>1.378 ± 0.843</td>
<td>1.423 ± 0.594</td>
<td>No change</td>
<td>0.8644</td>
</tr>
<tr>
<td>3,4-Dihydroxyphenylalanine‡</td>
<td>84.2 ± 29.2</td>
<td>74.6 ± 16.8</td>
<td>No change</td>
<td>0.2643</td>
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<tr>
<td>3-Nitrotyrosine‡</td>
<td>80.8 ± 16.0</td>
<td>67.5 ± 21.9</td>
<td>Increase</td>
<td>0.0477</td>
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</table>

* Methanesulfonic acid hydrolysis was used for amino acid and methionine sulfoxide analyses, with detection by derivatization with o-phthalaldehyde and fluorometric detection. Amino acid and methionine sulfoxide concentrations are expressed as the mean ± SD moles of amino acid/mole of isoleucine. Statistical analyses were performed using a 1-tailed t-test when an increase or decrease was predicted before analysis was performed; otherwise, a 2-tailed t-test was used. SLE = systemic lupus erythematosus; HPLC = high-performance liquid chromatography.

† HCl hydrolysis was used for analysis of tyrosine (3-chlorotyrosine, dityrosine, 3,4-dihydroxyphenylalanine, and 3-nitrotyrosine) and phenylalanine (o-tyrosine) oxidation products, with ultraviolet and fluorescence detection as described in Materials and Methods.

**DISCUSSION**

The results presented here are consistent with an increased extent of protein oxidation in the serum of patients with SLE as compared with controls, as evidenced by decreased serum protein thiol levels, increased protein-bound carbonyl levels, decreased Met and total Met plus MetSO levels, and increased MetSO levels. The change in concentration of some of these species correlated with disease activity and with anti-dsDNA antibody positivity, with enhanced oxidation occurring in the presence of more severe disease. This may indicate direct causality. Analysis of protein-bound amino acids also revealed significant losses of 2 other amino acids, Arg and Phe, and an increase in Gly residues.

The sulfur-containing amino acids Cys and Met are particularly susceptible to oxidation (for review, see refs. 29 and 30), making them sensitive markers of protein modification. Oxidation of Cys and, to a lesser extent, Met may be of particular significance, since these residues play an important role in the catalytic activity of many enzymes (31). Unlike other protein oxidation products, the oxidation of Cys and Met residues can be at least partly reversed. The formation of cystine from Cys can be readily reversed by reductase and isomerase enzymes (15, 30), although there is no evidence for the repair of oxyacids, such as RSO₂H and RSO₃H, which are formed in competition with cystine (15, 30). MetSO can be reduced to Met by methionine sulfoxide reductase enzymes (15, 32), although such repair does not occur in serum or plasma. Further oxidation of MetSO to the sulfone MetSO₂ is irreversible (15, 32), with the formation of this species probably accounting for the observed decrease in the total Met plus MetSO.

It has been hypothesized that the oxidation of Met to MetSO and its subsequent reduction by methionine sulfoxide reductases may be important in the regulation of the biologic activity of proteins and an important antioxidant defense mechanism (32, 33). Oxidation of Met residues in proteins can bring about changes in hydrophobicity and protein unfolding, with resulting loss of function (15, 34). Although free methionine can spontaneously oxidize to MetSO (35), this is unlikely to be a significant problem in the current study.
since both the control and the patient samples were treated in the same manner, and this process is known to be modulated by plasma proteins (35). Even if selective artifactual oxidation of Met to MetSO was occurring in the SLE patient samples, the decrease in total levels of Met plus MetSO and the increased loss seen with increased disease activity are still consistent with an enhanced level of oxidative stress in the SLE patients.

Oxidation of Cys residues has been observed in other diseases in which oxidative stress has been implicated, including adult respiratory distress syndrome (36) and coronary artery disease (37). Previous studies have reported decreased serum thiols in SLE patients compared with controls. In early studies (38,39) a significant decrease in serum thiols was detected in SLE patients, with the latter study also reporting a correlation with increasing disease activity. These studies, however, were conducted before the development of a uniform classification system for SLE or systematic disease activity scales; thus, comparison with the results of the current study is difficult. A later study (12) also reported a loss of thiols, with the values in SLE patients being only one-third those in healthy controls. However, the expression of thiol levels in micromolar concentrations in this study makes the determination of protein thiol levels problematic, owing to variations in serum protein concentrations between patients. An inverse correlation between xanthine oxidase levels and thiols was also detected (12), which is consistent with this enzyme being the cause of the observed oxidative stress.

Low molecular mass thiols are unstable over short periods at physiologic temperatures, as well as over longer periods when frozen (40). For this reason low molecular mass thiols were not assessed in the current study, and the minor contribution from these species to the total serum levels is assumed to be zero. Only the more stable high molecular mass protein thiols that make up the majority of the total thiols in serum were analyzed here. Thiol-conserving agents (41) were not used in the current study because they are not consistent with the other assays we used. Similarly, total thiol analysis after reduction (40) was not considered because this methodology does not provide information on the extent of oxidation.

Figure 4. Serum methionine oxidation levels in individual control subjects and patients with systemic lupus erythematosus (SLE) and correlation with disease activity, as determined by A, C, and E, the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) score (<6 indicates low disease activity; ≥6 indicates high disease activity) and by B, D, and F, anti–double-stranded DNA (anti-dsDNA) antibody levels (≤4.2 IU ml⁻¹ indicates negative and >4.2 IU ml⁻¹ indicates positive anti-dsDNA antibodies). Serum proteins were hydrolyzed with methanesulfonic acid, with subsequent amino acid analysis by high-performance liquid chromatography and fluorometric detection of derivatized amino acids (see Materials and Methods for details). Concentrations of amino acids (methionine [Met], methionine sulfoxide [MetSO], and Met plus MetSO combined) are expressed as moles of the given amino acid per mole of Ile. * = P < 0.05; ** = P < 0.01, by one-way analysis of variance with Newman-Keuls post hoc test.

Elevated levels of protein-bound carbonyls have previously been detected in patients with diabetes mellitus (42) and in plasma and tracheal aspirates from preterm infants (43,44). The increase in carbonyls detected in SLE patients in the current study is small when compared with the findings of some previous studies, possibly as a result of the absence of fibrinogen in these serum samples, since this protein has previously been reported to be highly susceptible to carbonyl formation in in vitro studies (45). A correlation between protein
carbonyl levels and MPO concentrations in preterm infants has been reported previously (44), suggesting that this enzyme plays a role in the observed oxidation. In contrast, in the current study, a small, but significant, decrease in MPO levels was observed in the SLE patients, suggesting that the activity of this enzyme is not the cause of the observed enhanced oxidation. A previous study of patients with vasculitis and patients with autoimmune diseases associated with vasculitis including SLE, also reported a decrease in serum MPO levels in SLE patients compared with controls, although no statistical analysis was performed (46).

The increase in protein-bound carbonyls reported here may be an underestimation of the total yield of carbonyls formed, since it is known that protein oxidation yields both protein-bound and low molecular mass released carbonyls (47,48); only the former were quantified here. The ratio of bound to released carbonyls is dependent on the oxidant (48), and so, the total carbonyl yield cannot be extrapolated from previous data, since the oxidants involved in SLE are not known. The released carbonyls arise from fragmentation reactions of alkoxyl radicals generated on aliphatic side chains on proteins (47,48). When such reactions occur at the β-carbon (the first carbon of the side chain), an α-carbon radical is formed. Subsequent reaction of this species with a hydrogen atom donor results in the formation of an additional Gly residue. This type of reaction may account for the increase in protein-bound Gly residues detected in the SLE patients.

The lack of significant increases in the Tyr oxidation products DOPA, di-Tyr, and 3Cl-Tyr are consistent with the total amino acid analysis data (Table 2), where no significant loss of parent tyrosine (p-Tyr) was observed. Levels of serum 3NO2-Tyr have been reported to be significantly increased in SLE patients compared with controls (49,50). In the current study, the observed increase in this product did not correlate with increasing disease activity. The nonsignificant increase in the Phe oxidation product o-Tyr is in contrast to the significant loss of the parent amino acid, as determined by total amino acid analysis. This is consistent with previous studies that have shown that o-Tyr is not the sole oxidation product from this species, with multiple hydroxylated isomers and dimeric species being detected (30). Although Arg residues react rapidly with some oxidants (e.g., hydroxyl radicals [51]), it is likely that the observed decrease in this side chain arises via other mechanisms, since other residues that also react rapidly with hydroxyl radicals (e.g., Tyr, Trp, and His) (51) did not decrease in concentration. One potential route to loss of Arg residues is via reaction with carbonyl compounds generated by glycation/glycoxidation reactions or lipid oxidation (19); some of these species react rapidly with Arg residues (52). This has not been explored further.

The correlation between the various markers of protein oxidation examined and anti-dsDNA antibody positivity is not as strong or as significant as the correlation between these markers and the SLEDAI scores. This is not surprising, given that only 50–80% of SLE patients have elevated levels of these autoantibodies (for review, see ref. 53). However, levels of these antibodies have been reported to be a good predictor of disease exacerbation over time (54), a factor that was not examined in the present study. Use of the SLEDAI score, in contrast, enables the disease activity to be assessed at a fixed point in time and allows superior comparison between patients. Future studies should include correlations of SLEDAI scores and anti-dsDNA antibody levels with protein oxidation markers in a longitudinal followup.

The changes in protein-bound amino acids detected in this study suggest either that oxidation is occurring continuously in SLE patients at an elevated level compared with controls or that oxidation occurs in acute bursts, with inefficient repair of these lesions once they are formed. Since the major protein in serum is albumin and since this has a rapid turnover (15–20 days [55]), the current data are consistent with a chronically elevated level of oxidative stress in patients with SLE. This suggestion is consistent with the enhanced levels of oxidation detected in patients with higher levels of disease activity and suggests that measurement of protein oxidation parameters may be a useful surrogate marker of disease activity. Whether such measurements can be used in a prognostic manner remains to be established. Longitudinal studies of levels of protein oxidation with cumulative end-organ damage are essential to determine if protein oxidation is a major pathogenic mechanism in SLE.

ACKNOWLEDGMENTS

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BAFF Overexpression and Accelerated Glomerular Disease in Mice With an Incomplete Genetic Predisposition to Systemic Lupus Erythematosus

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Objective. To determine whether overexpression of BAFF can accelerate the development of systemic lupus erythematosus–associated end-organ disease in hosts with an underlying autoimmune diathesis.

Methods. We introduced a BAFF transgene (Tg) into autoimmune-prone B6.Sle1 and B6.Nba2 mice and evaluated these mice for serologic autoimmunity and renal pathology.

Results. B6.Sle1.BAFF and B6.Nba2.BAFF mice, but not non-Tg littermates, frequently developed severe glomerular pathology by 3 months of age. Age-matched B6.BAFF mice, despite renal Ig deposits and increases in B cells and Ig production similar to those in B6.Sle1.BAFF and B6.Nba2.BAFF mice, did not develop glomerular pathology. In B6.Sle1.BAFF and B6.Nba2.BAFF mice, severity of glomerular disease did not obligately correlate with circulating levels of IgG antichromatin and/or anti–double-stranded DNA antibodies or with amounts of these autoantibodies deposited in the kidneys. Even in mice with severe glomerular disease, renal tubulointerstitial infiltrates were very limited, and increased proteinuria was not detected.

Conclusion. BAFF-driven effects on glomerular pathology may be mediated, at least in part, by autoantibodies with specificities other than chromatin and/or by autoantibody-independent means. There is an uncoupling of BAFF-driven precocious glomerular pathology from concomitant development of clinically apparent renal disease, strongly suggesting that BAFF overexpression works in concert with other factors to promote overt renal disease.

Renal involvement in patients with systemic lupus erythematosus (SLE) is clinically apparent in ~50% of cases and leads to considerable morbidity. The vast majority of SLE patients with renal involvement develop increased proteinuria, sometimes to degrees great enough to adversely affect systemic fluid balance and hemodynamics (nephrotic syndrome). Glomerular disease is widely believed to be the major contributor to this increased proteinuria. Indeed, the World Health Organization (WHO) classification of lupus nephritis focuses almost entirely on glomerular lesions, with little attention to tubular, interstitial, or vascular lesions. Accordingly, factors which promote glomerular disease in SLE may be vital to the pathogenesis of lupus nephritis.
One such candidate factor is B cell–activating factor belonging to the tumor necrosis factor family (BAFF). BAFF, also known as B lymphocyte stimulator (BLyS), TALL-1, THANK, tumor necrosis factor superfamily member 13B, and zTNF4, is a 285–amino acid type II transmembrane protein member of the tumor necrosis factor ligand superfamily (1–6). It is expressed by myeloid lineage cells (1–3,5,7,8), bone marrow–derived radiation-resistant stromal cells (9), and T cells (10), and it is cleaved at the cell surface by a furin protease to release a soluble, biologically active 17-kd molecule (1,7).

BAFF plays a vital role in B cell survival (11–17). BAFF-deficient mice display substantial (albeit incomplete) reductions in mature B cells and in baseline serum Ig levels and Ig responses to T cell–dependent and T cell–independent antigens (18–20). In contrast, constitutive overexpression of BAFF in BAFF-transgenic (Tg) mice leads to SLE-like features (elevated circulating titers of multiple autoantibodies and renal Ig deposits) (6,21,22). By 8 months of age (but not 5 months of age), BAFF-Tg mice may manifest increased proteinuria (22).

BAFF overexpression is associated with SLE in humans as well. Cross-sectional and longitudinal studies have documented increased circulating levels of BAFF in as many as 50% of SLE patients. At the population level, circulating levels of BAFF correlate with circulating levels of anti–double-stranded DNA (anti-dsDNA) antibodies (23–25) and with clinical disease activity (26).

Importantly, elevated circulating levels of BAFF are not specific to SLE. Many patients with rheumatoid arthritis and Sjögren’s syndrome also harbor elevated circulating levels of BAFF (23,24,27,28). Indeed, elevated circulating levels of BAFF do not necessarily result in autoimmune disease. For example, circulating BAFF levels are elevated in a large percentage of human immunodeficiency virus (HIV)–infected individuals (29,30), but these subjects do not manifest features of SLE or related autoimmune disorders.

The common uncoupling of BAFF overexpression from the development of SLE-like illness suggests that BAFF overexpression may remain clinically silent in the absence of an independent underlying SLE diathesis. The prediction is that the greater the underlying SLE diathesis, the greater the effects of BAFF overexpression on the phenotypic expression of disease. To test this hypothesis, we utilized 2 distinct mouse lines congenic with C57BL/6 (B6) mice. B6 mice homozygous for the NZW mouse–derived Sle1 region (B6.Sle1 mice) (31) or for the NZB mouse–derived Nba2 region (B6.Nba2 mice) (32) each have an incomplete genetic predisposition to SLE, in that they spontaneously develop elevated circulating titers of IgG antichromatin autoantibodies but rarely develop renal disease (32–34). Full-blown disease is realized in these mice when other genetic insults are superimposed, such as expression of the Sle2 or Sle3 regions in the case of B6.Sle1 mice or by crossing with NZW mice in the case of either B6.Sle1 or B6.Nba2 mice (32,35–37).

In this study, we demonstrate that the introduction of a BAFF Tg into B6.Sle1 or B6.Nba2 mice often led to the precocious development of severe glomerular pathology by 3 months of age. BAFF-Tg mice that bore neither the Sle1 nor the Nba2 region did not develop glomerular pathology at this young age. Glomerular lesions in this model did not immutably correlate with circulating levels of IgG autoantibodies against chromatin and/or dsDNA or with the amounts of these autoantibodies deposited in the kidneys, which raises the possibility that the BAFF-driven effects are mediated, at least in part, via nonchromatin autoantibodies and/or via autoantibody-independent means. Of note, little renal tubulointerstitial infiltration and no increased proteinuria were observed even among mice with severe glomerular disease. This points to an uncoupling of BAFF-driven precocious glomerular pathology from the concomitant development of clinically apparent renal disease and implies that factors in addition to BAFF contribute vitally to lupus nephritis in the BAFF-Tg model of SLE.

MATERIALS AND METHODS

Mice. All mice were maintained at the University of Southern California (USC), and the experiments were approved by the Institutional Animal Care and Use Committee. Mice transgenic for murine BAFF (21) that had been backcrossed to B6 mice for >9 generations (B6.BAFF mice) were maintained as hemizygotes for the BAFF Tg. B6.Sle1.BAFF and B6.Nba2.BAFF mice were generated by crossing B6.Sle1 and B6.Nba2.BAFF mice generated by crossing B6.Sle1 and B6.Nba2 mice (31,32,38), respectively, with B6.BAFF mice and screening the F1 progeny (obligate heterozygotes for the Sle1 and Nba2 regions) for the BAFF Tg by polymerase chain reaction (PCR) (see below). F1 mice bearing the BAFF Tg were backcrossed to B6.Sle1 and B6.Nba2 mice, and the resulting pups were screened for homozygosity for the Sle1 and Nba2 regions, respectively, by PCR (38) and for the BAFF Tg.

Parental B6.Sle1.BAFF and B6.Nba2.BAFF mice were mated with B6.Sle1 and B6.Nba2 mice, respectively, to yield experimental BAFF-Tg mice and control non-Tg littermates of either sex. As additional controls, B6.BAFF mice were mated with B6 mice to yield BAFF-Tg and non-Tg mice of either sex that bore neither the Sle1 nor the Nba2 region. No significant differences in the measured parameters were identified be-
between male and female mice of any strain; therefore, the results for males and females were pooled.

**Detection of the BAFF Tg.** Genomic DNA extracted from mouse tail clippings was PCR-amplified for 29 cycles each at 95°C for 1 minute, 64°C for 1 minute, and 72°C for 1 minute. The primer sequences used were 5′-GCAGTTTCA-CAGCGATGTCTC-3′ and 5′-GTCTCCGGTGCTGAAA-TCTG-3′. The PCR products were subjected to electrophoresis in 1.5% agarose gels containing ethidium bromide, and bands were visualized under ultraviolet light. The band size for the BAFF Tg was ~700 bp.

**Cell surface staining.** Murine spleen mononuclear cells were stained singly or in combination with fluorescein isothiocyanate–conjugated, phycoerythrin-conjugated, or Cy-Chrome–conjugated monoclonal antibodies specific for murine CD3, CD4, CD8, CD44, CD62L, CD45R (B220), CD21, or CD23 (BD PharMingen, San Diego, CA) and then analyzed by flow cytometry (39).

**Serum immunoglobulins and spleen Ig-secreting cell determinations.** Sera were assayed for levels of total IgG and total IgM by enzyme-linked immunosorbent assay (ELISA) (39). Spleen cells were assayed for numbers of total Ig-secreting cells by the reverse hemolytic plaque assay (40,41). Each plaque-forming cell was taken as an Ig-secreting cell.

**Serum autoantibody determinations.** Serum levels of IgG autoantibodies to chromatin and dsDNA were determined by ELISA (38). Quantitative values were obtained by use of standard curves obtained with monoclonal antibodies to the appropriate nuclear antigen (42). Samples from (NZB × NZW)F1 (NZB/NZW) and B6 mice were run in every assay as positive and negative controls, respectively.

**Serum BAFF determination.** Serum BAFF levels were determined by a sandwich ELISA. Quantitative values were calculated from a standard curve of known concentrations of recombinant soluble murine BAFF (Biogen Idec, Cambridge, MA). The lower level of detection is 0.01 μg/ml.

**Assessment of proteinuria.** Reagent strips for urinary protein (Albustix; Bayer, Elkhart, IN) were dipped in mouse urine. Results were assigned a score of 0–4 by visual color comparison to the standard color key that was supplied.

**Kidney histology.** Individual sections of formalin-fixed kidneys were stained with hematoxylin and eosin or with Masson’s trichrome and were examined by light microscopy by one of us (MNK), who was blinded to the genotype of the mouse. Each case was assessed for the presence of glomerulonephritis (GN) using a modification of the WHO classification (30). Class IV was assigned for diffuse proliferative GN (50% of glomeruli), 2 = staining in the mesangium only (50% of glomeruli), 3 = strong staining in the mesangium (50% of glomeruli) with occasional staining of capillary loops, and 4 = strong staining in the mesangium (>50% of glomeruli) with widespread staining of capillary loops.

Ig elution from kidneys. One-half of a snap-frozen kidney from each mouse was thawed and minced with a clean razor blade. Kidneys from mice of a given cohort were pooled, and Ig was eluted (43). The eluates were dialyzed at 4°C against distilled water for 24 hours and then against phosphate buffered saline for an additional 24 hours. The total eluted IgG in each group was ~2–5 μg, as determined by ELISA. The antigenic specificities of the eluted antibodies were measured by antigen-specific ELISAs, using eluates adjusted to a total IgG concentration of 500 ng/ml (44,45).

**RESULTS**

**Numbers of splenic T cells and B cells and global Ig production in 3-month-old BAFF-Tg and non-Tg B6, B6.Sle1, and B6.Nba2 mice.** The numbers of splenic CD3+, CD4+, CD8+, and total B (B220+) cells were each modestly greater (geometric mean 31–68%) in non-Tg 3-month-old B6.Sle1 and B6.Nba2 mice than in age-matched non-Tg B6 mice ($P \leq 0.032$ for each comparison) (Figures 1A–D), although the numbers of splenic marginal zone B cells were essentially the same (Figure 1E).

Introduction of the BAFF Tg had similar effects on the numbers of CD3+, CD4+, CD8+, total B (B220+), and marginal zone B cells in B6, B6.Sle1, and B6.Nba2 mice. Splenic CD3+, CD4+, and CD8+ cell numbers in B6.Sle1.BAFF and B6.Nba2.BAFF mice remained modestly greater (geometric mean 58–116%) than those in B6.BAFF mice ($P \leq 0.004$ for each comparison), with CD4+ and CD8+ cells displaying a more activated phenotype (greater percentages of CD44+,62L– cells and/or lesser percentages of CD44+,62L+ cells) in all BAFF-Tg mice than in their non-Tg
littermates (data not shown). The numbers of splenic total B cells and marginal zone B cells in BAFF-Tg mice were each considerably greater (geometric mean 168–188% and 336–713%, respectively) than those in the corresponding non-Tg littermates ($P < 0.001$ for each comparison) (Figures 1D–E).

The numbers of splenic Ig-secreting cells were similar among non-Tg B6.Sle1, B6.Nba2, and B6 mice (Figure 2A). Serum total IgM levels in non-Tg B6.Sle1 and B6.Nba2 mice were lower (geometric mean 41–72%) than in non-Tg B6 mice ($P < 0.001$) (Figure 2B). Serum total IgG levels in non-Tg B6.Sle1 and B6.Nba2 mice were greater (geometric mean 29–96%) than in non-Tg B6 mice ($P < 0.001$) (Figure 2C). These serologic results paralleled those previously observed for corresponding mice tested at 8–9 months of age (38).

Introduction of the BAFF Tg resulted in increased numbers of splenic Ig-secreting cells (geometric mean 4.6–14.0-fold), increased levels of serum total IgM (geometric mean 2.4–8.5-fold), and increased levels of serum total IgG (geometric mean 1.8–3.5-fold) in B6, B6.Sle1, and B6.Nba2 mice ($P \leq 0.010$ for each comparison).

Serum levels of BAFF were measured in all but 3 of the mice. There was marked variability in serum BAFF levels among the BAFF-Tg mice, with some mice harboring vastly elevated levels and others harboring levels that were not much greater than those in their non-Tg littermates (Figure 3). This variability among BAFF-Tg mice has been observed both in the colony at USC and in the colony at Biogen Idec. The basis for this variability is not known, but it likely reflects the inherent biologic properties of BAFF-Tg mice, rather than some unappreciated unique local environmental issue. In any case, the great variability did permit us to assess the in vivo consequences of differences in serum BAFF levels across a very wide range.

When results were pooled from BAFF-Tg mice and their non-Tg littermates within a given cohort, splenic B cell numbers and all the tested parameters of global Ig production, including splenic Ig-secreting cell numbers, serum total IgM levels, and serum total IgG
levels, uniformly correlated strongly with serum BAFF levels (Table 1). Among BAFF-Tg mice within a given cohort (excluding non-Tg littermates), serum BAFF levels did not necessarily correlate with each of these B cell parameters, indicating that factors in addition to circulating BAFF levels are critical determinants of B cell numbers and global Ig production.

**Disparate effects of constitutive overexpression of BAFF on circulating autoantibody levels among B6, B6.Sle1, and B6.Nba2 mice.** By 8–9 months of age, large percentages of B6.Sle1 and B6.Nba2 mice harbor detectable circulating IgG antichromatin autoantibodies (32–34,38). By 3 months of age, there was already a significant difference in serum levels of IgG antichromatin in the B6 mice compared with the B6.Sle1 and the B6.Nba2 mice ($P < 0.001$) (Figure 2D). Serum levels of IgG anti–double-stranded DNA in B6.Nba2 mice were significantly greater than those in either the B6 or the B6.Sle1 mice ($P < 0.001$) (Figure 2E). Of note, 3-month-old B6.BAFF mice harbored no detectable circulating IgG antichromatin or anti–double-stranded DNA autoantibodies (Figures 2D and E).

Unexpectedly, the increases in serum levels of total IgG observed among B6.Sle1.BAFF mice in comparison to their non-Tg littermates were not paralleled by increases in serum levels of IgG antichromatin or anti–double-stranded DNA autoantibodies (Figures 2D and E). We
observed no significant correlations between serum levels of IgG antichromatin or anti-dsDNA antibodies and serum levels of BAFF among all the Sle1-bearing mice or among just the B6.Sle1.BAFF mice (Table 1). In contrast, serum levels of IgG anti-dsDNA antibodies were significantly greater in B6.Nba2.BAFF mice than in their non-Tg littermates (P < 0.001) (Figure 2E), and these levels correlated significantly with serum BAFF levels regardless of whether all the Nba2-bearing mice or just the B6.Nba2.BAFF mice were considered (Table 1). However, serum IgG antichromatin levels in B6.Nba2.BAFF mice were not significantly greater than those in their non-Tg littermates (Figure 2D), and no significant correlations were appreciated between serum levels of IgG antichromatin antibodies and BAFF among all the Nba2-bearing mice or among just the B6.Nba2.BAFF mice (Table 1). The differences in autoantibody profiles between Sle1-bearing and Nba2-bearing mice are consistent with previous observations (58) and reinforce the assertion that these mice are genetically distinct.

Constitutive overexpression of BAFF and precocious glomerular pathology in B6.Sle1 and B6.Nba2 mice, but not B6 mice. The variable effects on serum IgG autoantibody levels notwithstanding, introduction of the BAFF Tg into B6.Sle1 or B6.Nba2 mice had a dramatic effect on the development of glomerular disease. Kidney sections from 12 B6.Sle1.BAFF mice, 12 B6.Sle1 non-Tg littermates, 12 B6.Nba2.BAFF mice, 12 B6.Nba2 non-Tg littermates, 11 B6.BAFF (without the Sle1 or Nba2 regions), and 13 B6 non-Tg littermates were analyzed. Despite their young age (3 months), 4 B6.Sle1.BAFF mice and 5 B6.Nba2.BAFF mice already had class III (focal proliferative) GN, and 1 B6.Sle1.BAFF mouse and 5 B6.Nba2.BAFF mice already had class IV (diffuse proliferative) GN (Figure 3).

Massive mesangial and subendothelial deposits, with or without glomerular hypercellularity, were frequently observed in kidneys from B6.Sle1.BAFF and B6.Nba2.BAFF mice (Figure 4). In contrast, such histologic changes were rare and, when present, were very mild in B6.Sle1 and B6.Nba2 non-Tg mice. Only 1 B6.Sle1 and 2 B6.Nba2 non-Tg mice displayed class III GN, and no B6.Sle1 or B6.Nba2 mice displayed class IV GN. Among the 24 Sle1-bearing mice and the 24 Nba2-bearing mice, the severity of GN correlated strongly with serum levels of BAFF (Table 1). Among the limited numbers of B6.Sle1.BAFF and B6.Nba2.BAFF mice analyzed (without consideration of their non-Tg littermates), a trend between the severity of GN and the serum levels of BAFF was still appreciated in B6.Sle1.BAFF mice but not in B6.Nba2.BAFF mice. Among B6.Sle1 and B6.Sle1.BAFF mice, the severity of GN correlated with neither the serum levels of IgG antichromatin antibodies (r = −0.055, P = 0.793) nor the serum levels of IgG anti-dsDNA antibodies (r = 0.221, P = 0.294). Among B6.Nba2 and B6.Nba2.BAFF mice, the severity of GN correlated with serum levels of IgG anti-dsDNA antibodies (r = 0.645, P < 0.001) but not with serum levels of IgG antichromatin antibodies (r = 0.248, P = 0.240).

Importantly, all B6 and B6.BAFF mice displayed normal histologic features (WHO class I) or showed only mild, focal mesangial hypertrophy (class IIb) without any relationship to serum BAFF levels (Figure 3). Thus, at 3 months of age, the only BAFF-Tg mice to manifest substantial glomerular pathology were those that bore the Sle1 or Nba2 regions.
Of note, histologic changes in the kidneys did not faithfully parallel renal IgG deposition. IgG deposits were not detected in the kidneys of B6 mice (median immunofluorescence score 0.0 [n = 6]) but were present to a modest degree in the kidneys of B6.Sle1 (median immunofluorescence score 0.75 [n = 6]) and B6.Nba2 (median immunofluorescence score 0.75 [n = 5]) mice (P = 0.007). Importantly, renal IgG deposits were pronounced not only in the kidneys of B6.Sle1.BAFF (n = 6) and B6.Nba2.BAFF mice (n = 5), but also in the kidneys of B6.BAFF mice (n = 6) (Figure 5). Despite the lack of a statistically significant difference in the median immunofluorescence scores (2.75, 3.0, and 2.0, respectively) among these groups of mice, the B6.Sle1.BAFF and B6.Nba2.BAFF mice displayed considerable glomerular pathology, whereas the B6.BAFF mice displayed none (Figures 3 and 4).

This discordance between renal Ig deposits and pathology cannot be reconciled solely on the basis of differences in specificities of the deposited Ig (Figure 6).
Although eluates from B6.Nba2.BAFF kidneys contained more IgG autoantibodies against chromatin, dsDNA, laminin, and α-actinin than did eluates from the kidneys of B6.Nba2 non-Tg littermates, a similar pattern was not observed for eluates from kidneys of B6.Sle1.BAFF mice and their non-Tg littermates. A greater amount of IgG anti-α-actinin antibodies was eluted from kidneys of B6.Sle1.BAFF mice than from kidneys of B6.Sle1 non-Tg littermates, but no differences were appreciated in the amounts of IgG antichromatin or anti-dsDNA antibodies eluted from the kidneys of these groups of mice, and a considerably lesser amount of IgG antilaminin antibodies was eluted from kidneys of B6.Sle1.BAFF mice than from kidneys of B6.Sle1 non-Tg littermates. Similar amounts of IgG anti-type IV collagen antibodies were eluted from the kidneys of each mouse cohort.

Absence of increased proteinuria in B6.Sle1.BAFF and B6.Nba2.BAFF mice despite severe glomerular disease. None of the BAFF-Tg mice (including those with class III or class IV GN) had increased proteinuria (≥3+ by dipstick) relative to that in their non-Tg littermates (data not shown). Increased proteinuria has also not been observed in the limited numbers of 6-month-old B6.Sle1.BAFF and B6.Nba2.BAFF mice examined to date. Moreover, routine histologic and immunohistologic analyses revealed little tubulointerstitial inflammatory cell infiltrates even in kidneys with severe glomerular disease (Figure 4).

DISCUSSION

Using 2 distinct mouse lines congenic with B6 (B6.Sle1 and B6.Nba2) that each bears a genetic diathesis to SLE but rarely develops end-organ disease (32–34), we have demonstrated that constitutive overexpression of BAFF frequently leads to the development of severe glomerular pathology by as early as 3 months of age (Figure 3). When all Sle1-bearing mice or Nba2-bearing mice were considered, precocious glomerular pathology correlated strongly with serum levels of BAFF (Table 1). The greater prevalence of class IV GN among B6.Nba2.BAFF mice than among B6.Sle1.BAFF mice may reflect, in part, the greater serum BAFF levels in the former than in the latter group. Of note, a trend between serum BAFF levels and severity of GN was noted among B6.Sle1.BAFF mice but not among B6.Nba2.BAFF mice. This demonstrates that circulating BAFF levels, although certainly critical, are not obligately the dominant factor in determining the degree of glomerular pathology.

Importantly, similar constitutive overexpression of BAFF in B6 mice without the autoimmune proclivity imposed by the Sle1 or Nba2 regions does not lead to detectable changes in renal histology at this age, despite readily detectable IgG deposits in the kidneys (Figures 4 and 5). This indicates that BAFF overexpression per se is insufficient for the development of precocious glomerular disease and indicates that renal IgG deposits are not
synonymous with histologic changes. BAFF-driven glomerular pathology fails to develop in age-matched B6 mice, despite similar BAFF-driven changes in T cells, B cells, and global Ig production among B6, B6.Sle1, and B6.Nba2 mice (Figures 1 and 2).

Regardless of whether all Sle1-bearing or Nba2-bearing mice were considered or whether just the respective BAFF-Tg mice were considered, serum BAFF levels correlated strongly with serum levels of total IgG (and total IgM) but not with serum levels of IgG antichromatin antibodies (Table 1). For the Sle1-bearing mice, the lack of correlation extended to serum levels of IgG anti-dsDNA antibodies as well. In humans with SLE or rheumatoid arthritis, circulating BAFF levels correlate significantly with anti-dsDNA antibodies and rheumatoid factor antibodies, respectively (23,24). Nevertheless, the absence of a positive correlation between circulating levels of BAFF and some autoantibody has precedent. Despite one group of investigators documenting a correlation between circulating levels of BAFF and anti-SSA/Ro antibodies in patients with Sjögren’s syndrome (28), no such correlation was seen by another group of investigators (27). Since BAFF can form heterotrimers with APRIL (46), it is possible that the lack of correlation between serum levels of BAFF and IgG autoantibodies may be due to differential biologic effects of BAFF homotrimers compared with those of BAFF/APRIL heterotrimers. Although we did not detect circulating APRIL in our mice (Stohl W, et al: unpublished observations), our ELISA may not be able to recognize APRIL complexed to BAFF. Thus, the relationship between circulating autoantibody levels and circulating BAFF levels is not straightforward, and further investigation will be necessary to clarify this.

In addition to serum levels of IgG antichromatin and IgG anti-dsDNA antibodies not immutably correlating with serum BAFF levels, serum levels of each of these autoantibodies did not correlate with the severity of glomerular disease among B6.Sle1 and B6.Sle1.BAFF mice, and serum levels of IgG antichromatin antibodies did not correlate with the severity of glomerular disease among B6.Nba2 and B6.Nba2.BAFF mice. This may indicate that IgG antichromatin or anti-dsDNA antibodies are not the pathogenic autoantibodies in these mice. The specificity of the true pathogenic autoantibodies may be something other than chromatin or dsDNA and, thus, remained unmeasured.

There is precedent for this notion. Although severe renal disease develops in association with substantial Ig deposition in the kidneys of both NZM 2328 and congenic NZM.C57Lc4 mice, the prevalence of detectable circulating IgG anti-dsDNA or IgG antihistone/DNA antibodies is high only in NZM 2328 mice but is very low in NZM.C57Lc4 mice (47). Moreover, IgG anti-dsDNA antibodies are plentiful in eluates of diseased kidneys from NZM 2328 mice but are virtually absent from eluates of diseased kidneys from NZM.C57Lc4 mice. However, multiple antibodies with reactivities to autoantigens in kidney and liver extracts are readily detectable in both, which raises the possibility that IgG autoantibodies with nonchromatin specificities are pathogenic.

While the amounts of IgG antichromatin, anti-dsDNA, antihistamin, and anti-α-actinin antibodies deposited in the kidneys of B6.Nba2.BAFF mice were uniformly greater than those in the kidneys of B6.Nba2 non-Tg littermates, the amounts of 3 of these autoantibodies deposited in the kidneys of B6.Sle1.BAFF mice were either equal to or were actually less than the amounts deposited in the kidneys of B6.Sle1 non-Tg littermates (Figure 6). An important caveat in regard to these experiments is that the antigenic specificities of these antibodies in vivo may not necessarily be the same as those measured in the in vitro assays. Nevertheless, the amounts of IgG antichromatin, anti-dsDNA, antihistamin, and anti-α-actinin antibodies deposited in the disease-free kidneys of B6.BAFF mice were very similar to the amounts deposited in the diseased kidneys of B6.Sle1.BAFF mice, so it is doubtful that any of these autoantibodies is truly pathogenic in Sle1-bearing or Nba2-bearing mice. This may explain why B6.Sle1 and B6.Nba2 mice do not develop renal disease despite having high titers of circulating antichromatin and anti-dsDNA antibodies. Functional testing of the isolated autoantibodies from B6.Sle1 and B6.Nba2 mice will be required to validate this thesis.

It is plausible that the pathogenesis of BAFF-driven glomerular disease includes autoantibody-insensitive and/or autoantibody-independent mechanisms in addition to autoantibody-dependent mechanisms. There are precedents for this from other models.

First, STAT-4–deficient NZM 2328 mice develop accelerated renal disease and mortality despite a modest decrease in circulating anti-dsDNA levels, whereas congenic STAT-6–deficient NZM 2328 mice develop markedly attenuated renal disease and improved survival despite serum anti-dsDNA levels equal to or greater than those of wild-type NZM 2328 mice (48).

Second, in related NZM 2410 mice, treatment with anti–interleukin-4 antibodies or the genetic ablation of STAT-6 was shown to ameliorate renal disease and enhance survival, whereas the genetic ablation of
STAT-4 hastened renal disease and premature death. Despite the profound effects on morbidity and mortality, none of these interventions had appreciable effects on circulating levels of anti-dsDNA antibodies (49).

Third, treatment of NZB/NZW mice with one BAFF antagonist (TACI-Ig) had a dramatic salutary effect on renal disease and survival without measurable effects on circulating anti-dsDNA antibody levels (6) (although treatment of such mice with another BAFF antagonist [BAFF-R-Ig] did reduce circulating levels of anti-dsDNA antibodies in parallel with clinical improvement [50]).

Fourth, although B cell–deficient MRL-lpr/lpr mice do not develop nephritis or vasculitis (51), MRL-lpr/lpr mice bearing B cells that are incapable of secreting Ig (and, hence, harbor no circulating autoantibodies) do develop such disease (albeit in a milder form than that developed by wild-type MRL-lpr/lpr mice) (52). Thus, B cells clearly effect a disease-promoting function that is independent of their ability to produce autoantibodies but may be dependent upon the ability of B cells to promote the activation of autoreactive T cells (52,53). By extension, the effects of BAFF overexpression on the precocious development of glomerular disease in B6.Sle1 and B6.Nba2 mice may relate more to the effects of BAFF on B cell numbers (Figure 1D) than to its effects on circulating autoantibody levels. Although Ig-secreting cells are present in the kidneys of diseased NZB/NZW mice (54), it remains unknown whether the local antibody production per se is critical to pathogenesis. Experiments are presently being designed to assess the effects of BAFF overexpression on the development of disease in mice bearing B cells incapable of secreting any Ig.

We postulate that BAFF overexpression amplifies an underlying latent diathesis to SLE, rather than creating an SLE-like disease de novo. In hosts with a relatively “high” latent SLE diathesis, such as B6.Sle1 and B6.Nba2 mice, the development of end-organ (glomerular) pathology requires only short-term overexpression of BAFF. Pathologic changes in hosts with an “intermediate” latent SLE diathesis would require longer-term BAFF overexpression, and in hosts with a “low” latent SLE diathesis, even long-term BAFF overexpression might be insufficient to induce pathology.

We suggest that wild-type B6 mice have an underlying “intermediate” latent SLE diathesis. In a large cohort of (B6 × 129)F2 mice, a B6-derived locus on chromosome 3 was shown to augment circulating levels of IgG antinuclear and antichromatin antibodies (55). Indeed, B6 mice deficient in Fcγ receptor IIb (FcγRIIB) spontaneously develop circulating antichromatin and anti-dsDNA autoantibodies along with immune complex GN and pulmonary vasculitis, whereas FcγRIIB-deficient BALB/c mice do not (56). Moreover, B6 mice deficient in programmed death 1 (PD-1) develop a clinical phenotype highlighted by arthritis and GN (57), whereas BALB/c mice deficient in PD-1 develop no signs of arthritis or GN but do develop a lethal dilated cardiomyopathy (58). Thus, discrete immune disturbances elicit SLE-like features in B6 mice, but the identical immune disturbances do not do so in BALB/c mice. Accordingly, it is not surprising that BAFF-Tg mice bearing a B6 or B6-mixed genetic background can develop SLE-like features with advancing age (6,21,22).

A final issue is the absence of severe proteinuria (≥3+ by dipstick) even in mice with class III or class IV GN. The discordance between proteinuria and glomerular pathology at 3 months of age strongly suggests that proteinuria may not be an adequate marker for (early) lupus nephritis, even when it is severe. Given the paucity of tubulointerstitial infiltrates even in mice with class IV GN (Figure 4), it may be that the combination of glomerular disease along with tubulointerstitial infiltrates is necessary for the development of severe proteinuria. We have not observed increased proteinuria in the limited number of 6-month-old B6.Sle1.BAFF and B6.Nba2.BAFF mice studied to date (data not shown), which strongly suggests that BAFF overexpression works in concert with other factors to promote the development of clinically apparent renal disease. Expression of these unidentified contributory factors likely requires neither the Sle1 nor the Nba2 region, since BAFF-Tg mice bearing neither region develop proteinuria by 8 months of age (22). Studies have been initiated to elucidate the nature of these factors.

ACKNOWLEDGMENTS

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Development and Validation of a Clinical Index for Assessment of Long-Term Damage in Juvenile Idiopathic Arthritis

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Objective. To develop and validate a clinical measure of articular and extraarticular damage in patients with juvenile idiopathic arthritis (JIA).

Methods. The Juvenile Arthritis Damage Index (JADI), which is derived from physical examination and a brief review of the patient’s clinical history, is composed of 2 parts: assessments of articular damage (JADI-A) and extraarticular damage (JADI-E). Instrument validation was accomplished by evaluating 158 JIA patients with disease duration of at least 5 years, seen consecutively over 21 months. The instrument’s feasibility, face and content validity, construct and discriminative ability, internal consistency, and interrater reliability were examined.

Results. Among the 158 JIA patients, 47% and 37% had articular and extraarticular damage, respectively. The JADI was found to be feasible and to possess both face and content validity. The JADI-A score correlated highly with the number of joints with limited range of motion (Spearman’s $r_s = 0.72$) and correlated moderately with the Childhood Health Assessment Questionnaire score ($r_s = 0.41$), Steinbrocker functional classification ($r_s = 0.50$), and Poznanski’s score of radiographic damage ($r_s = -0.54$), thereby demonstrating good construct validity. Correlations with the JADI-E score were lower, owing to the heterogeneity of its items. The JADI-A discriminated well among different levels of disability. The internal consistency (Chronbach’s alpha) of the JADI-A and JADI-E was 0.93 and 0.59, respectively. The intraclass correlation coefficients between pairs of independent observers ranged from 0.85 to 0.97.

Conclusion. The JADI exhibited good reliability, construct validity, and discriminative ability and is therefore a valid instrument for the assessment of long-term damage in patients with JIA, in the context of both clinical management and research settings.

Juvenile idiopathic arthritis (JIA) is a chronic and heterogeneous disease characterized by prolonged synovial inflammation that may lead to permanent alterations in joint structures. Permanent changes may also develop in extraarticular organs/systems, such as the eye (as a complication of chronic anterior uveitis) or the kidney (due to systemic amyloidosis), or may result from side effects of medications (1). This morbidity may have a relevant impact on the quality of life of patients and their families (2,3).

In the outcome studies published so far (for review, see refs. 4 and 5), the long-term morbidity in JIA patients has been most frequently evaluated in terms of functional disability. Currently, the most widely used tool for assessment of functional status is the Childhood Health Assessment Questionnaire (C-HAQ) (6). However, despite its advantages and widespread use, the C-HAQ has been shown to have specific limitations in research and clinical settings. First, it has been demonstrated to have a ceiling effect, with a tendency for scores to cluster at the normal end of the scale, particularly in patients with fewer joints involved (7,8). Second, its estimation of physical disability in patients with active disease can be inflated by symptoms of inflammation, particularly joint pain (9,10). Third, the parent’s observation of the child’s physical function has been found to

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be frequently inaccurate, being affected by both the severity of arthritis and the level of pain (11). Finally, the C-HAQ may not capture information on several possible forms of damage that may develop in JIA patients over time, such as micrognathia, height retardation, localized growth disturbances, pubertal delay, or visceral organ failure.

Damage in the joints of patients with JIA is assessed by radiographs, which may show the destruction of bone and cartilage. Despite the usefulness of radiographs in studying disease progression, there are some drawbacks. First, radiographs do not fully reflect the biologic outcome of the disease, because they represent mainly cartilage and osseous changes, whereas part of the articular damage in JIA is in the soft tissues surrounding the bones. In addition, radiographs do not measure damage in extraarticular systems or visceral organs. Second, the few available methods for scoring radiographic damage in JIA patients concentrate on the wrists or knees (12–14), whereas damage in other joints may be of equal importance for a patient’s functional ability. Third, the cost of measuring radiographic damage and the related radiation exposure make these methods less suitable for studying large numbers of patients or for use in developing countries.

To monitor the course of the disease effectively and to address multiple outcomes over the long term, there is a need for an adjunctive clinical instrument that encompasses all forms of damage that may accumulate in patients with JIA over time. Several attempts to design a method of scoring clinical damage in adult rheumatoid arthritis have been reported (15–18), but such a measure does not exist for JIA. In order to provide a clinical measure that reflects the overall biologic outcome of JIA, we have devised a simple and easy-to-apply clinical index, the Juvenile Arthritis Damage Index (JADI), to assess the total amount of articular and extraarticular damage. In this report, we provide evidence of the reliability and validity of this scale in a large cohort of JIA patients with longstanding disease.

**PATIENTS AND METHODS**

**Patient selection.** The present cross-sectional study comprised all patients seen consecutively between September 2002 and May 2004 at the Departments of Pediatrics of Genoa and Pavia Universities in Italy. The patients met the following entry criteria: 1) diagnosis of JIA in accordance with the 2001 International League of Associations for Rheumatology (ILAR) revised criteria (19); 2) disease duration of at least 5 years; and 3) provision of informed consent. Patients were excluded if they had enthesitis-related arthritis.

**Clinical assessment.** At the time of the study visit, the following information was obtained for each patient: sex, age at disease presentation, ILAR category of JIA, disease duration and age at study visit, and previous use of systemic corticosteroid and second-line drug therapies. The following clinical assessments were made by the attending pediatric rheumatologist (AR or SV in Genoa and SMM in Pavia): physician’s global assessment of overall disease activity measured on a 10-cm visual analog scale (VAS) (0 = no activity, 10 = maximum activity), number of swollen joints, number of joints with pain on movement/tenderness, number of joints with limited range of motion (ROM), and number of joints with active arthritis (defined as joints with swelling, or if no swelling present, joints with limitation of movement with either pain on motion or tenderness). The articular indices were assessed in a total of 67 joints (those that are included in the standard articular examination). The attending physician also assigned the Steinbrocker functional classification (20).

A parent of each patient was asked to make a global assessment of the child’s overall well-being on a 10-cm VAS (0 = very good, 10 = very poor), to assess the degree of the child’s pain on a 10-cm VAS (0 = no pain, 10 = very severe pain), and to complete the Italian version of the C-HAQ (21) (0 = best, 3 = worst). For purposes of the analysis, the C-HAQ score was divided into the following 4 categories: 0 = no disability, >0 and ≤0.5 = mild disability, >0.5 and ≤1.5 = moderate disability, and >1.5 = severe disability (22).

The parent was also asked to evaluate the child’s health-related quality of life (HQOL) through the Italian parent version of the Child Health Questionnaire (CHQ) (21). Briefly, the CHQ (23) is a generic instrument that is designed to capture the physical, emotional, and social components of health status of children of at least 5 years of age. It comprises 15 subscales and yields 2 summary measures: the physical score (PhS) and the psychosocial score (PsS). These scores have been standardized in healthy Italian children to have a mean of 50 and an SD of 10. Higher scores in the scales indicate better HQOL. The laboratory assessment of JIA activity included the erythrocyte sedimentation rate (ESR) determined with the Westergren method, and the C-reactive protein (CRP) level determined with nephelometry.

**Radiographic assessment.** In patients with wrist involvement, standard radiographs of both wrists in the posteroanterior view were obtained. Radiographic damage was scored according to the method described by Poznanski et al (12), as previously reported (13). Briefly, this method is based on the measurement of the radiometacarpal (RM) length, which is the distance from the base of the third metacarpal bone to the midpoint of the distal growth plate of the radius, and of the maximal length of the second metacarpal bone (M2). All radiographs were evaluated by the same observer (FR), who has specific experience in the assessment of Poznanski’s score. For each wrist, the number of standard deviations between the expected and the observed RM length for the measured M2 was calculated. The RM/M2 score, which represents the carpal length and constitutes Poznanski’s score, reflects the amount of radiographic damage in the wrist. A more negative score indicates more severe radiographic damage. For each pair of wrists, the mean score was used in the analyses.
Damage assessment. The amount of articular and extraarticular damage was assessed using the JADI. This index was devised by a group of 6 experienced pediatric rheumatologists (AR, SV, AB, NR, SMM, and AM) based on their previous clinical experience, as well as on pediatric rheumatology and physiotherapy textbooks (1,24–26) and on similar efforts undertaken in adult rheumatoid arthritis (15–18). After extensive discussion of the relative importance of each potential item, an item was retained only when there was agreement among the group components indicating that it should be kept in the index. Thus, content validity was provided by the members of the group. To ensure face validity, the instrument was shown to 10 physicians in the study centers who were not part of the JADI group and to 4 physiotherapists, and their opinion on the suitability of the instrument was obtained.

The index was designed to be quick and easy to score, using information obtained by physical examination and by a brief review of the patient’s clinical history. The definitions for scoring each item are concise and simple, in order to make the method accessible to inexperienced assessors. The JADI is intended to rate the extent of damage, defined as persistent changes in anatomy, physiologic status, pathologic processes, or function, that is the result of prior active disease, complications of therapy, or comorbid conditions, that is not due to currently active arthritis, and that is present for at least 6 months despite previous therapies, including exercise and rehabilitation. Damage is often irreversible and cumulative, and thus, damage scores are most frequently expected to increase or remain stable over time. However, because some forms of damage may improve or even resolve in pediatric patients, scores may decline in some cases. The index is composed of 2 parts, one devoted to the assessment of articular damage (JADI-A) and one devoted to the assessment of extraarticular damage (JADI-E) (see Appendices A and B).

In the JADI-A, 36 joints or joint groups are assessed for the presence of damage. The damage observed in each joint is scored on a 2-point scale (1 = partial damage, 2 = severe damage, ankylosis, or prosthesis). The only tool needed is a goniometer, although most joints can be assessed without one. The maximum total score is 72.

The JADI-E includes 13 items in 5 different organs/systems. Each item is scored as either 0 or 1 according to whether damage is absent or present, respectively. Due to the relevant impact of ocular damage on the child’s health, it was decided to give a score of 2 for each eye when the patient has had ocular surgery, and a score of 3 when the patient has developed legal blindness. A glossary of terms is included in the JADI-E (see Appendix B) to provide more specific definitions of each single item. The maximum total score is 17.

The amount of damage was determined independently by 3 observers (AR, SV, and AB) in patients seen in Genoa, and by 2 observers (SMM and MB) in patients seen in Pavia. Damage was assessed on the same day at which the other assessments were performed.

Statistical analysis. To validate the JADI, we used the filter of the Outcome Measures in Rheumatology Clinical Trials (27,28). Feasibility or practicality of the JADI was determined by addressing the issues of brevity, simplicity, and ease of scoring and from the percentage of missing values (29). Face and content validity have been discussed above.

Criterion validity is a measure of the extent to which values on an instrument agree with those of a gold standard. However, there is no reference measure against which to test the validity of the JADI. For this reason, convergent construct validity was investigated. Construct validity is a form of validation that seeks to examine whether the construct in question, in this case the JADI, is related to other measures in a manner consistent with a priori prediction. Given that the JADI-A was devised to measure cumulative articular damage, it was predicted that the correlation of the JADI-A score with joint counts (number of joints with limited ROM) would be high, since both are measures of closely related constructs. Correlations with measures of physical disability and radiographic damage were predicted to be moderate, since both are important components of cumulative damage, and correlations with disease activity parameters were predicted to be low. Since the JADI-E measures cumulative damage not only in the musculoskeletal system, but also in some extraskeletal organs/systems, the correlations of the JADI-E score with the extent of physical disability and radiographic damage were predicted to be low to moderate; as for the JADI-A, the correlations of the extraarticular component of the JADI with disease activity measures were predicted to be low. In the validation process, we also evaluated the correlation between the JADI scales and the HQOL assessment. In this case, no prediction was attempted, because HQOL is a multidimensional concept that can be affected by several other factors in addition to damage. Correlations were assessed using Spearman’s rank correlation coefficients (rS). For the purpose of this analysis, correlations >0.7 were considered high, correlations ranging from 0.4 to 0.7 were considered moderate, and correlations <0.4 were considered low (30). Agreement between predicted and observed correlations was taken as evidence of construct validity.

To determine whether the JADI exhibited different characteristics in mildly and more severely affected subjects, the group of patients with moderate-to-severe disability was identified as those with a score >0.5 on the C-HAQ. Key correlations were then recalculated and compared with those obtained in the complete population. Furthermore, we compared the Spearman’s correlation of JADI-A and C-HAQ scores with the Steinbrocker functional classification, Poznanski’s score of radiographic damage, and the HQOL score. The discriminative ability of the JADI was assessed through one-way analysis of variance, by comparing JADI scores from patients belonging to different ILAR categories or having different levels of disability as measured by the Steinbrocker functional classification or the C-HAQ.

Interrater reliability was assessed by calculating the intraclass correlation coefficients (ICCs) (31) between 2 independent, blinded observers who completed the JADI scales in the same patients on the same day. An ICC value higher than 0.8 was considered indicative of excellent reliability. The mean of the results of JADI assessment obtained from the 2 observers was used in all validation analyses.

The internal consistency of the scales was determined by calculating Cronbach’s alpha coefficient (32). A value of 0.80 was considered acceptable (33). The responsiveness of the instrument could not be assessed due to the cross-sectional nature of the study. It will be done in a future prospective study, but this will take at least 5 years.

All statistical tests were 2-sided, and a P value less than
0.05 was considered significant. The statistical package used was Statistica (StatSoft, Tulsa, OK).

RESULTS

Patient characteristics. A total of 158 patients, 141 from Genoa and 17 from Pavia, were included in the study; their main clinical features are presented in Table 1. None of the eligible patients seen in the study period refused to participate or were excluded for other reasons. Of the 107 patients who had received second-line drug therapies, 103 had received methotrexate, 40 cyclosporin A, 14 etanercept, 11 sulfasalazine, 4 azathioprine, 3 hydroxychloroquine, 1 colchicine, and 1 infliximab.

Articular and extraarticular damage. The results of articular and extraarticular damage assessments are shown in Table 2, together with the assessments of physical disability and HQOL. Forty-seven percent of patients had damage in at least one articular site and 37% of patients had damage in at least one extraarticular domain. Fifty-two percent of patients had disability according to the C-HAQ (score >0), while 38% had

Table 1. Clinical features of the 158 study patients

<table>
<thead>
<tr>
<th></th>
<th>Total</th>
<th>Median</th>
<th>Minimum</th>
<th>Maximum</th>
</tr>
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<tbody>
<tr>
<td>No. (%) male/no. (%) female</td>
<td>35 (22.1)/123 (77.8)</td>
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<tr>
<td>ILAR category, no. (%)</td>
<td></td>
<td></td>
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<tr>
<td>Systemic arthritis</td>
<td>20 (12.6)</td>
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<tr>
<td>Rheumatoid factor-negative polyarthritis</td>
<td>28 (17.7)</td>
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<tr>
<td>Rheumatoid factor-positive polyarthritis</td>
<td>5 (3.2)</td>
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<tr>
<td>Oligoarthritis, extended</td>
<td>47 (29.7)</td>
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<tr>
<td>Oligoarthritis, persistent</td>
<td>52 (32.9)</td>
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<tr>
<td>Psoriatic arthritis</td>
<td>6 (3.8)</td>
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<tr>
<td>Age at disease onset, years</td>
<td>3.1 0.5 14.8</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at study visit, years</td>
<td>11.8 5.5 25.6</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Disease duration, years</td>
<td>7.3 5.0 24.5</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Physician’s global assessment of overall disease activity†</td>
<td>2.5 0.0 10.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Parent’s global assessment of the patient’s overall well-being (n = 151)‡</td>
<td>0.7 0.0 9.5</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Parent’s assessment of the patient’s pain (n = 148)†</td>
<td>1.0 0.0 9.9</td>
<td></td>
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<tr>
<td>No. of swollen joints</td>
<td>1.0 0.0 30.0</td>
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<td></td>
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<tr>
<td>No. of joints with pain on motion/tenderness</td>
<td>1.0 0.0 28.0</td>
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<tr>
<td>No. of joints with limited range of motion</td>
<td>1.0 0.0 61.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>No. of joints with active arthritis</td>
<td>2.0 0.0 39.0</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Duration of morning stiffness, minutes (n = 148)</td>
<td>0.0 0.0 240.0</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Poznanski’s score, units (n = 75)‡</td>
<td>-1.4 -7.0 1.5</td>
<td></td>
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<tr>
<td>Erythrocyte sedimentation rate, mm/hour (n = 147)§</td>
<td>15.0 2.0 108.0</td>
<td></td>
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</tr>
<tr>
<td>C-reactive protein, mg/dl (n = 147)¶</td>
<td>0.1 0.1 9.9</td>
<td></td>
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<tr>
<td>Previous second-line drug therapy, no. (%)</td>
<td>107 (67.7)</td>
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<tr>
<td>Previous systemic corticosteroid therapy, no. (%)</td>
<td>68 (43.0)</td>
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</table>

* ILAR = International League of Associations for Rheumatology.
† Range 0 (best) to 10 (worst).
‡ Abnormal score: less than −2.0.
§ Normal <15.
¶ Normal <0.3 (all values below the threshold were equalized to 0.1 mg/dl).
disability according to the Steinbrocker classification (classes II–IV). The percentage of patients with severe disability was 1.3% by the C-HAQ (score \( \geq 1.5 \)) and 0.6% by the Steinbrocker classification (class IV). The wrist was the most frequently damaged joint (16%), followed by the elbow (14%) and the interphalangeal joints (14%), whereas the cervical spine (6%) and the metacarpophalangeal joints (6%) were the least commonly affected sites. Ocular damage (6% and 10% in the right eye and left eye, respectively), growth failure (11%), and muscle atrophy (9%) were the most frequently reported extraarticular items, whereas avascular necrosis of bone, diabetes mellitus, secondary amyloidosis, malignancy, and other organ failure were not observed.

Feasibility. The JADI appeared to be easy to apply. After a short learning period, it took 5–15 minutes for each patient, depending on the amount of damage. There were no missing responses for either of the JADI scales.

Face and content validity. As stated above, content validity was established by the members of the group who devised the index. Face validity was confirmed by 10 physicians and 4 physiotherapists who have specific experience in the field, all of whom provided their agreement. Nevertheless, several points were raised regarding the definitions of the items, and these were discussed and partially incorporated in the final version.

Construct validity. The Spearman’s correlation coefficients used to assess convergent construct validity of the JADI scales are summarized in Table 3. As predicted, correlation of the JADI-A score with the number of joints with limited ROM was high. Moreover, as predicted, correlations with the C-HAQ score, Steinbrocker functional classification, and Poznanski’s score of radiographic damage were moderate. Correlations between the JADI-A score and measures of disease activity, including physician’s and parent’s global assessments, swollen and painful joint counts, duration of morning stiffness, the ESR, and CRP level, were low; the sole exception was a moderate correlation with the active joint count, perhaps reflecting the close correlation between the JADI-A score and the number of joints with limited ROM, the latter of which is one of the components of the definition of active joints.

All Spearman’s correlation coefficients for associations between the outcome measures and the JADI-E score were low. All correlations of damage scores with the CHQ PhS and PsS scores were low, although there was a tendency toward better correlations with the physical component (PhS) of the CHQ.

When only patients with moderate-to-severe disability (C-HAQ score \( \geq 0.5 \); \( n = 31 \)) were analyzed, convergent construct validity showed some differences with respect to the entire population. In this subset of patients with more severe disability, correlations of the JADI-A score with the number of joints with limited ROM \( (r_S = 0.79) \), with Poznanski’s score of radiographic damage \( (r_S = -0.65) \), and with the CHQ PhS \( (r_S = 0.50) \) were higher, and correlations of the JADI-E
score with the Steinbrocker functional classification ($r_S = 0.49$) were higher. In contrast, correlations of the JADI-A score with the active joint count ($r_S = 0.33$) were lower.

**Discriminative validity.** The property of discriminative validity was assessed by comparing JADI scores among patients belonging to different ILAR categories or having different levels of disability. The JADI-A discriminated well among patients on the basis of ILAR category of JIA or C-HAQ score category (data not shown) and on the basis of Steinbrocker functional class (Figure 1).

**Internal consistency.** Chronbach’s alpha was calculated to measure the internal consistency of the scales. For the JADI-A, $\alpha = 0.93$; for the JADI-E, $\alpha = 0.59$.

**Interrater reliability.** The ICC for JADI assessments between pairs of independent observers ranged from 0.85 to 0.97, indicating very good interrater reliability.

**Relationship of the JADI and C-HAQ with Steinbrocker classification, radiographic damage, and HQOL.** The JADI-A and the C-HAQ score were found to be correlated to a similar extent with the Steinbrocker functional classification, whereas the JADI-A proved to be more strongly correlated with the number of joints with limited ROM ($r_S = 0.72$ versus $r_S = 0.55$ with the C-HAQ) and with Poznanski’s score of radiographic damage ($r_S = -0.54$ versus $r_S = -0.21$ with the C-HAQ). In contrast, the C-HAQ score was better correlated with both the CHQ PhS ($r_S = -0.56$ versus $r_S = -0.19$ with the JADI-A) and the CHQ PsS ($r_S = -0.19$ versus $r_S = 0.04$ with the JADI-A).

**DISCUSSION**

We have described the development of a new clinical measure of articular and extraarticular damage...
in patients with JIA. It is simple, easy to use, and is quick, taking only 5–15 minutes to score, which makes it practical for use in the clinical setting. The instrument was found to be feasible and to possess both face and content validity; furthermore, it exhibited good convergent construct validity, excellent reliability (intrarater agreement and internal consistency), and strong discriminative validity in a large cohort of JIA patients with longstanding disease. The lower performance of the JADI-E as compared with the JADI-A in terms of construct validity and internal consistency was expected, because the former scale addresses a heterogeneous set of organ systems. By documenting these key measurement properties, we have shown that the JADI is a valid instrument for the assessment of accumulated damage in this patient population and is, therefore, potentially applicable in both clinical and research contexts.

The articular component of the JADI has been designed to assess 3 main forms of joint damage that are persistent for at least 6 months and are not due to currently active arthritis: limited ROM, deformity, and previous surgical interventions such as prosthetic replacement, arthrodesis, arthroplasty, or fusion. Although all main joints of the body are assessed, the scale does not require the measurement of all individual joint angles by a goniometer; this would be quite tedious and time-consuming. Instead, for each joint, only the movements that are known to be affected more frequently and precociously in JIA patients (being, thus, a surrogate measure of whole-joint movements) have been included. On the basis of current knowledge of a joint’s normal ROM, an experienced examiner may visually estimate, for most joints, whether the ROM is normal or limited by the threshold indicated in the JADI-A. In some joints, particularly the cervical spine, shoulder, and hip, it may be difficult to distinguish damage from reversible impairment due to inflammation. In the case of impairment of shoulder or hip movement, the examiner has to decide whether it is fixed impairment or one that might improve after a corticosteroid injection. In the case of uncertainty, a second assessment (i.e., after 6–12 months) will help to clarify the issue.

Like its articular counterpart, the JADI-E is designed to assess the sources of extraarticular damage most frequently observed in JIA patients. The list of damage items is not intended to be exhaustive, but may be modified or enlarged after the application of the index to other populations of patients seen in different clinical or research settings. In general, we anticipate that both components of the JADI may undergo a process of refinement as we and other investigators incorporate new data, including information on the score change over time. Furthermore, it might be worth investigating whether weighting the JADI-A items differently, depending on the relative importance of each joint to a child’s function, would improve the clinical relevance of the overall score. We found that item weighting using a recently developed weighted joint score (34) did not increase the correlations of the JADI-A with the other JIA severity measures (data not shown).

The JADI has been found by us to be a useful and practical tool. This does not mean, however, that it should be the only instrument used for the assessment of long-term outcomes in JIA patients. When we evaluated the Spearman’s correlation between the JADI-A and the C-HAQ, we found that the 2 instruments were only moderately correlated. This means that the JADI and the C-HAQ both provide complementary and nonredundant information that facilitates the measurement of long-term morbidity in JIA patients. Notably, the JADI-A and the C-HAQ provided different levels of correlation with the radiographic score and with the HQOL, which are other key measures in JIA outcome studies. The closer relationship of the C-HAQ with the HQOL, particularly with its physical component, is not surprising, because the 2 measures address closely related constructs; likewise, the superior correlation of the JADI-A with the radiographic score was not unexpected, because both are objective measures of joint damage. Taken together, these findings lead us to recommend that both the JADI and the C-HAQ be incorporated, together with a radiographic score, an HQOL tool, and the traditional indicators of disease activity and severity, in a core set of measures that should be used in every longitudinal observational study in JIA. This would provide a framework to investigate the full range of factors that can promote long-term morbidity and disability in JIA.

Some limitations to this study need mentioning. The validation analysis was cross-sectional and therefore issues of causality, predictive validity over time, and responsiveness to clinically meaningful change remain to be examined. Although the index was designed to be sufficiently comprehensive to cover all JIA subtypes, it may not detect all possible forms of damage in the juvenile spondyloarthropathies. Notably, the study sample was composed of consecutive patients who continued to receive care at a tertiary pediatric rheumatology care facility at 5 years after disease onset, leading to a potential overrepresentation of patients with more active disease. However, although many of the patients
whose disease entered remission in more recent years were probably discharged, the 21-month time frame for study enrollment led us to include most of the patients with mild disease who attended the study units for their annual review.

Therefore, although our study was not designed as an outcome survey, and thus does not reflect outcomes in JIA patients in general, it provides useful information on the disease status of a large population of JIA patients with longstanding disease who are likely to have benefited from the recent advances in the treatment of the disease, such as the widespread use of methotrexate and intraarticular corticosteroids, the aggressive early introduction of these drugs and/or other disease-modifying antirheumatic medications, and, in recent years, the availability of the newer biologic agents. Our finding that only ~1% of the patients had severe disability confirms the tendency toward a marked improvement in functional outcome seen in recent studies (4,5,35). Nonetheless, the degree of impaired function and irreversible damage observed is still considerable and needs to be improved.

In summary, we have developed a new instrument for the assessment of damage to joints and other organs in patients with JIA that we believe is feasible for measuring long-term outcome in large cohorts and for comparing the long-term effectiveness of diverse treatment strategies in different centers and in different countries. This measure is likely to increase current understanding of the natural history of the disease.

REFERENCES


### APPENDIX A: THE JUVENILE ARTHRITIS DAMAGE INDEX FOR ASSESSMENT OF ARTICULAR DAMAGE (JADI-A) IN PATIENTS WITH JUVENILE IDIOPATHIC ARTHRITIS

#### THE JUVENILE ARTHRITIS DAMAGE INDEX - ARTICULAR DAMAGE

<table>
<thead>
<tr>
<th>Joint Type</th>
<th>Definition of articular damage</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Temporomandibular</td>
<td>Micrognathia or face asymmetry that are severe enough to cause relevant aesthetic compromise and/or malalignment of the teeth arcades</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Severe restriction of mouth opening (not allowing introduction of three superimposed hand fingers) with radiographic joint changes</td>
<td>2</td>
</tr>
<tr>
<td>Cervical spine</td>
<td>Extension &lt;50% of the normal range or cervical subluxation demonstrated radiographically</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ankylosis or history of mediullary compression or surgical fusion</td>
<td>2</td>
</tr>
<tr>
<td>Shoulder</td>
<td>External rotation &lt;50% of the normal range and/or abduction &lt;180° (not above the shoulder plane)</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ankylosis or prosthesis</td>
<td>2</td>
</tr>
<tr>
<td>Elbow</td>
<td>Flexion contracture &lt;30°</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Flexion contracture &gt;30°, ankylosis or prosthesis</td>
<td>2</td>
</tr>
<tr>
<td>Wrist</td>
<td>Extension or flexion &lt;50% of the normal range or subluxation or volar, ulnar or radial deviation</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ankylosis or prosthesis</td>
<td>2</td>
</tr>
<tr>
<td>Metacarpophalangeal</td>
<td>Flexion contracture, ulnar or radial deviation 1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Subluxation, ankylosis or prosthesis - 2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>1</td>
</tr>
<tr>
<td>Proximal interphalangeal</td>
<td>Flexion contracture 1</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Swan neck or en boutonnière deformity or ankylosis - 2</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>III</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>IV</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>V</td>
<td>1</td>
</tr>
<tr>
<td>Hip</td>
<td>Internal rotation &lt;10°</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Ankylosis or prosthesis</td>
<td>2</td>
</tr>
<tr>
<td>Knee</td>
<td>Valgus deviation &gt;15° due to arthritis or flexion contracture &lt;25°</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Flexion contracture &gt;25° or prosthesis</td>
<td>2</td>
</tr>
<tr>
<td>Ankle</td>
<td>Fixed valgus deformity &lt;20°</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Fixed valgus deformity &gt;20°, ankylosis, arthrodesis or prosthesis</td>
<td>2</td>
</tr>
<tr>
<td>Metatarsophalangeal</td>
<td>Visible deformity due to arthritis</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>Arthroplasty of the forefoot</td>
<td>2</td>
</tr>
</tbody>
</table>

**Total score (max 72)**
### Glossary of terms:

**Cataract**: a lens opacity (cataract), ever, whether due to corticosteroid therapy or uveitis, documented by ophthalmoscopy.

**Ocular complications of uveitis other than cataract**: synechiae, band keratopathy, glaucoma, or phthisis bulbi documented by an ophthalmologist, resulting in a loss of vision of at least 1/10.

**Muscle atrophy**: decreased muscle mass demonstrated on clinical examination.

**Osteoporosis with fractures or vertebral collapse**: demonstrated by an imaging technique.

**Avascular necrosis of bone**: demonstrated by any imaging technique.

**Significant abnormality of the vertebral curve due to leg-length discrepancy or hip contracture**: demonstrated on clinical examination or by any imaging technique.

**Significant leg-length discrepancy or growth abnormality of a bone segment**: inequality of at least 1 cm in the length of the legs or growth defect or overgrowth of any bone segment due to arthritis, demonstrated radiographically.

**Striae rubrae**: widespread cutaneous purple striae with scarring resulting from steroid toxicity.

**Subcutaneous atrophy resulting from intraarticular corticosteroid injection**: significant and persistent subcutaneous atrophy in the site of a previous intraarticular corticosteroid injection.

**Growth failure**: defined as the presence of two of the following three features:
1) Lower than the 3rd percentile height for age.
2) Growth velocity over 6 months lower than the 3rd percentile for age.
3) Crossing at least 2 centiles (5%, 10%, 25%, 50%, 75%, 95%) on growth chart.

**Pubertal delay**: delay in development of secondary sexual characteristics greater than 2 standard deviations beyond the mean for age in Tanner staging.

**Diabetes mellitus**: diabetes mellitus requiring therapy, but regardless of treatment.

**Secondary amyloidosis**: symptomatic amyloidosis confirmed by examination of tissue sections by Congo red dye.

### Appendix B: The Juvenile Arthritis Damage Index for Assessment of Extraarticular Damage (JADI-E) in Patients with Juvenile Idiopathic Arthritis

<table>
<thead>
<tr>
<th>Item</th>
<th>Absent</th>
<th>Present</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Ocular</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cataract and/or other complications of uveitis with loss of vision (score 2 in case of ocular surgery; score 3 in case of legal blindness)</td>
<td>0</td>
<td>1 2 3</td>
</tr>
<tr>
<td><strong>Musculoskeletal non-articular</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Severe muscle atrophy</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Osteoporosis with fractures or vertebral collapse</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Avascular necrosis of bone</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Significant abnormality of the vertebral curve due to leg-length discrepancy or hip contracture</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Significant leg-length discrepancy or growth abnormality of a bone segment</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Cutaneous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Striae rubrae</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Subcutaneous atrophy resulting from intraarticular corticosteroid injection</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Endocrine</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Growth failure</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Pubertal delay</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td><strong>Secondary Amyloidosis</strong></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td><strong>Total score (max 17)</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Anti–Tumor Necrosis Factor α Blockade in the Treatment of Juvenile Spondylarthropathy

Shirley M. L. Tse,1 Ruben Burgos-Vargas,2 and Ronald M. Laxer1

Objective. Persistent inflammation refractory to standard antirheumatic therapy in children with juvenile spondylarthropathy (SpA) leads to morbidity and reduced quality of life. Tumor necrosis factor α (TNFα) plays an important role in the pathogenesis of synovitis and enthesitis. This study was undertaken to examine the impact of anti-TNFα agents on juvenile SpA that is refractory to nonsteroidal antiinflammatory drugs (NSAIDs), disease-modifying antirheumatic drugs, and corticosteroids.

Methods. Ten juvenile SpA patients with a mean ± SEM age of 15.0 ± 0.7 years and disease duration of 4.4 ± 0.8 years, all of whom were HLA–B27 positive, were followed up for 1 year after initiation of either infliximab (n = 8) or etanercept (n = 2). Outcomes examined were within-subject differences in the tender entheseal count (TEC) and active joint count (AJC), markers of inflammation, functional assessments (Childhood Health Assessment Questionnaire [C-HAQ] score), and requirements for antirheumatic drugs.

Results. At baseline, all patients exhibited active arthritis and enthesitis that were resistant to NSAIDs (n = 10), methotrexate (n = 6), sulphasalazine (n = 8), corticosteroids (oral n = 6, intravenous pulse n = 3, and intraarticular n = 6), and bisphosphonates (n = 2). In 2 patients, sulphasalazine (n = 2), corticosteroids (n = 1), and bisphosphonates (n = 1) were stopped after initiation of the anti-TNFα agent. In all patients, the arthritis and enthesitis significantly improved as evidenced by remission of the TEC and AJC by 6 months that was sustained during the 1-year followup, markers of inflammation and C-HAQ scores normalized, and there was a reduction in requirements for antirheumatic drugs (reduced dosage or discontinuation of NSAIDs n = 10, methotrexate n = 5, sulphasalazine n = 6, corticosteroids n = 4, and bisphosphonates n = 1).

Conclusion. Anti-TNFα therapy is a potential novel treatment for refractory juvenile SpA. Further prospective studies are required to examine the effectiveness and long-term outcomes of anti-TNFα therapy in this cohort.

The juvenile spondylarthropathies (SpA) comprise a group of HLA–B27–associated disorders with a disease onset in children younger than age 16 years. Under the International League of Associations for Rheumatology classification of juvenile idiopathic arthritis (JIA), the juvenile SpA population is referred to as those with enthesitis-related arthritis (ERA) (1). The disease is characterized by manifestations of arthritis and enthesitis, particularly with involvement of the lower limbs and, in some cases, the sacroiliac and spinal joints. Peripheral enthesitis, seen more frequently in children than in adults with SpA, can result in significant functional disability. Currently there are no effective remittive therapies for juvenile SpA. Treatment with nonsteroidal antiinflammatory drugs (NSAIDs) and corticosteroids may provide symptomatic improvement but do not alter disease progression. Sulfasalazine (SSZ) was found not to be significantly better than placebo in a randomized controlled trial (2). Methotrexate (MTX) has an uncertain effect and has not been demonstrated to modify the course of disease progression.

Children with juvenile SpA are potentially at significant risk of morbidity, impaired function, and reduced quality of life (3,4). The presence of ongoing disease activity lasting more than 5 years is predictive of disability (5), and disease remission has been reported to occur in only 17% of patients after 5 years, with moder-
ate to severe functional limitations found in up to 60% of patients at 10 years (6). Therefore, more efficacious therapies administered early in the disease course are required.

One promising treatment is directed against tumor necrosis factor α (TNFα). As demonstrated in adult-onset ankylosing spondylitis (AS), TNFα may play a pivotal role in the pathogenesis of juvenile SpA. At sites of synovitis and enthesitis in AS patients, as well as in the knees of children with juvenile SpA, there is evidence of T lymphocyte and macrophage infiltrates, activated CD8 cells, and expression of TNFα and TNFβ, as well as expression of interferon-γ, interleukin-2 (IL-2), IL-4, and IL-6 (7–12). This serves as a rationale for the use of TNFα antagonists. Improvement with such therapy has been noted both clinically and radiologically in AS and in undifferentiated SpA, in open and double-blind studies (13–19). Preliminary studies have shown promising results in juvenile SpA or ERA (20–24). To date, TNFα antagonists have been established as safe and tolerable in children receiving treatment for polyarticular juvenile rheumatoid arthritis/JIA (25–29) as well as inflammatory bowel disease (30,31). Two anti-TNFα agents that have been used are etanercept, a fusion protein of the p75 TNF receptor and human Fc IgG1, and infliximab, a chimeric humanized monoclonal anti-TNF antibody.

In this open-label, pilot cohort study, we demonstrate that anti-TNFα agents are both tolerable and efficacious in children with juvenile SpA that is refractory to NSAIDs and disease-modifying antirheumatic drugs (DMARDs).

**PATIENTS AND METHODS**

Ten patients with juvenile SpA, whose diagnosis was in accordance with the European Spondylarthropathy Study Group classification criteria (32) and who also fulfilled the diagnostic criteria for the JIA subgroup of ERA (1), were selected from The Hospital for Sick Children, Toronto, Canada and Hospital General de Mexico, Mexico City, Mexico for inclusion in this observational study. All patients had persistent arthritis and enthesitis despite treatment with maximum doses of NSAIDs and other drugs (corticosteroids, MTX, and SSZ), and were initiating anti-TNFα therapy. The choice of anti-TNF agent administered was based on drug availability and coverage from each patient’s medical insurance plan. Patients received either infliximab (infusions of 5 mg/kg at weeks 0, 2, and 6 and then every 8 weeks) or etanercept (0.4 mg/kg subcutaneously [SC] twice weekly to a maximum dose of 25 mg). This study was approved by the Research Ethics Board of The Hospital for Sick Children.

Data compiled from a retrospective chart review of all study patients were extracted through the use of specially prepared data forms at baseline, 6 weeks, 6 months, and 1 year. The information extracted, where available, included demographic data, medication history, active joint count, tender enthesal count, markers of inflammation, and functional assessment by the Childhood Health Assessment Questionnaire (C-HAQ) (33). Active joints were defined by the presence of T lymphocyte and macrophage infiltrates, activated CD8 cells, and expression of TNFα and TNFβ, as well as expression of interferon-γ, interleukin-2 (IL-2), IL-4, and IL-6 (7–12). This serves as a rationale for the use of TNFα antagonists. Improvement with such therapy has been noted both clinically and radiologically in AS and in undifferentiated SpA, in open and double-blind studies (13–19). Preliminary studies have shown promising results in juvenile SpA or ERA (20–24). To date, TNFα antagonists have been established as safe and tolerable in children receiving treatment for polyarticular juvenile rheumatoid arthritis/JIA (25–29) as well as inflammatory bowel disease (30,31). Two anti-TNFα agents that have been used are etanercept, a fusion protein of the p75 TNF receptor and human Fc IgG1, and infliximab, a chimeric humanized monoclonal anti-TNF antibody.

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**RESULTS**

The demographic characteristics of the patients at baseline are summarized in Table 1. The 10 juvenile SpA patients had a mean ± SEM age of 15.0 ± 0.7 years at the initiation of anti-TNFα therapy. There were 8 boys and 2 girls. All patients were HLA–B27 positive and had a mean ± SEM disease duration of 4.4 ± 0.8 years at the time of starting anti-TNF treatment. In 5 of the patients, there was a positive family history of HLA–B27–related disease. All patients had active disease (arthritis and/or enthesitis) that persisted despite treatment with NSAIDs (n = 10), MTX (up to 1 mg/kg

| Table 1. Demographic and clinical characteristics of the study patients (n = 10)* |
|------------------|------------------|
| Demographic      |                  |
| Sex, no. male/no. female | 8/2 |
| Age at TNF initiation, years | 15.0 ± 0.7 |
| Disease duration, years   | 4.4 ± 0.8 |
| HLA–B27 positive, no.   | 10 |
| ANA positive, no.       | 0 |
| RF positive, no.        | 0 |
| Family history of HLA–B27–related disease, no. | 5 |

*Except where indicated otherwise, values are the mean ± SEM.

TNF = tumor necrosis factor (antagonist); ANA = antinuclear antibody; RF = rheumatoid factor; NSAIDs = nonsteroidal anti-inflammatory drugs; IV = intravenous; C-HAQ = Childhood Health Assessment Questionnaire.
to a maximum of 25 mg SC) (n = 6), SSZ (n = 8), corticosteroids (oral n = 6, intravenous pulse n = 3, intraarticular n = 6), and bisphosphonates (n = 2). At baseline, 4 patients had sacroiliac and axial involvement. In total, 8 patients were treated with infliximab and 2 patients received etanercept. In addition to the anti-TNFα agents, concomitant therapy reported in these patients included NSAIDs (n = 10), MTX (n = 4), SSZ (n = 4), combination of MTX and SSZ (n = 2), oral corticosteroids (n = 5), and bisphosphonates (n = 1).

The arthritis in all patients showed improvement (Figure 1) in terms of a significant reduction in the active joint count, tender entheseal count, markers of inflammation, C-HAQ scores, and concomitant antirheumatic medications. The response was rapid, occurring as early as 6 weeks, and sustained in all cases. This is in keeping with the response seen in AS patients treated with anti-TNFα agents, and is consistent with preliminary data on 8 patients with juvenile AS treated with etanercept who showed improvements in the number of active joints, morning stiffness, and erythrocyte sedimentation rate (23). In contrast to the patients in the study by Henrickson and Reiff (23), our study cohort comprised patients who were all HLA–B27 positive, had active arthritis, and had a much higher reported frequency of enthesitis (90%). In addition, since patients with juvenile SpA or ERA have a propensity to have either a few joints/entheses or many joints/entheses involved, the data were reported as individual trends rather than as cumulative mean scores to better observe the response to anti-TNFα agents.

The 10 study patients with juvenile SpA all demonstrated improvement with anti-TNFα therapy, as evidenced by a reduction in their active joint count, tender entheseal count, markers of inflammation, C-HAQ scores, and concomitant antirheumatic medications. The response was rapid, occurring as early as 6 weeks, and sustained in all cases. This is in keeping with the response seen in AS patients treated with anti-TNFα agents, and is consistent with preliminary data on 8 patients with juvenile AS treated with etanercept who showed improvements in the number of active joints, morning stiffness, and erythrocyte sedimentation rate (23). In contrast to the patients in the study by Henrickson and Reiff (23), our study cohort comprised patients who were all HLA–B27 positive, had active arthritis, and had a much higher reported frequency of enthesitis (90%). In addition, since patients with juvenile SpA or ERA have a propensity to have either a few joints/entheses or many joints/entheses involved, the data were reported as individual trends rather than as cumulative mean scores to better observe the response to anti-TNFα agents.

Although the American College of Rheumatology pediatric core criteria definition of improvement in juvenile arthritis (34) has currently not been validated...
for juvenile SpA or ERA, our study patients met the requirements for improvement. This response is significant, since the condition in all patients in this study was previously refractory to NSAIDs, combination DMARDs, and corticosteroids. Further studies need to be done to validate a measure of improvement or disease activity in this pediatric population. Improvement criteria for AS (i.e., the ASsessment in Ankylosing Spondylitis improvement criteria [35]) focus on spinal involvement, which is not a prevalent manifestation in juvenile SpA or ERA, and its use in children is therefore limited. Furthermore, all patients fulfilled the proposed JIA criteria for inactive disease and medication-induced clinical remission (36). The preliminary criteria for clinical remission in JIA were developed by consensus by pediatric rheumatologists and originally focused mainly on the oligoarticular (persistent, extended), polyarticular (rheumatoid factor–negative or –positive), and systemic JIA subtypes. Further studies will be required to standardize and validate these criteria to define remission and to be applicable to all subtypes of JIA.

Table 2. Outcomes and antirheumatic drug requirements after treatment with anti–tumor necrosis factor α agents*

<table>
<thead>
<tr>
<th></th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
<th>Patient 5</th>
<th>Patient 6</th>
<th>Patient 7</th>
<th>Patient 8</th>
<th>Patient 9</th>
<th>Patient 10</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C-HAQ score</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baseline†</td>
<td>0.3</td>
<td>0.9</td>
<td>0</td>
<td>0.25</td>
<td>1.4</td>
<td>0.7</td>
<td>1.0</td>
<td>0.8</td>
<td>1.7</td>
<td>1.4</td>
</tr>
<tr>
<td>6 weeks</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.3</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6 months</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.12</td>
<td>0.2</td>
<td>0</td>
<td>0</td>
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<tr>
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<td>NC</td>
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* ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; ND = not done; Y = yes; D = discontinued; R = reduced; NC = no change; N = no (see Table 1 for other definitions).
† P < 0.02, baseline versus 3 followup time points.
‡ P < 0.001, baseline versus 3 followup time points.
Anti-TNF agents regarding this approach in children have been reported. Preliminary results suggesting that anti-TNFα agents may be efficacious in the treatment of peripheral enthesitis were reported by D’Agostino et al (37), who noted clinical and radiologic improvement in 2 HLA-B27–positive adult patients with refractory, erosive, calcaneal enthesitis. No studies regarding this approach in children have been reported.

Although more patients in this study received infliximab compared with etanercept, both anti-TNFα agents seemed to be equally efficacious. The medications were well tolerated in all cases and no severe adverse events were reported. There were no reports of serious infections with either anti-TNFα agent. All patients were prescreened to exclude the presence of tuberculosis prior to the initiation of anti-TNFα therapy.

Anti-TNFα appears to be safe and efficacious in the treatment of refractory synovitis and enthesitis. The response to anti-TNFα agents in juvenile SpA patients appears to be as efficacious as that seen in open and double-blind studies in AS patients. Our study highlights the improvements made in both arthritis and enthesitis in response to anti-TNFα agents in the largest cohort of juvenile SpA patients studied to date, who fulfilled the criteria for ERA and had previously exhibited an inadequate response to standard antirheumatic drug therapy. Further prospective studies are required to examine the long-term outcomes of anti-TNFα blockade in children with juvenile SpA.

REFERENCES

24. Horneff G, Schmeling H, Moebius D, Foeldvari I. Efficacy of etanercept in active refractory juvenile spondyloarthropathy: pro-
Dysregulation of Chemokine Receptor Expression and Function by B Cells of Patients With Primary Sjögren’s Syndrome

Arne Hansen,1 Karin Reiter,1 Till Ziprian,1 Annett Jacobi,1 Andreas Hoffmann,1 Mirko Gosemann,1 Jürgen Scholze,1 Peter E. Lipsky,2 and Thomas Dörner1

Objective. To assess whether abnormal chemokine receptor expression and/or abnormal responsiveness to the cognate ligands might underlie some of the disturbances in B cell homeostasis characteristic of primary Sjögren’s syndrome (SS).

Methods. Chemokine receptor expression by CD27−/H11546 naive and CD27+ memory B cells from patients with primary SS and healthy control subjects was analyzed using flow cytometry, single-cell reverse transcriptase–polymerase chain reaction (RT-PCR), and migration assays.

Results. In contrast to healthy subjects, significantly higher expression of both surface CXCR4 and CXCR4 messenger RNA (mRNA) was seen in peripheral blood B cells from patients with primary SS. These differences were most prominent in CD27− naive B cells (P < 0.0006). In addition, significantly higher frequencies of CD27− naive B cells from patients with primary SS expressed mRNA for the inhibitory regulator of G protein signaling 13 (P < 0.001). Expression of CXCR5 by peripheral CD27+ memory B cells was moderately diminished in patients with primary SS compared with healthy controls (P = 0.038). No significant differences were noted in the expression of CXCR3, CCR6, CCR7, and CCR9 between B cells from healthy controls and those from patients with primary SS. Transmigration assays of blood B cells from patients with primary SS and healthy controls showed comparable responses of CD27− naive B cells but significantly diminished responses of activated primary SS CD27+ memory B cells to the ligands of CXCR4 and CXCR5, CXCL12 (P = 0.032), and CXCL13 (B lymphocyte chemoattractant; B cell–attracting chemokine 1; P = 0.018), respectively, when compared with those from healthy controls. Finally, compared with controls, peripheral reduction but glandular accumulation of CXCR4+, CXCR5+, CD27+ memory B cells was identified in patients with primary SS.

Conclusion. In primary SS, overexpression of CXCR4 by circulating blood B cells does not translate into enhanced migratory response to the cognate ligand, CXCL12. This migratory response may be modulated by intracellular regulators. Retention of CXCR4+, CXCR5+, CD27+ memory B cells in the inflamed glands seems to contribute to diminished peripheral CD27+ memory B cells in primary SS.

Primary Sjögren’s syndrome (SS) is characterized by chronic focal lymphocytic inflammation of the lacrimal and salivary glands, resulting in keratoconjunctivitis sicca and xerostomia. Both interaction of activated glandular epithelial cells with infiltrating lymphoid and dendritic cells and systemic lymphocyte derangement are thought to contribute to the pathogenesis of primary SS (for review, see refs. 1 and 2). The lymphoid infiltrates within the inflamed glands often contain germinal center (GC)–like structures consisting of T and B cell aggregates with proliferating lymphocytes and a network of follicular dendritic cells and activated endothelial cells (3,4). Besides antigen-driven clonal proliferation of B cells (3,5), analyses of inflamed glandular tissue from patients with primary SS also reveal a polyclonal accumulation of CD27+ memory B cells and CD27high plasma cells (6,7). Moreover, immunophenotyping studies indicate that there is disturbed B cell homeostasis in...
patients with primary SS, with diminished frequencies and absolute numbers of peripheral CD27+ memory B cells (6,8,9). More recently, a single-cell messenger RNA (mRNA) study showed further abnormalities, especially in the mechanisms of heavy chain switch recombination (10).

Chemokines and their corresponding chemokine receptors play an important role in lymphopoiesis, differentiation, homing, recirculation, and immune responses of lymphocyte subsets under physiologic and pathologic conditions (11–14). The inflamed glands seen in primary SS have been shown to express a unique profile of adhesion molecules, cytokines, and chemokines, including overexpression of CXCL13 (B lymphocyte chemoattractant [BLC]; B cell–attracting chemokine 1 [BCA-1]) mRNA and protein, a central chemokine involved in B cell homing (15–17), as well as of CCL19, CCL18, CXCL9 (monokine induced by interferon-γ), and CXCL10 mRNA (17,18,19). Moreover, CXCR5-expressing B cells have been detected in the glandular infiltrates of patients with primary SS (15,16). Thus, it has been proposed that disturbances in chemokine expression may selectively guide and regulate lymphoid subsets into or within the target tissues as well as the (re)circulation between blood and secondary lymphoid organs of patients with primary SS.

In order to delineate these disturbances in greater detail and to determine whether these abnormalities might contribute to the disturbed B cell homeostasis in patients with primary SS, we analyzed the expression of chemokine receptors known to provide critical positioning clues for B cells and plasma cells during development and/or immune responses, including CXCR3, CXCR4, CXCR5, CCR6, CCR7, and CCR9 (11,12,20).

**PATIENTS AND METHODS**

**Patients.** After the local ethics committee granted approval and the patients provided informed consent, heparinized whole blood samples (10 ml) were obtained from 21 patients with primary SS (20 women; mean ± SD age 57.6 ± 14.6 years, age range 25–79 years, 1 man; age 44 years) at the Department of Medicine, University Hospital Charité, Berlin. The mean ± SD disease duration was 7.1 ± 3.8 years (range 1–13 years). The patients fulfilled both the American College of Rheumatology (21) and the revised American–European Consensus Group (22) classification criteria for primary SS. All patients tested positive for antinuclear antibodies (fine speckled pattern) as well as for anti-Ro and/or anti-La antibodies and/or rheumatoid factor. All had focal lymphocytic sialadenitis of the minor salivary glands (focus score >1/4 mm²) and a positive Schirmer I test result. The patients received no glucocorticoids or immunosuppressive drugs. As controls, heparinized blood samples from apparently healthy subjects and patients with systemic lupus erythematosus (SLE), matched by age and sex with the primary SS patients were also analyzed.

Peripheral blood mononuclear cells (PBMCs) were obtained by centrifugation on Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) gradients, as previously described (23). In addition, PBMCs were also analyzed, mononuclear cells were prepared, as previously described (6,7), from minor salivary gland biopsy samples from 4 patients with primary SS and 1 female patient with nonspecific sialadenitis.

**Fluorescence-activated cell sorting.** For flow cytometric analysis of chemokine receptor expression on peripheral CD19+,CD27− naived and CD19+,CD27+ memory B cells, PBMCs from 16 patients with primary SS, 10 healthy control subjects, and 12 SLE patients were stained with a fluorescein isothiocyanate (FITC)–conjugated monoclonal antibody (mAb) to CD19 (clone HD37; Dako, Glostrup, Denmark), with a Cy5-labeled mAb to CD27 (clone 2E4; a kind gift from Dr. René van Lier, Department of Immunobiology, Academic Medical Center, Amsterdam, The Netherlands), and with phycoerythrin (PE)–labeled mAb specific for one of the following chemokine receptors: CXCR3 (clone 1C6; BD Pharmingen, San Diego, CA), CXCR4 (clone 12G5; BD Pharmingen), CXCR5 (FAB 190F; R&D Systems, Minneapolis, MN), CCR6 (clone 11A9; BD Pharmingen), CCR7 (FAB 197F; R&D Systems), or CCR9 (FAB 179F; R&D Systems). PE-conjugated IgG2a (clone G155-178; BD Pharmingen) and IgG2b (clone 133303; R&D Systems) (as negative controls) were used in conjunction with the respective chemokine receptor–specific antibodies. Incubation with antibodies was performed in phosphate buffered saline (PBS)/0.5% bovine serum albumin (BSA)/5 mMEDTA at 4°C for 15 minutes. Subsequently, cells were washed twice in PBS/2% BSA/4 mMEDTA. Propidium iodide (1 µg/ml; Sigma, Munich, Germany) was added immediately before flow cytometric analysis to exclude dead cells. Flow cytometric analyses were performed using a FACSan Calibur and CellQuest software (Bector Dickinson, San Jose, CA). For analysis of CXCR4 and CXCR5 coexpression, streptavidin–peridin chlorophyll protein–labeled/biotinylated anti-CD19 (clone 1D3; BD Pharmingen) and FITC-labeled anti-CXCR5 (FAB 190F; R&D Systems) mAb were used in combination with anti-CXCR4 and anti-CD27 mAb (shown above).

**Single-cell reverse transcriptase–polymerase chain reaction (RT-PCR).** Altogether, 720 single-sorted CD19+,CD27− and CD19+,CD27+ B cells from 4 patients with primary SS (168 CD27− naive cells, 168 CD27+ memory cells) and 4 healthy controls (192 CD27− naive cells, 192 CD27+ memory cells) were analyzed. Individual B cells were sorted (FACS Vantage; Bector Dickinson) into single wells containing modified 1× RT-PCR buffer (5 mM dithiothreitol, 400 ng oligo[dT]18, 0.2 mM dNTP, 1% Triton X-100, 10 units RNAse, 40 units avian myoblastosis virus reverse transcriptase), as previously described (10,24). First-strand complementary DNA (cDNA) was generated at 42°C for 60 minutes. Transcripts for the chemokine receptors CXCR3, CXCR4, CXCR5 splice variant 1, CXCR5 splice variant 2, and the inhibitory regulator of G protein signaling 13 (RGS13) (25) were amplified by specific nested PCR protocols using 5 µl cDNA in the first round and 5-µl aliquots of the external PCR

**...**
mixtures in the second round. GAPDH-specific transcripts were analyzed as internal controls. The PCR conditions included a 5-minute denaturation at 94°C, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing for 45 seconds, and extension at 72°C for 1 minute. Oligonucleotide sequences are shown in Table 1. The PCR products were separated on 1.2% agarose gel. Following column purification, several PCR products from all primer combinations were directly sequenced using the BigDye Termination Sequencing kit (Perkin Elmer, Emeryville, CA) and analyzed with an automated sequencer (ABI 377; Perkin Elmer). Sequence alignments were performed by BLASTN searches against nucleotide databases (National Center for Biotechnology Information, Bethesda, MD; online at www.ncbi.nlm.nih.gov/blast). To calculate the sensitivity of each specific nested PCR protocol (e.g., for CXCR4, CXCR5, or GAPDH), limiting dilution experiments with purified target DNA were performed, which indicated that as few as 1–10 cDNA copies could be detected (e.g., for CXCR4, CXCR5, or GAPDH), limiting dilution experiments with purified target DNA were performed, which indicated that as few as 1–10 cDNA copies could be detected.

**Table 1.** Oligonucleotides used for specific nested polymerase chain reaction protocols*

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* NCBI = National Center for Biotechnology Information; F = forward; R = reverse; N = nested; V1 = splice variant 1; V2 = splice variant 2; RGS13 = regulator of G-protein signaling 13.

**RESULTS**

Analysis of chemokine receptor expression by peripheral blood B cells using flow cytometry. Using 4-color flow cytometry, CD19+ B cells were analyzed for the expression of CD19 as a marker of memory B cells.
cells and for the expression of chemokine receptor CXCR3, CXCR4, CXCR5, CCR6, CCR7, or CCR9. Dead cells were excluded by propidium iodide staining. Frequencies of positive cells and the geometric mean fluorescence intensity of anti–chemokine receptor staining were calculated according to statistical thresholds set in reference to staining with negative control antibodies.

The frequency of peripheral CD27\(^{-}\)/CD19\(^{+}\)/H11001/CD19\(^{+}\)/CD27\(^{+}\)/H11001/CD27\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001 memory B cells was significantly reduced in patients with primary SS compared with healthy control subjects (mean SD 13.3 ± 7.2% versus 25.6 ± 7.2%; \(P = 0.0014\)), whereas the frequency of CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD27\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001 naive B cells was significantly enhanced in patients with primary SS compared with healthy controls (mean SD 62.6 ± 18.6% versus 74.4 ± 14.6%; \(P = 0.0021\)) as reported previously (6,8,9).

To ensure that these alterations in patients with primary SS did not further influence the analyses, either CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{-}\)/CD27\(^{+}\)/H11001 or CD19\(^{-}\)/CD27\(^{-}\)/H11001/CD19\(^{+}\)/CD27\(^{+}\)/H11001 B cells were gated, and the chemokine receptor expression was subsequently analyzed within each subpopulation (Figures 1A and B). Significantly higher percentages of CXCR4\(^{+}\)/CD27\(^{-}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001 naive B cells (mean SD 95.2 ± 2.9% versus 87.7 ± 4.2%; \(P = 0.0003\)) and CXCR4\(^{+}\)/CD27\(^{-}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{+}\)/H11001 expressing CD27\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001 memory B cells (78.5 ± 10.1% versus 63.6 ± 17.8%; \(P = 0.0251\)) were found in patients with primary SS compared with healthy controls. Moreover, the geometric mean fluorescence intensity of anti–CXCR4 staining on CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{+}\)/H11001 naive B cells (mean SD 189.5 ± 75.8 versus 95.1 ± 30.4; \(P = 0.0021\)) and CD27\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{+}\)/H11001 memory B cells (62.6 ± 26.1 versus 28.7 ± 14.6; \(P = 0.0021\)) was significantly enhanced in patients with primary SS as compared with healthy controls (Figures 2A and B).

To evaluate whether this alteration is specific for primary SS or is a general feature of systemic autoimmune diseases, peripheral blood B cells from SLE patients were also analyzed for surface expression of CXCR4. The frequency of CXCR4\(^{+}\)/CD27\(^{-}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001 naive B cells (mean SD 95.2 ± 2.9 in primary SS versus 84.5 ± 9.5 in SLE; \(P = 0.0017\)) and CD27\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{+}\)/H11001 memory B cells (78.5 ± 10.1 in primary SS versus 52.0 ± 14.5 in SLE; \(P = 0.0001\)) was significantly enhanced in patients with primary SS as compared with SLE patients, whereas there were no significant differences between SLE patients and healthy subjects. The density of CXCR4 expression on CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{+}\)/H11001 naive B cells (geometric mean

![Figure 1. Comparison of the frequencies of A, CXCR3\(^{+}\)/CD19\(^{+}\)/H11001/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001/CD27\(^{-}\)/CD19\(^{+}\)/CD27\(^{+}\)/H11001 peripheral B cells, and B, CCR6\(^{+}\)/H11001/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001 peripheral B cells from patients with primary Sjögren’s syndrome (SS) and healthy control subjects, as determined by flow cytometry. CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{+}\)/CD27\(^{-}\)/CD19\(^{-}\)/CD27\(^{+}\)/H11001 or CD19\(^{-}\)/CD27\(^{-}\)/H11001/CD19\(^{+}\)/CD27\(^{+}\)/H11001 B cells were gated, and chemokine receptor expression of each subpopulation was analyzed separately. Significant differences between patients with primary SS (pSS) and normal healthy subjects (NHS) are indicated. In addition, the following were significantly different by Mann-Whitney U test: in healthy controls, CD27\(^{-}\)/CXCR3\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{-}\)/CD27\(^{+}\)/H11001 versus CD27\(^{-}\)/CXCR3\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{-}\)/CD27\(^{+}\)/H11001 (\(P < 0.0001\) for both), and CD27\(^{-}\)/CXCR4\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{-}\)/CD27\(^{+}\)/H11001 (\(P = 0.0007\)); in patients with primary SS, CD27\(^{-}\)/CXCR3\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{-}\)/CD27\(^{+}\)/H11001 versus CD27\(^{-}\)/CXCR3\(^{-}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{-}\)/CD27\(^{+}\)/H11001 (\(P < 0.0001\) for both), and CD27\(^{-}\)/CXCR5\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{-}\)/CD27\(^{+}\)/H11001 versus CD27\(^{-}\)/CXCR5\(^{+}\)/CD19\(^{+}\)/CD27\(^{-}\)/H11001/CD19\(^{-}\)/CD27\(^{+}\)/H11001 (\(P = 0.0013\)). Bars indicate the median.
fluorescence intensity ±SD 189.5 ± 75.8 in primary SS versus 85.3 ± 78.6 in SLE; P = 0.0015) and CD27+ memory B cells (62.6 ± 26.1 in primary SS versus 19.3 ± 12.9 in SLE; P = 0.0001) of patients with primary SS was found to be significantly enhanced compared with those in patients with SLE. Again, there were no significant differences in CXCR4 expression between SLE patients and healthy controls. Notably, the density of CXCR4 expression was significantly higher on CD27− naive B cells than on CD27+ memory B cells in all 3 groups analyzed (healthy controls, and patients with primary SS and SLE; P ≤ 0.0007 for all comparisons) (Figure 2A).

The frequency of CXCR5-expressing CD27+ memory B cells (mean ± SD 79.6 ± 14.8% in patients versus SD 89.8 ± 4.1% in controls; P = 0.043) (Figure 1A) and the density of CXCR5 expression on CD27+ memory B cells (geometric mean fluorescence intensity ± SD 259.6 ± 159.4 in patients versus 388.9 ± 60.4 in controls; P = 0.038) were significantly diminished in patients with primary SS as compared with healthy controls. No further differences in chemokine receptor expression on blood B cells between patients with primary SS and healthy controls were identified, neither in the CXCR5 expression on CD27− B cells nor in the expression of CXCR3, CCR6, CCR7, and CCR9 on CD27− or CD27+ B cells.

Experiments were performed to examine the cellular distribution and chemokine receptor expression by B cells in salivary glands of patients with primary SS. Comparison of peripheral and glandular B cells from 4 patients with primary SS revealed an accumulation of CD27− naive B cells in minor salivary gland infiltrates. The vast majority of these glandular CD27− memory B cells expressed both CXCR4 and CXCR5 (an example is shown in Figure 3B). Conversely, analysis of peripheral CD27− memory B cells from patients with primary SS revealed a markedly diminished proportion of CXCR4+,CXCR5+ cells as compared with healthy controls (Figure 3A). In contrast, there was no reduction of peripheral CD27− naive B cells in patients with primary SS compared with healthy controls (data not shown).

Amplification of chemokine receptor transcripts from individual B cells by single-cell RT-PCR. The cDNA samples from all individual cells sorted in the current study were tested for their integrity by amplification of the “housekeeping” gene GAPDH. Each of the subsets manifested a comparable high frequency of positive cells (mean ± SD 46.4 ± 2.5% in healthy subjects versus 46.1 ± 7.7% in patients with primary SS). Notably, a significantly enhanced frequency of CD27− naive B cells that expressed CXCR4 transcripts was
found in patients with primary SS (60 of 168 cells; 35.7%) compared with healthy controls (26 of 144 cells; 18.1%) (P = 0.0006) (Figures 4A and B). Furthermore, in patients with primary SS, the frequency of CXCR4-transcript–positive B cells was significantly enhanced in CD27− naive B cells (60 of 168 cells; 35.7%) compared with CD27+ memory B cells (37 of 168 cells; 22.0%) (P = 0.0079). A significantly increased percentage of CD27+ memory B cells expressing CXCR4-specific mRNA transcripts was also found in patients with primary SS (37 of 168 cells; 22.0%) compared with healthy controls (33 of 240 cells; 13.8%) (P = 0.033).

Both known CXCR5–mRNA splice variants (variant 1 NM_001716 and variant 2 NM_032966; National Center for Biotechnology Information database [28,29]) were analyzed in healthy controls and in patients with primary SS. It was found that individual peripheral B cells expressed either variant 1 (which...

Figure 3. CXCR4 and CXCR5 coexpression on CD27+ memory B cells in patients with primary Sjögren’s syndrome (SS) and in healthy controls. A, CD19+,CD27+ memory B cells from the peripheral blood of 3 patients with primary SS (pSS) and of 3 normal healthy subjects (NHS) were analyzed by flow cytometry according to their coexpression of CXCR4 and CXCR5. B, Flow cytometric analysis of peripheral blood and glandular CD19+ B cells from a patient with nonspecific sialadenitis (control) and a patient with primary SS assessed for the coexpression of CD27. CD19+,CD27+ memory B cells from the primary SS patient were further gated and analyzed for their coexpression of CXCR4 and CXCR5. Data are representative of results from 4 primary SS patients. Gates were set according to isotype controls.
encodes a protein that is 45 amino acids longer at the N-terminus than isoform 2 or variant 2. However, it is currently not known whether there is a functional difference between the variants. Importantly, no differences in CXCR5–mRNA expression were found between patients with primary SS and healthy subjects. Finally, when the expression of mRNA transcripts for the chemokine receptor–signaling regulator protein RGS13 (25) was examined, a significantly enhanced percentage of CD27 naïve B cells expressing RGS13 transcripts was found in patients with primary SS (28 of 168 cells; 16.7%) compared with healthy controls (7 of 144 cells; 4.9%) (P = 0.001), whereas the portion of CD27 memory B cells expressing RGS13 mRNA in patients with primary SS was not significantly different from that in healthy controls (Figure 4A).

Migration of CD27 naïve and CD27+ memory B cells in vitro. To demonstrate the functionality of chemokine receptor expression, peripheral CD19+ B cells from 5 patients with primary SS and 5 healthy subjects were analyzed using transmigration assays. No significant differences in response to either CXCL12 or CXCL13 were found between patients with primary SS and healthy controls when unstimulated B cells were analyzed (Figure 5A). However, both in patients with primary SS and in healthy controls, the transmigratory capacity of B cells was significantly enhanced by LPS stimulation (P < 0.0001). After stimulation, significantly higher percentages of CD27+ memory B cells than of CD27 naïve B cells migrated in response to CXCL12 and CXCL13 in both groups. Of note, there were significantly diminished responses of CD27+ memory B cells from patients with primary SS to both CXCL12 (mean ± SD 76.8 ± 7.8% versus 86.0 ± 3.8% in controls; P = 0.032) and CXCL13 (76.6 ± 7.2% versus 88.0 ± 1.8% in controls; P = 0.018), respectively, as compared with those from healthy subjects (Figure 5B).

DISCUSSION

Recent studies have shown disturbances in peripheral B cell populations in primary SS, with significantly enhanced CD27 naïve and diminished CD27+ memory B cells (6,8,9). This was confirmed in the
present study. An accumulation of CD27 memory B cells in inflamed tissue (6,10), altered recirculation of B cell subsets from these sites (7), and/or altered B cell differentiation (30) may contribute to these disturbances. The underlying assumption of the present study was that the expression of chemokine receptors on peripheral B cells might reflect a distinct B cell pattern in primary SS, with specific functional consequences. Overall, a differential expression of chemokine receptors by peripheral blood B cells from patients with primary SS was identified.

First, there was overexpression of CXCR4 by blood B cells from patients with primary SS that was most prominent in CD27 naive B cells. In particular, significantly higher frequencies of CXCR4-expressing B cells were detected in patients with primary SS compared with healthy controls, both in CD27 naive B cells ($P = 0.0003$) and in CD27+ memory B cells ($P = 0.0251$). Moreover, the density of CXCR4 surface expression was significantly enhanced in patients with primary SS as compared with healthy controls ($P = 0.0021$) for both CD27 naive and CD27+ memory B cells. Remarkably, these differences were also evident when blood B cells from patients with primary SS were compared with those from patients with SLE ($P = 0.0015$ for CD27 naive cells and $P = 0.0001$ for CD27+ memory cells), whereas there was no significant difference in CXCR4 expression between healthy subjects and SLE patients.

Thus, this abnormality appeared to be specific to primary SS rather than being common in systemic autoimmunity. Moreover, when individual B cells were analyzed for chemokine receptor mRNA, significantly enhanced frequencies of CD27 naive B cells ($P = 0.0006$) and CD27+ memory B cells ($P = 0.033$) expressing CXCR4 transcripts were found in patients with primary SS compared with healthy controls. However, CXCR4 overexpression by blood B cells from patients with primary SS did not translate into an enhanced migratory response to the corresponding chemokine, CXCL12, as compared with those from healthy controls. These results suggest that there was intracellular modulation of the migratory response in primary SS B cells.

To assess the discrepancy between CXCR4 expression and migratory response to the corresponding chemokine (CXCL12) in greater detail, mRNA expression of RGS13 (25,31) as one potential influencing factor was examined in individual CD27 naive and CD27+ memory B cells. RGS13 belongs to the family of RGS proteins (for review, see refs. 25 and 31) that are

Figure 5. Transmigration assays showing the frequencies of in vitro–migrated peripheral CD19+,CD27 naive and CD19+,CD27+ memory B cells from 5 patients with primary Sjögren’s syndrome (SS) and 5 normal healthy subjects in response to either 50 nM CXCL12 or 250 nM CXCL13 (B lymphocyte chemoattractant; B cell–attracting chemokine 1). A, Unstimulated B cells and B, lipopolysaccharide-stimulated B cells from patients with primary SS (pSS) and healthy controls (NHS). Values are the mean and SEM. Significant differences between CD19+,CD27 naive and CD19+,CD27+ memory B cells as determined by Mann-Whitney U test are indicated.
thought to be responsible for the fine-tuning of the intracellular signaling of G protein–coupled receptors, especially chemokine receptors. Thereby, they establish thresholds for responsiveness, provide stop signals for migration, and/or contribute to receptor desensitization to corresponding chemokines (25,31). RGS13 has recently been shown to modulate signaling through CXCR4 and CXCR5 in murine and human germinal center B cells possessing one of the most limited patterns of expression of known RGS (25). Moreover, cotransfection with RGS13 inhibited the migrational response of CXCR4-transfected Chinese hamster ovary cells toward CXCL12 in vitro (25). In the present study, significantly enhanced expression of RGS13 mRNA by CD27− naive blood B cells from primary SS patients (P = 0.001) was found. Thus, the combined data suggest that CXCR4 overexpression by blood B cells from patients with primary SS might be partly compensated by up-regulation of the inhibitory regulator protein RGS13 and, thereby, might contribute to the discrepancy between CXCR4 expression and migratory response to its corresponding ligand, CXCL12.

In this context, it is well established that surface expression of chemokine receptors does not necessarily indicate their migratory functionality (32–34). Indeed, the responsiveness of chemokine receptors for their respective ligands is differentially regulated (e.g., by RGS proteins) during the orchestration of the migration of lymphoid subpopulations into anatomic compartments, their development, activation, and immune response (26,27,31–36). B cells from different developmental stages, e.g., developing bone marrow B cells (36), B cells leaving GC structures (33), and medullary plasmablasts leaving lymph nodes (34), have been found to express high levels of surface CXCR4 but were unresponsive to CXCL12. In this regard, there is some evidence that CXCR4 might fulfill additional functions besides chemotaxis, e.g., cell growth, proliferation, and transcriptional activation (11,33,37,38). In accordance, CXCL12 treatment has been found to increase NF-κB activity in nuclear extracts from CXCR4-transfected murine pre-B lymphoma cells (37). Moreover, it has been shown that CXCL12–CXCR4 interaction stimulates G protein–mediated activation processes in peripheral T cells (39). Although it is currently unclear whether CXCR4 also fulfills such additional functions in human blood B cells, it might be speculated that CXCR4 and RGS13 (over)expression might contribute to or, alternatively, reflect abnormal B cell stimulation in primary SS, which warrants further studies.

Compared with healthy controls, flow cytometric analysis revealed a moderately diminished frequency of CXCR5+,CD27+ memory B cells (P = 0.0425) combined with a lower density of CXCR5 surface expression on CD27+ memory B cells (P = 0.038) in patients with primary SS. In this context, the CXCL13–CXCR5 pairing has been shown to be critically involved in the homing of B cells into lymphoid follicles, as well as in the development of organized lymphoid follicles (28,29,40,41). The formation of ectopic lymphoid tissue in chronic inflammatory disease, such as primary SS, is a complex process regulated by an array of cytokines, adhesion molecules, and chemokines (4,13), partly mimicking signals found in normal lymphoid organogenesis (42). Whether expression of CXCL12 and CXCL13 in the target tissues of patients with primary SS is closely associated with the development of GC-like structures or, rather, is a feature of the entire inflammatory process is still controversial (4,15).

However, it has been suggested that CXCL13 overexpression in the inflamed glands of patients with primary SS plays an active role in the recruitment of lymphoid cells as infiltrating cells, mostly B cells, which express the cognate receptor CXCR5. Thus, in patients with primary SS, overexpression of CXCL13 in inflamed glands with consequent local retention of CXCR5-bearing B cells (15,16) might also lead to reduced frequencies of peripheral CD27+ memory B cells expressing lower levels of surface CXCR5. This assumption has been supported by recent studies of primary SS indicating accumulation of memory B cells in glandular infiltrates (6,10). In accordance with this, simultaneous analyses in this study of B cells from peripheral blood and minor salivary gland infiltrates of patients with primary SS also revealed an accumulation of CD27+ memory B cells in the inflamed glands. The vast majority of these infiltrating CD27+ memory B cells coexpressed CXCR5, along with CXCR4. Conversely, diminished frequencies of peripheral blood CD27+ memory B cells coincide with a striking reduction of the peripheral CXCR4+,CXCR5+ memory B cell subpopulation in patients with primary SS. Thus, glandular coexpression of both CXCL12 and CXCL13 (15–18) seems to navigate this subpopulation of peripheral CD27+ memory B cells into the inflamed glands, where it resides. Consistent with this, residual circulating peripheral CD27+ memory B cells from patients with primary SS showed a diminished migratory response to the corresponding ligands of CXCR4 and CXCR5, CXCL12 and CXCL13, respectively, after stimulation. This suggests that memory B cells with less migratory capacity remain in the blood as a result of the selective migration and retention
of CXCR4+, CXCR5+ memory B cells into the inflamed glands.

In conclusion, peripheral B cells in primary SS manifest specific abnormalities in chemokine receptor expression and function of both memory and naive subpopulations. The abnormally expressed receptors, CXCR4 and CXCR5, specifically bind the chemokines, CXCL12 and CXCL13 (BLC; BCA-1), respectively, which are important for navigating lymphocytes in lymphoid tissues, and, thereby, for lymphocyte homeostasis (11, 12, 42). Migration/retention of CXCR4+, CXCR5+, CD27+ memory B cells in the inflamed target tissues of patients with primary SS appears to account for the diminished number of these cells in the peripheral blood. However, the increased number of naive B cells in the peripheral blood does not appear to reflect an alteration in chemotaxis. Rather, the increased expression of CXCR4 appears to be offset by intracellular modulation with resultant normal migratory responsiveness. Both differences might reflect an abnormality in activation status of the naive subpopulation. Thus, disturbed B cell differentiation, activation, and/or (re)circulation between immune compartments may contribute to the disturbed B cell homeostasis in primary SS (10, 30). Detailed understanding of the impact of chemokines and their cognate receptors, including their regulation, may allow the development of future therapeutic interventions in primary SS, a disease unresponsive to classic immunosuppression.

ACKNOWLEDGMENT

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REFERENCES


Requirement of Activation of Complement C3 and C5 for Antiphospholipid Antibody–Mediated Thrombophilia

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Objective. Antiphospholipid antibodies (aPL) have been shown to induce thrombosis, activate endothelial cells, and induce fetal loss. The pathogenesis of aPL-induced thrombosis, although not completely understood, may involve platelet and endothelial cell activation as well as procoagulant effects of aPL directly on clotting pathway components. Recent studies have shown that uncontrolled complement activation leads to fetal death in aPL-treated mice. In this study, we tested the hypothesis that aPL are responsible for activation of complement, thus generating split products that induce thrombosis.

Methods. To study thrombus dynamics and adhesion of leukocytes we used in vivo murine models of thrombosis and microcirculation, in which injections of aPL were used.

Results. Mice deficient in complement components C3 and C5 were resistant to the enhanced thrombosis and endothelial cell activation that was induced by aPL. Furthermore, inhibition of C5 activation using anti-C5 monoclonal antibodies prevented thrombophilia induced by aPL.

Conclusion. These data show that complement activation mediates 2 important effectors of aPL, induction of thrombosis and activation of endothelial cells.

Antiphospholipid syndrome (APS) is characterized by increased risk of vascular thrombosis, involving the venous, arterial, and placental circulatory systems. The pathogenic mechanisms for antiphospholipid antibody (aPL)–induced thrombosis are incompletely understood. Passive transfer of IgG from aPL-positive sera (IgG-APS) has been found to induce fetal loss, thrombosis, and endothelial cell activation in mice, suggesting a direct pathogenic role of aPL (1–3). Complement activation is a necessary intermediary event in the pathogenesis of fetal loss associated with aPL in this model (4,5).

It is well established that activated complement fragments themselves have the capacity to bind and activate inflammatory and endothelial cells, as well as induce a prothrombotic phenotype either directly through C5b–9 (membrane attack complex [MAC]) or through C5a receptor (C5aR)–mediated effects (6,7). Endothelial cells can release tissue factor in response to C5a activation (8). Inflammatory cells, when triggered by complement proteolytic products C5a and C3a, respond with the production of selected procoagulant activities, thereby initiating the coagulation pathways. MAC has also been associated with thrombosis. Studies performed in rats showed that CD59, an inhibitor of C5b–9 assembly and insertion, serves a protective role in a rat model of thrombotic microangiopathy, demonstrating that C5b–9 plays a critical role in the pathogenesis of thrombosis (9).

In previous studies, we demonstrated that the complement C3 convertase inhibitor, Crry, inhibited IgG-APS–induced thrombosis, suggesting that complement activation is required in IgG-APS–induced thrombophilia (4). Moreover, Girardi and coworkers proposed that heparin, the current standard treatment in patients with APS, prevents obstetric complications by blocking activation of complement, as opposed to preventing placental thrombosis (10). We therefore tested the hypothesis...
that complement activation mediates endothelial cell activation and the thrombogenic effects of IgG-APS.

MATERIALS AND METHODS

Mice. C3-deficient mice were obtained from V. M. Holers (University of Colorado Health Sciences, Denver) (11). The mice were generated by intercrossing C3−/− mice at F1 during a backcross to C57BL/6 and then propagating C3−/− progeny. C5−/− mice (B10.D2-H2b2-H2-T18+ Hc9/o2Sn) and the C5+/− background strain of mice (B10.D2-H2b2-H2-T18+ Hc9/oSnJ) were obtained from the Jackson Laboratories (Bar Harbor, ME). CD1 male mice (weight ~30 grams) were obtained from Charles River Laboratories (Wilmington, DE). All animals were housed in the animal care (American Association of Laboratory Animal Care–approved) facilities of the Morehouse School of Medicine. Animals were handled by trained personnel according to Institutional Animal Care and Use Committee guidelines.

Preparation of IgG-APS. Antiphospholipid antibodies from APS patients (IgG-APS) were affinity-purified using cardiolipin liposomes and protein G-Sepharose chromatography as previously described (2,3). Human IgG from a non-autoimmune healthy individual (IgG-NHS) was purified by an identical method. The sterile-filtered IgG fractions were determined to be free of endotoxin contamination by the limulus amoebocyte lysate assay (E-Toxate; Sigma, St. Louis, MO). Protein concentration was determined using the method of Lowry (12). Levels of human anticardiolipin (aCL) and anti-β2-glycoprotein I antibodies were measured by standard enzyme-linked immunosorbent assay, performed as previously described (2,3).

Analysis of thrombus dynamics and leukocyte adhesion in complement-deficient mice. To investigate the role of C3 and C5 in thrombophilia induced by aPL, male CD1 mice (weight 25–30 grams; Charles River Laboratories) were injected intraperitoneally with 500 µg of IgG-APS or IgG-NHS (n = 10 mice per group) at time 0 and at 48 hours later. Half of the mice in each group received 1 mg of anti-C5 mAb (BB5.1) and half received 1 mg of murine IgG control at 30 minutes before each injection with IgG-APS or IgG-NHS. Surgical procedures to study thrombus dynamics were performed 72 hours after the first IgG-APS (or IgG-NHS) injection, as previously described (2,3).

Statistical analysis. An independent t-test was used to compare the antibody levels in different groups of mice. Student’s unpaired t-test was used to compare the mean thrombus size and number of adhering WBCs between treated and control groups. P values of less than 0.05 were considered significant.

RESULTS

Protection of C3- and C5-deficient mice from IgG-APS–induced thrombophilia in vivo. Consistent with our previous findings, IgG-APS significantly enhanced thrombus size (3.3-fold increase) in C3+/+ mice when compared with the effects of treatment with IgG-NHS in C3+/+ mice (2,4). In C3−/− mice treated with IgG-APS, there was a significant reduction (P = 0.0002) in the size of injury-induced thrombi (67% decrease) when compared with the effects of IgG-APS in C3+/+ mice (Table 1). This reduction in thrombus size in C3−/− mice treated with IgG-APS was similar to that in C3−/− or C3+/+ mice treated with IgG-NHS (Table 1). In C5+/+ mice, IgG-APS induced a significant, 2.3-fold increase in thrombus size (P = 0.003) when

| Table 1. Effects of IgG-APS on thrombus size and leukocyte adhesion to endothelial cells in C3−/− mice* |
|-----------------|----------------|-----------------|
| **Mouse type** | **No. of animals** | **Thrombus size, μm²** | **WBC adhesion** | **aCL titer, GPL units†** |
| WT IgG-NHS | 9 | 802 ± 390 | 14.0 ± 5.0 | <10 |
| IgG-APS | 7 | 2,646 ± 980 | 35.0 ± 12.0 | 78.0 ± 8.6 |
| WT C3−/− IgG-NHS | 7 | 1,083 ± 443 | 4.7 ± 0.9 | <10 |
| IgG-APS | 7 | 873 ± 425 | 4.7 ± 2.0 | 86.3 ± 10.4 |

* Values are the mean ± SD. IgG-APS = IgG from antiphospholipid-positive sera; WBC = white blood cell (expressed as no. of leukocytes adhering within 5 different venules); aCL = anticardiolipin; GPL = IgG phospholipid; WT = wild-type; IgG-NHS = IgG from non-autoimmune healthy sera; C3−/− = deficient in C3.
† Negative titer considered <10 GPL units.
comparing with the effects of IgG-NHS in C5\(^{+/−}\) mice. The increase in thrombus size induced by IgG-APS was reduced by 54\% (P = 0.011) in C5\(^{−/−}\) mice (Table 2). The thrombus size in C5\(^{−/−}\) mice treated with IgG-APS was not different from that in C5\(^{−/−}\) or C5\(^{+/+}\) mice treated with IgG-NHS (Table 2).

Protection of C3- and C5-deficient mice from IgG-APS–induced endothelial cell activation in vivo. To determine whether complement is required for aPL induction of in vivo endothelial cell activation, we examined WBC adhesion to the endothelium of cremaster muscle. IgG-APS induced an increase in WBC adhesion in both C3\(^{+/+}\) and C5\(^{+/+}\) mice (2.5-fold [P = 0.01] and 4.6-fold [P = 0.0025], respectively) when compared with the effects of treatment with IgG-NHS (Tables 1 and 2). In contrast, adhesion of WBCs to cremaster endothelium was significantly reduced after treatment with IgG-APS in C3- and C5-deficient mice (reduction of 87\% [P = 0.002] and 74\% [P = 0.003], respectively) (Tables 1 and 2).

The titer of aCL antibodies in C3\(^{+/+}\) mice injected with IgG-APS was not different from the aCL titers in C3\(^{−/−}\) mice treated with IgG-APS (mean ± SD 78 ± 8.6 IgG phospholipid [GPL] units versus 86.3 ± 10.4 GPL units). Similarly, the levels of aCL antibodies in C5\(^{+/+}\) mice treated with IgG-APS were not different from the aCL levels in C5\(^{−/−}\) mice treated with IgG-APS (115.7 ± 74.2 GPL units versus 102.3 ± 12.2 GPL units). These findings exclude the possibility that the protective effects of IgG-APS in reducing proinflammatory and thrombogenic events in C3\(^{−/−}\) and C5\(^{−/−}\) mice were related to lower aCL titers in these mice.

Prevention of IgG-APS–induced thrombosis by anti-C5 mAb. To confirm that C5 activation is required for the induction of thrombosis by aPL, mice treated with IgG-APS were injected with anti-C5 mAb to inhibit C5 cleavage. In control mice, IgG-APS caused an increase in thrombus size (mean ± SD 3.577 ± 1.129 \(\mu\)m\(^2\) with IgG-APS versus 712.0 ± 272 \(\mu\)m\(^2\) with IgG-NHS; P = 0.001) (Figure 1), whereas in mice treated with anti-C5 mAb and IgG-APS, the thrombus size was significantly smaller (838 ± 222 \(\mu\)m\(^2\); P = 0.005) when compared with mice treated with control IgG and IgG-APS (Figure 1). The mouse IgG used as the control for the anti-C5 mAb did not affect thrombus size in animals treated with IgG-APS or IgG-NHS. The aCL titer in IgG-APS–treated mice was 152.2 ± 14.8 GPL units and was not different from the aCL titer in mice treated with IgG-APS and anti-C5 mAb (135.3 ± 20.3 GPL units), thus excluding the possibility that the protective effects of anti-C5 mAb were due to a diminution in aCL titers because of interferences between IgG-APS and anti-C5 mAb.

DISCUSSION

Using a model of surgically induced thrombus formation, we demonstrated that complement activation plays an important role in thrombosis induced by aPL in mice. Specifically, we identified C3 and C5 as the critical intermediaries linking pathogenic aPL to WBC adhesion and development of thrombosis. Our conclusions are based on the prevention of thrombophilia observed in C3\(^{−/−}\) and C5\(^{−/−}\) mice and the protective effects of anti-C5 mAb. In previous studies from our group, the findings suggested that C3 activation is required for
aPL-induced thrombosis. We demonstrated that Crry-Ig, an inhibitor of C3 convertase, blocks thrombosis initiated by aPL (4). Subsequently, Girardi et al showed that complement activation, specifically C5a–C5aR interaction, is required for aPL-induced pregnancy loss and suggested that C5a promotes neutrophil infiltration of decidual tissue (5). Evidence that neutrophils activated by C5a release procoagulant substances and that monocytes activated by C5a release tissue factor suggests that infiltrating leukocytes stimulated by complement split products can initiate placental infarction and ultimately cause fetal death (8).

In the current study, we have extended our findings by demonstrating that complement is required for aPL-mediated thrombosis and for increased leukocyte adhesion to endothelium. In the absence of C3 or C5, we observed neither enhanced leukocyte adherence nor increased thrombosis associated with aPL treatment. Furthermore, we found that anti-C5 mAb prevented aPL-mediated thrombosis, emphasizing the role of C5 (either C5a, C5aR, or C5b–9) in induction of thrombophilia. C5a binding to endothelial cells results in increased expression of P-selectin and markedly increases neutrophil adhesion (14), and binding of C5b to target surfaces initiates assembly of the MAC that triggers proinflammatory signaling pathways and induces a prothrombotic phenotype in vascular tissue (6). Observations that blockade of C5aR prevents thrombus formation and leukocyte accumulation in a rat model of antibody-mediated thrombotic glomerulonephritis underscore the linkage between complement activation and thrombophilia (15).

Thrombosis in APS is sporadic and may occur in any vein or artery of the body. In this study, we used a mouse model of thrombosis induced by a standardized pinch injury in the femoral vein to define the mediators of thrombophilia associated with aPL (2,3). Recently, other investigators have demonstrated enhancement of thrombosis by aPL in an experimental model of photochemically induced vascular injury in hamsters (16). Patients with APS often have aPL for prolonged periods of time without clinical manifestations, and thrombosis occurs after a triggering event such as an infection, immobilization, or surgery. Therefore, our experimental model of injury-induced thrombosis, although artificial, simulates a “second hit” that triggers thrombotic episodes in susceptible patients and mimics sporadic clotting as observed clinically in APS.

We recognize that there are differences between the human and the murine complement systems. However, the anti-C5 mAb (BB5.1) used in these studies has been shown to effectively block C5 activation in vitro and in vivo in mice and in humans (13,17,18). Independent of the initiator of the complement cascade, this mAb prevents C5 activation and thus prevents the generation of the potent proinflammatory factors, C5a and C5b–9. Anti-C5 mAb precipitates the 2 chains of C5 from normal mouse serum and inhibits C5-dependent hemolysis in a functional complement test. It has been shown to prevent aPL-induced pregnancy loss, in which thrombosis plays an important role (5).

Anti-C5 biologic therapy has been extensively investigated in several other animal models of complement-mediated diseases, including collagen-induced arthritis and lupus-like autoimmune disease in (NZB/NZW)F1 mice (19,20). Eculizumab (5G1.1), the humanized anti-C5 mAb, is considered a potential treatment for several chronic inflammatory diseases, including rheumatoid arthritis and nephritis, and phase II trials have been initiated for these indications. Furthermore, eculizumab has been shown to prevent C5 activation in humans and to have beneficial effects in patients with paroxysmal nocturnal hemoglobinuria; specifically, it reduces intravascular hemolysis, hemoglobinuria, and the need for transfusion in these patients, providing a proof-of-concept that blockade of complement activation is feasible and tolerable in patients with chronic disease (18).

We propose that pathogenic aPL, in addition to their direct effects on platelet and endothelial cell targets, induce complement activation, and thus generate complement split products that attract inflammatory cells and initiate thrombosis and tissue injury. Our finding that blockade of C5 is effective in preventing thrombosis in a mouse model of APS has important therapeutic implications. Blockade of complement activation may be a valuable target for interventions that prevent, arrest, or modify the thrombogenic effects of aPL.

REFERENCES


The Prevalence of Undiagnosed Pulmonary Arterial Hypertension in Subjects With Connective Tissue Disease at the Secondary Health Care Level of Community-Based Rheumatologists (the UNCOVER Study)

Fredrick M. Wigley,¹ Joao A. C. Lima,¹ Maureen Mayes,² David McLain,³ J. Lincoln Chapin,⁴ and Clive Ward-Able⁴

**Objective.** Most of the data about the prevalence of pulmonary arterial hypertension (PAH) are from tertiary centers that are biased toward seeing more severe cases; therefore, the true prevalence of PAH among patients with connective tissue disease is unknown. We sought to determine the point prevalence of undiagnosed PAH in community-based rheumatology practices.

**Methods.** The study design was a multicenter, prospective and retrospective survey and analysis of clinical cases in 50 community rheumatology practices. We evaluated a total of 909 patients with either scleroderma (systemic sclerosis [SSc]) or mixed connective tissue disease (MCTD). If a subject had not been diagnosed as having PAH, then a new Doppler echocardiogram was obtained to measure cardiac parameters, including estimated right ventricular systolic pressure (ERVSP), and a full review of medical records was done.

**Results.** Of 909 screened patients, 791 were evaluable and completed the study; 669 had not previously been studied for PAH. Of these 669 patients, 89 (13.3%) were found by Doppler echocardiography to have an ERVSP of ≥40 mm Hg. Of these 89 patients, 82 (92.1%) had SSc and 7 (7.9%) had MCTD. The total prevalence of PAH in the survey was 26.7% (211 of 791 patients, including 122 with known PAH and 89 newly diagnosed as having PAH). Doppler echocardiographic data showed 20 of 89 patients (22.5%) with ERVSP of ≥50 mm Hg, 20 of 89 patients (22.5%) with increased RV dimension, and 25 of 89 patients (28.1%) with right atrial enlargement. Patients with ERVSP ≥40 mm Hg had decreased exercise tolerance compared with those with ERVSP <40 mm Hg (27% compared with 9.5%, respectively, were severely symptomatic).

**Conclusion.** A significant number of patients with SSc or MCTD (13.3%) followed up in a community rheumatology practice setting have undiagnosed elevated ERVSP consistent with PAH.

Pulmonary arterial hypertension (PAH) is a major cause of morbidity and mortality among patients with scleroderma (systemic sclerosis [SSc]) and mixed connective tissue disease (MCTD) (1–3), yet physicians often do not detect its presence until the late stages of disease. The reported range for the prevalence of PAH is 5–50% among SSc patients (4), while it has a prevalence of 25% in patients with MCTD and 20% in those with systemic lupus erythematosus (5). This wide range in the reported frequency of PAH is probably due to differences in the methods and criteria used to make a diagnosis, as well as to differences in the patient populations. Some studies rely solely on heart catheterization studies in selected patients, while others have used clinical and/or Doppler echocardiography criteria. Most of the data about the prevalence of PAH come from

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The presence of PAH may be underdiagnosed, because awareness of the preclinical asymptomatic phase is low among physicians and because early symptoms are frequently attributed to the underlying connective tissue disease. PAH in SSc patients has an extremely poor prognosis, with a reported median survival of 12 months following diagnosis (2,7–9). Most agree that the next challenge in managing patients with PAH is detection of the disease process at an early or presymptomatic stage with the goal of preventing or delaying disease progression.

While diagnosis of PAH by heart catheterization still remains the gold standard and is highly recommended before commencing therapy, Doppler echocardiography is the most practical and reliable noninvasive tool to survey for disease (10,11). We sought to determine the point prevalence of undiagnosed PAH in community-based rheumatology practices by conducting a survey using Doppler echocardiography technology.

PATIENTS AND METHODS

Subjects. Centers were eligible as community-based rheumatology practices if they were geographically and legally distinct from a tertiary, teaching rheumatology center. Investigators identified all of their living patients with SSc or MCTD who had visited the practice at least once in the previous 12 months. Patients had to be at least 18 years old and fulfill 1 of the following 3 conditions: 1) have disease meeting the American College of Rheumatology (ACR; formerly, the American Rheumatism Association) classification criteria for SSc (12); 2) have at least 3 of 5 features of the CREST syndrome (calcinosis, Raynaud’s phenomenon, esophageal motility, sclerodactyly, telangiectasias); or 3) have a diagnosis of MCTD as defined by the Alarcon-Segovia and Cardiel criteria (13). Patients were excluded if they had a connective tissue disorder other than SSc or MCTD, sarcoidosis, human immunodeficiency virus, a history of congenital heart disease or left-sided heart failure, chronic thromboembolic pulmonary hypertension, or severe chronic obstructive pulmonary disease. The study was approved by the appropriate Institutional Review Boards.

Allocation to study groups and data collected. At the time of chart review, if the patient had an existing diagnosis of PAH according to the current rheumatologist, this patient was allocated to the retrospective group, and any further data were obtained from the patient’s existing medical record. If the patient did not have an existing diagnosis of PAH, he/she was allocated to the prospective group and invited to join the study.

After informed consent was obtained, patients in the prospective group underwent Doppler echocardiography of the right and left chambers of the heart (unless one had been performed within the previous 6 months). They completed a questionnaire about dyspnea and were examined for the presence of digital ulcers. Additional clinical and serologic data were collected from the medical records.

Doppler echocardiography. Each patient without an existing diagnosis of PAH (prospective group) underwent Doppler echocardiography if one had not been performed within the previous 6 months. The echocardiography was performed at a site of the investigator’s choice, with a specific request for the ascertainment of estimated right ventricular systolic pressure (ERVSP) as measured by the peak tricuspid regurgitant flow velocity using the modified Bernoulli equation, \( P = 4v^2 \), where \( P \) represents the pressure in mm Hg and \( v \) represents the maximal regurgitant velocity in meters per second. The addition of the estimated right atrial (RA) pressure was left to the discretion of the echocardiographer at the individual sites. If a tricuspid regurgitant flow was not adequately identified, the pressure was recorded as “indeterminate.” Evidence of right ventricular (RV) dysfunction was assessed by the following features: 1) increased RV dimension, defined as the RV diameter at the level of the tricuspid annulus being >4 cm in the 4-chamber view; 2) RA enlargement, defined as a space of >4 cm between the interatrial septum and the lateral RA wall; and 3) abnormal intraventricular septal motion in any view, defined by a thickening of the interventricular septum during systole, but with no movement toward the left ventricle (LV), or with septal motion toward the RV. We considered an ERVSP ≥40 mm Hg to be consistent with the presence of PAH.

Dyspnea questionnaire. Each patient in the prospective group was asked to complete a dyspnea questionnaire rating the severity of dyspnea at a single time point. A score was calculated based on the rating for 3 different categories: functional impairment, magnitude of task, and magnitude of effort, with 5 grades of dyspnea per category (from 0 [severe] to 4 [unimpaired]). These categories were further simplified to classify patients with a dyspnea score of 4 as asymptomatic, those with grades 2 or 3 as mildly symptomatic, and those with grades 0 or 1 as severely symptomatic.

Statistical analysis. The final population was used for all statistical analyses except for those involving demographics, which were derived from the population of evaluable patients. The goal of the primary analysis was to estimate the point prevalence of undiagnosed PAH, expressed as a simple proportion, in patients with SSc or MCTD who were attending community-based rheumatology clinics. Proportions were also used for the majority of the secondary analyses, and comparisons between groups were tested for differences using chi-square tests or Student’s \( t \)-tests, in which \( P \) values less than 0.05 were considered to be significant.

Since there was a potential for selection bias, physicians were asked to obtain the medical records of their known patients with SSc or MCTD and to screen those patients for inclusion at the time of data entry into the Web-based electronic data capture system. In order to minimize excessive enrollment of patients from individual sites, a limit of 25 screened patients was allowed until the last 100 patients were required, at which time patient entry was allowed on a
competitive basis. A proportion of sites were monitored for data verification.

Role of the funding source. Actelion Pharmaceuticals US, Inc. funded this investigation. Employees of Actelion Pharmaceuticals participated in the design of the study, in the collection, analysis, and interpretation of the data, and in the decision to report the findings.

RESULTS

Centers. Fifty sites (46 in the US and 4 in Canada) participated in the study, with an average of 18 patients per site (range 2–55). A list of investigators in the study (called the UNCOVER [The Prevalence of Undiagnosed Pulmonary Arterial Hypertension in Subjects with Connective Tissue Disease at the Secondary Health Care Level of Community-Based Rheumatologists] Study) is shown in Appendix A.

Patient populations (Table 1). A total of 909 patients were screened, and 815 patients were considered evaluable in that they met the inclusion criteria and were not lost to followup. Of these 815 patients, 693 had no previous diagnosis of PAH (the prospective group), and 122 were known to have PAH (the retrospective group). Twenty-four patients in the prospective group did not undergo Doppler echocardiography, which left 669 patients (82.1% of the evaluable patients) with complete Doppler echocardiographic and questionnaire data. Of the 693 patients in the prospective group, only 190 (27.4%) had previously been evaluated for PAH, and only 78 (11.3%) had undergone Doppler echocardiography in the previous 6 months. The 2 centers with the highest levels of enrollment entered 55 and 51 patients. However, the ratio of the number of retrospectively studied patients to the number of prospectively studied patients in each of these 2 centers was similar to that in the group as a whole.

Demographics. In the population of 815 evaluable patients, 731 (89.7%) were women. The mean ± SD age of patients in the retrospective (n = 122) and prospective (n = 693) groups was 62.4 ± 13.0 years and 55.5 ± 13.1 years, respectively, and the mean ± SD time since disease onset was 8.5 ± 6.5 years and 7.8 ± 6.7 years, respectively. The mean ± SD time since the onset of Raynaud’s phenomenon was 10.4 ± 7.9 years for the total population of 815 evaluable patients. SSc was diagnosed in 715 of the 815 evaluable patients (87.7%). Of the 693 patients in the prospective group, 604 were diagnosed as having SSc (38.1% of whom met the ACR criteria and 61.8% of whom met the criteria for the CREST syndrome). Of the 604 prospectively studied patients diagnosed as having SSc, 382 (63.2%) had limited disease, 220 (36.4%) had diffuse disease, and 2 were not classified. Of the total of 815 evaluable patients, 100 had MCTD (11 of 122 [9.0%] in the retrospective group and 89 of 693 [12.8%] in the prospective group).

Prevalence of PAH. Doppler echocardiographic data were obtained on 669 of 693 patients in the prospective group because 24 patients did not complete the study. Eighty-nine of these 669 patients (13.3%) had evidence of PAH as defined by an ERVSP of ≥40 mm Hg. The total prevalence of PAH in the survey was 122 known cases in the retrospective group and 89 newly diagnosed cases (yielding 211 patients with PAH in a total of 791 patients with SSc or MCTD [26.7%] attending community-based rheumatology clinics). Of these 89 patients, 82 (92.1%) had SSc and 7 (7.9%) had MCTD.

Echocardiographic findings. In the final prospective group, 282 of 669 patients (42.2%) had an ERVSP ≥30 mm Hg (Table 2). Twenty of 669 patients (3.0%) had an ERVSP ≥50 mm Hg. Of the 669 patients in the final prospective group, 127 (19.0%) had echocardiograms that could not be used to evaluate ERVSP, since

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<th>Table 1. Patients participating in the survey*</th>
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|* We originally screened a population of 909 patients. The evaluable population consisted of patients who passed the original screening and were not lost to followup. The final population consisted of the evaluable patients who underwent Doppler echocardiography and completed the dyspnea questionnaire. PAH = pulmonary arterial hypertension; SSc = systemic sclerosis; MCTD = mixed connective tissue disease.

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<th>Table 2. ERVSP by Doppler echocardiography in 669 patients with unknown PAH status (the prospective group)*</th>
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|* ERVSP = estimated right ventricular systolic pressure; PAH = pulmonary arterial hypertension.
the tricuspid regurgitant flow could not be identified on
Doppler echocardiography. For purposes of compari-
son, echocardiography findings among 453 patients in
the prospective group with an ERVSP <40 mm Hg and
89 with an ERVSP ≥40 mm Hg, respectively, showed
increases in the following parameters with increasing
ERVSP values: RV dimension (61 of 453 patients
[13.5%] versus 20 of 89 patients [22.5%; P = 0.03]), RA
enlargement (43 of 453 patients [9.5%] versus 25 of 89
patients [28.1%; P < 0.0001]), abnormal septal motion
(11 of 453 patients [2.4%] versus 3 of 89 patients [3.4%;
P = 0.61]), and ≥1 aspect of RV dysfunction (85 of 453
patients [18.8%] versus 34 of 89 patients [38.2%; P <
0.0001). There was no difference between patients with
ERVSP <40 mm Hg and those with ERVSP ≥40 mm
Hg in the mean ± SD LV ejection fractions (63.0 ±
8.3% and 61.8 ± 10.8%, respectively), LV end-diastolic
dimension (4.4 ± 0.6 cm³ and 4.5 ± 0.6 cm³, respec-
tively), and LV wall thickness (10.6 ± 1.9 mm and 9.6 ±
1.9 mm, respectively).

**Retrospectively studied patients.** Of the 122 pa-
tients with a preexisting diagnosis of PAH, 111 had SSc
and 11 had MCTD. The diagnosis of PAH in the
retrospectively studied group was “as defined by the
investigator,” the rheumatologist’s opinion. One hun-
dred fifteen patients (94.3%) were diagnosed as having
PAH by Doppler echocardiography, and only 7 (5.7%)
were diagnosed by right heart catheterization. Of the
115 patients diagnosed by Doppler echocardiography,
only 14 (12.2%) underwent right heart catheterization
following the echocardiography, yielding a total of 21 of
122 patients (17.2%) diagnosed as having PAH who had
undergone a confirmatory right heart catheteriza-

ization. Of the 115 patients diagnosed by Doppler echocardiography, 13 (11.3%) had ERVSP <35 mm Hg, 25 (21.7%) had ERVSP <40 mm Hg, and 90 (78.3%) had ERVSP ≥40 mm Hg.

Of the 122 patients with PAH, only 51 (41.8%) were receiving specific treatment for PAH. Of these 51 patients, 36 (70.6%) were receiving bosentan, 5 (9.8%) were receiving epoprostenol, and 10 (19.6%) were receiving other types of treatment for PAH.

Dyspnea. Of the 693 patients in the prospective group, 686 (99.0%) completed the dyspnea questionnaire, while 669 (96.5%) completed both the questionnaire and the prospective Doppler echocardiography study. Of the 89 patients with an ERVSP ≥40 mm Hg, 76 (85%) demonstrated either mild or severe functional impairment compared with 320 of the 580 patients with ERVSP <40 mm Hg (55%) (Figure 1). Compared with patients with an ERVSP <40 mm Hg, a larger proportion of patients with an ERVSP ≥40 mm Hg also had impairments in magnitude of task and effort.

Digital ulcers. Among all patients (retrospectively plus prospectively studied patients), there was no association between having a history of digital ulcers and the presence of PAH as defined by an ERVSP of ≥40 mm Hg (79 patients with PAH of 270 patients with ulcers [29.3%] versus 132 patients with PAH of 521 patients without ulcers [25.3%]; P = 0.237 by chi-square test).

Pulmonary function tests (PFTs). Among all 791 patients, results of PFTs were reported for 401, of whom 86 of 122 patients (70.5%) were in the retrospective group and 315 of 669 patients (47.1%) were in the prospective group. Only 29 of 89 patients (32.6%) in the prospective group with ERVSP ≥40 mm Hg had PFT results available for review. In the prospective group, the percent predicted diffusing capacity for carbon monoxide (DLCO) was significantly lower in patients with ERVSP ≥40 mm Hg than in patients with ERVSP <40 mm Hg (58.9 ± 24.1% versus 71.9 ± 19.7%; P = 0.005 by t-test) (Table 3). In the entire population (retrospectively plus prospectively studied patients) for whom results of PFTs were available, the DLCO was significantly lower in patients with ERVSP ≥40 mm Hg (58.9 ± 24.1% versus 71.9 ± 19.7%; P = 0.0001 by t-test) (Table 3). In the entire population (retrospectively plus prospectively studied patients) for whom results of PFTs were available, the DLCO was significantly lower in patients with ERVSP ≥40 mm Hg (Table 4). It should be pointed out that in the prospective group, 47 of 141 patients (33.3%) with no impairment according to the dyspnea questionnaire had PFT results available for review compared with 268 of 528 patients (50.8%) with moderate or severe impairment (P = 0.0002), indicating that patients with dyspnea were more likely to have been sent for PFTs.

Serology. A positive antinuclear antibody titer was found in 615 of the 669 evaluable patients in the prospective group (91.9%). Of these 615 patients, anticentromere antibodies (ACAs) were found in 197 (32%), antitopoisomerase antibodies were found in 38 (6.2%), and anti-U1 RNP was found in 100 (16.3%). There was no association of any of these antibodies (either antibody positivity or antibody negativity) with the presence of PAH or with ERVSP ≥40 mm Hg.

**DISCUSSION**

The main finding in this study is that a significant number (point prevalence 13.3%) of patients with SSce
and MCTD followed up in 50 different community-based rheumatology practices had an undiagnosed elevated ERVSP, as measured by Doppler echocardiography, consistent with PAH. The prevalence of these Doppler echocardiographic abnormalities in the present study is similar to those found in surveys of patients followed up in hospital-based or tertiary centers, and until now it was suspected that those prevalences were inflated by referral bias (3,6,7). In addition, 22.5% of the 89 patients with ERVSP ≥40 mm Hg had a very high estimated pressure of ≥50 mm Hg; 22.5% had an increase in RV dimension and 28.1% had RA enlargement, which are suggestive of advanced disease. We also found that patients with ERVSP ≥40 mm Hg had a decreased exercise tolerance compared with those whose pressure was <40 mm Hg. These data indicate that significant numbers of both asymptomatic and symptomatic patients with SSc or MCTD in a community rheumatology practice have undiagnosed PAH.

While it is well established that there is an increased risk of pulmonary hypertension associated with connective tissue disease, especially SSc and MCTD, this is the first attempt to define the prevalence of unrecognized cases in a community setting, where one might expect a milder disease case mix. The importance of this finding is emphasized by studies demonstrating a substantial increased mortality risk associated with the development of PAH in patients with SSc and other connective tissue diseases (2,3,8,9). MacGregor et al report that a single Doppler echocardiographic pressure reading of ≥30 mm Hg is associated with a 20% mortality rate in 20 months (9). Similarly, it is reported that the survival rates in SSc are 81%, 63%, and 56% at 1, 2, and 3 years, respectively, after the diagnosis of PAH (3). Among patients with SSc, the retrospective mortality risk ratios (relative to patients without lung disease) were 2.9, 2.4, and 1.6 for patients with isolated PAH, restrictive lung disease combined with PAH, and restrictive lung disease alone, respectively (7). In a survey of serial Doppler echocardiographic studies in 282 SSc patients, it was reported that an increase of 10–20 mm Hg in the ERVSP from the previous study was associated with an increased risk of death within 7–8 months (14). This suggests that Doppler echocardiographic findings compatible with PAH in SSc patients, even without confirmatory heart catheterization data, are a risk factor for poor survival.

The other reason detection of PAH is important is that we now have therapeutic options that improve quality of life and exercise capacity in patients with class III and class IV functional status (15–17). However, it is not yet clear whether therapy improves survival in SSc patients with advanced PAH. Kuhn et al report that among 91 patients with PAH who were treated with epoprostenol, survival was poor in SSc patients (hazard ratio 2.32, 95% confidence interval 1.08–4.99) compared with that in patients with other forms of PAH, including those with primary pulmonary hypertension and congenital heart disease (18). The challenge for the future is to determine whether early diagnosis and intervention will prevent progression to advanced disease that is irreversible or difficult to treat.

Previous estimates of the prevalence of PAH among patients with SSc or other connective tissue disease vary widely, ranging from 5% to 50% (4). In a Canadian prospective study, only 4.9% of SSc patients were diagnosed as having PAH using Doppler echocardiography (2). Similar surveys in the UK found prevalences of 13% and 12% (3,9), and surveys in the US found prevalences of 35% and 38.6% (19,20). This variation is probably due to differences in the definition of PAH and in the populations of patients studied. Patients with mild or moderate PAH often exhibit no or nonspecific symptoms, or they may only have minimal shortness of breath on exertion. Physical findings may be absent in early disease, and chest radiographs are usually nondiagnostic. There are risk factors that should make the physician more suspicious of the presence of PAH, including a diagnosis of connective tissue disease (especially limited SSc), a late age at onset of SSc (20), progressive decline in the DLCO in SSc (21), and the presence of certain autoantibodies, including ACAs, anti-B23, anti–U3 RNP, anti–U1 RNP, or anti-Th/To antibodies (4). Rapidly progressive PAH presents more often as isolated PAH in patients with limited SSc (9).

Doppler echocardiography has emerged as a reliable means of assessing pulmonary pressure (ERVSP) noninvasively, as illustrated in this study. This method yields values that correlate well with those obtained using right heart catheterization (10,22), and it is currently the most commonly used screening tool for PAH. We recognize that patients with PAH defined by Doppler echocardiography may have normal values at the time of right heart catheterization; for that reason, we chose a conservative definition of a reading of ≥40 mm Hg. In fact, we found that more than 20% of our patients with unrecognized PAH had pressures ≥50 mm Hg, and, despite the presence of symptoms in many of these patients, previous investigations to diagnose PAH were lacking. In fact, only 27% of the patients with unknown PAH status had ever been evaluated for PAH prior to the present study.
Doppler echocardiography and PFTs appear to perform adequately for identifying patients with advanced PAH, but there are very few data showing the reliability of these techniques in patients without clinical symptoms. Mukerjee et al recently reported findings of a prospective study designed for early identification of SSc-related PAH through the use of Doppler echocardiography and DLCO, and they compared these data with those obtained from cardiac catheterization (23). ERVSP by Doppler echocardiography showed a moderate positive correlation (r² = 0.44, P < 0.005) with both mean pulmonary artery pressure and invasively determined tricuspid gradient (23). In their study, 97% of patients with a Doppler echocardiographic finding of ERVSP ≥45 mm Hg were found to have PAH at catheterization. A Doppler echocardiographic threshold of <40 mm Hg versus ≥40 mm Hg had a positive predictive value of 92% and a negative predictive value of 44% (23). Therefore, as an adjunct to clinical evaluation, Doppler echocardiography is a reasonable screening approach for identifying patients with PAH.

Methodologic considerations include the fact that 2 of our centers enrolled more patients than the others; however, the ratio of the number of retrospectively studied patients to the number of prospectively studied patients in each of these 2 centers was similar to that in the overall study, indicating the likely absence of selection bias. Another potential bias could have been introduced by the study design, in that participating rheumatologists were aware of the goal to discover patients who might have PAH. However, they were asked to survey all patients diagnosed as having SSc or MCTD in their practices who were being actively followed up (seen within the previous 12 months). Interestingly, we found that the majority of patients who had not been diagnosed as having PAH (503 of 693 [72.6%]) had not been studied for PAH in the past.

We did not use a central laboratory to perform or evaluate all the Doppler echocardiography studies, but we did use predefined criteria to guide the interpretation of the results. Investigators at each individual site estimated the RVSP using the Bernoulli equation with the addition of the estimated RA pressure according to their standard laboratory criteria. Right heart catheterization was used in only a small minority of patients, either as the primary diagnostic or confirmatory tool; these findings suggest the need for a lower threshold of referral to cardiologists or pulmonologists.

It is reported that digital ulcers are more common among SSc patients with PAH than among those without PAH (6,21). We did not confirm this finding, in that our patients with or without PAH had a similar history of digital ulcers. We did find an association between a reduced DLCO and the presence of PAH defined by Doppler echocardiography, as reported by others (1,21). However, Mukerjee et al found a weak correlation of DLCO with mean pulmonary artery pressure by heart catheterization. They suggest that the positive predictive accuracy of currently used noninvasive tests is adequate for the diagnosis of PAH, provided that sufficiently high thresholds are used (23). However, for those individuals with a mixed clinical picture who may have LV dysfunction, particularly diastolic dysfunction, direct measurements of pulmonary pressure and wedge pressures remain the gold standard.

In summary, this study is the first to address the prevalence of PAH in a community-based rheumatology practice setting. Using accepted Doppler echocardiography technology as a screening tool, we found that a significant number (13.3%) of SSc and MCTD patients from 50 different community-based practices had undiagnosed PAH. Many of these patients had Doppler echocardiographic evidence of RV dysfunction, an abnormally low DLCO, and decreased exercise tolerance suggestive of advanced disease. These data suggest that Doppler echocardiographic evaluation of SSc and MCTD patients followed up in the community is justified, irrespective of symptoms, to detect patients who may need further evaluation, close surveillance, and/or intervention for underlying PAH. These findings further support the recommendation of the American College of Chest Physicians’ Evidence-Based Clinical Practice Guidelines that for asymptomatic patients at high risk for PAH, Doppler echocardiography should be performed to detect elevated pulmonary arterial pressure (24).

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APPENDIX A: UNCOVER STUDY INVESTIGATORS

Investigators in the UNCOVER Study are as follows: Julio Aponte, MD (Cleveland, OH); Steven Baak, MD (Florissaint, MO); Philip A. Baer, MD (Scarborough, ON, Canada); Neil Birnbaum, MD (San Francisco, CA); Howard Busch, MD (Jupiter, FL); David Campbell, MD (Fort Wayne, IN); John Conradi, MD (Rochester, NY); Mary Ellen Couka, MD (Milwaukee, WI); Alfred Denio, MD (Virginia Beach, VA); Deborah Desir, MD (Hamden, CT); Justus Fiechtner, MD (Lansing, MI); John A. Goldman, MD (Atlanta, GA); Robert Griffin, MD (Reading, PA); Michael Gross, MD (Fair Lawn, NJ); Howard Hauptman, MD (Baltimore, MD); John A. Howland, MD (Bay City, MI); Joe Huftstetter, MD (Chattanooga, TN); Thomas Ignaczak, MD (Battle Creek, MI); Richard Jones, MD (Tuscaloosa, AL); Gurjit S. Kaeley, MD (Lakewood, WA); Albert R. Katz, MD (Tarzana, CA); Steven Ko, MD (Fort Wayne, IN); Steven Lauter, MD (St. Louis, MO); Sharon LeClercq, MD (Edmonton, Alberta, Canada); Wonil Lee, MD (North Hollywood, CA); Angela McCain, MD (Sugar Land, TX); David McLain, MD (Birmingham, AL); Carter Mulz, MD (San Jose, CA); Frederick Murphy, MD (Duncansville, PA); Gary Myerson, MD (Atlantic, GA); Michael Neuwell, MD (San Leandro, CA); Aileen Pangan, MD (Maywood, IL); Glenn Parris, MD (Lawrenceville, GA); Jeffrey Poiley, MD (Orlando, FL); Naveen Raja, MD (Whittier, CA); Daniel H. Rosler, MD (Milwaukee, WI); Alberto Santos-Ocampo, MD (Honolulu, HI); Brian Sayers, MD (Austin, TX); Gregory Schimizzi, MD (Wilmington, NC); William Sheryg, MD (Huntsville, AL); Yvonne Sherrer, MD (Ft. Lauderdale, FL); Douglas Smith, MD (Indianapolis, IN); Neil I. Stahl, MD (Burke, VA); Evelyn Sutton, MD (Halifax, Nova Scotia, Canada); Timothy Swartz, MD (Kalamazoo, MI); Peter Valen, MD (La Crosse, WI); Daniel Wallace, MD (Los Angeles, CA); Jay Warrick, MD (Knoxville, TN); Francis M. Williams, MD (Houston, TX); Michel Zummer, MD (Montreal, QC, Canada).
Involvement of Lysosomal Cathepsins in the Cleavage of DNA Topoisomerase I During Necrotic Cell Death

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Objective. Autoantibodies to DNA topoisomerase I (topo I) are associated with diffuse systemic sclerosis (SSc), appear to be antigen driven, and may be triggered by cryptic epitopes exposed during in vivo topo I fragmentation. These autoantibodies recognize topo I and fragments of this autoantigen generated during apoptosis and necrosis. We undertook this study to determine whether lysosomal cathepsins are involved in topo I fragmentation during necrosis.

Methods. Topo I cleavage during necrosis was assessed by immunoblotting of lysates from L929 fibroblasts exposed to tumor necrosis factor α (TNFα) and the broad caspase inhibitor Z-VAD-FMK, and by immunoblotting of lysates from endothelial cells treated with HgCl2. Purified topo I and L929 nuclei were incubated with cathepsins B, D, G, H, and L, and topo I cleavage was detected by immunoblotting. The intracellular localization of cathepsin L activity and topo I in necrotic cells was examined using fluorescence microscopy.

Results. Treatment of L929 cells with TNFα and Z-VAD-FMK induced caspase-independent cell death with necrotic morphology. This cell death involved topo I cleavage into fragments of approximately 70 kd and 45 kd. This cleavage profile was reproduced in vitro by cathepsins L and H and was inhibited by the cathepsin L inhibitor Z-FY-CHO. During necrosis, cathepsin L activity diffused from lysosomes into the cytoplasm and nucleus, whereas topo I partially relocalized to the cytoplasm. Z-FY-CHO delayed necrosis and partially blocked topo I cleavage. The topo I cleavage fragments were also detected in necrotic endothelial cells and recognized by SSc sera containing anti-topo I antibodies.

Conclusion. These results implicate cathepsins, particularly cathepsin L, in the cleavage of topo I during necrosis. This cleavage may generate potentially immunogenic fragments that could trigger anti-topo I immune responses in SSc.

Autoantibodies to DNA topoisomerase I (topo I) are associated with diffuse cutaneous involvement and pulmonary fibrosis in patients with systemic sclerosis (SSc) (1,2). Although the pathogenic role of these autoantibodies remains unclear, there is evidence that their serum levels correlate positively with disease severity and activity (2). The original molecular target of these autoantibodies was designated Scl-70 (scleroderma-associated autoantigen of 70 kd) because of its migration as a 70-kd band on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) (3,4). Subsequently, it was demonstrated that this 70-kd band was a proteolytic fragment corresponding to the catalytic C-terminal domain of topo I (4–6). Anti–topo I autoantibodies from SSc patients recognize epitopes in the central and C-terminal portions of the protein, but not in the N-terminus, which suggests that the 70-kd fragment is processed by antigen-presenting cells (APCs) to initiate an immune response to topo I in...
vivo (1). Consistent with this, fragmented topo I presented by dendritic cells (DCs) elicited a vigorous T cell response in vitro more efficiently than full-length topo I (7). These observations strongly suggest that cryptic epitopes generated by in vivo proteolytic fragmentation of topo I might drive the generation of anti–topo I responses in SSc.

There is compelling evidence indicating that dying cells, both apoptotic and necrotic, are reservoirs of fragmented or cleaved forms of intracellular autoantigens (8–12). The excessive accumulation of these cleavage products, which could potentially expose cryptic epitopes, might break immune tolerance in a proinflammatory microenvironment and elicit specific humoral and cellular immune responses in patients with systemic autoimmune diseases. Topo I appears to be highly susceptible to proteolytic fragmentation during cell death. In apoptotic cells, the protein is cleaved by caspases to generate C-terminal fragments of 70–80 kd which are recognized by autoantibodies from SSc patients and are catalytically active (12,13). Topo I fragments of 72–75 kd are also produced by granzyme B both in vitro and during cell death induced by cytotoxic T lymphocytes (14). Our group demonstrated previously that topo I is also cleaved into fragments of approximately 70 kd and 45 kd in cells undergoing primary or secondary necrosis (10,11).

The proteases responsible for topo I cleavage during necrosis have not been identified. We hypothesized that cathepsins, which are released from lysosomes during both apoptosis and necrosis (15–19), might be involved in this cleavage. In the present study, we show that topo I is cleaved into fragments of approximately 70 kd and 45 kd in mouse L929 fibroblasts and human endothelial cells undergoing necrotic cell death, and that cathepsins, particularly cathepsin L, are capable of generating these fragments. Furthermore, these fragments are recognized by most SSc sera containing anti–topo I antibodies.

**MATERIALS AND METHODS**

**Cells and reagents.** L929 cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM L-glutamine, 100 units/ml of penicillin, 100 μg/ml of streptomycin (all obtained from Cellgro, Herndon, VA), and 10% fetal bovine serum (Omega Scientific, Tarzana, CA). Human dermal microvascular endothelial cells (HDMECs) and bovine coronary artery endothelial cells (BCAECs) were obtained from Cambrex (Walkersville, MD) and cultured in EGM-MV and EGM-2 MV media (Cambrex), respectively. Actinomycin D, trypan blue, CA-074, acridine orange, 4’,6-diamidino-2-phenylindole (DAPI), and human and mouse tumor necrosis factor α (TNFα) were from Sigma (St. Louis, MO). Purified cathepsins B, D, G, H, L, and S, and the cathepsin L–specific inhibitor Z-FY-CHO were from Calbiochem (San Diego, CA). LysoSensor Green DND-189 was from Molecular Probes (Eugene, OR). The broad caspase inhibitor Z-VAD-FMK was from Biomol International (Plymouth Meeting, PA). Boc-D-FMK (broad caspase inhibitor), Z-YVAD-FMK (inhibitor of caspases 1 and 4), Z-VDVAD-FMK (caspase 2 inhibitor), Z-DEVD-FMK (caspase 3 inhibitor), Z-VEID-FMK (caspase 6 inhibitor), Z-IETD-FMK (caspase 8 inhibitor), and Z-LEHD-FMK (caspase 9 inhibitor) were from Enzyme Systems Products (Livermore, CA).

The cathepsin L fluorogenic substrate Magic Red MR-(FR)₂ and the DNA fluorescent binding dye Hoechst 33342 were from Immunochrometry (Bloomington, MN). Mouse anti-human topo I monoclonal antibody clone C-21 and recombinant caspase 3 were from BD PharMingen (San Diego, CA). Anti–lamin B goat polyclonal antibody C-20 and anti–cathepsin L goat polyclonal antibody M-19 were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti–poly(ADP-ribose) polymerase (anti–PARP) monoclonal antibody C2-10 was from R&D Systems (Minneapolis, MN). Nitrocellulose membrane was from VWR (Brisbane, CA). Human sera containing autoantibodies to topo I and lamin B were a kind gift from Drs. Eng M. Tan and Michael Pollard (The Scripps Research Institute, La Jolla, CA). Baculovirus-expressed human topo I was a kind gift from Dr. James J. Champoux (University of Washington, Seattle) and was purified as described previously (20).

**Induction of cell death.** Caspase-independent cell death with necrotic morphology was induced in L929 cells by preincubation with the broad caspase inhibitor Z-VAD-FMK (100 μM) for 1 hour followed by exposure to 10 ng/ml of human or mouse TNFα. There was no difference in the levels of cell death induced by human versus mouse TNFα. Necrosis was induced in HDMECs and BCAECs by treatment with 80 μM and 40 μM HgCl₂, respectively, for up to 12 hours. Apoptosis was induced in L929 cells by preincubation with 1 μg/ml actinomycin D 1 hour before exposure to TNFα. In some experiments, cells were pretreated with the cathepsin L inhibitor Z-FY-CHO (150 μM) or with individual caspase inhibitors 1 hour prior to addition of TNFα. Quantification of cytoplasmic membrane rupture, indicative of necrosis, was performed by trypan blue exclusion (11). Morphologic analysis of cells was performed using an Olympus IX70 inverted microscope (Olympus, Melville, NY) equipped with Hoffman modulation contrast.

**Preparation of cell lysates and immunoblotting procedures.** After treatment with cell death–inducing agents, cells were harvested for preparation of total cell lysates. Floating cells in the culture medium were pooled and combined with the attached cells and resuspended directly in lysis buffer containing 100 mM Tris HCl (pH 6.8), 4% SDS, 10% (volume/volume) glycerol, and the CØMPLETE protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). This cocktail inhibits a broad spectrum of serine, cysteine, and metallo proteases, and calpains (www.roche-applied-science.com). Lysates were passed sequentially through 18–27-gauge needles to shear DNA. The protein concentration was determined using...
the DC Protein Assay (Bio-Rad, Hercules, CA). Prior to electrophoresis, lysates were heated for 5 minutes at 95°C in the presence of 4% mercaptoethanol and 0.04% bromphenol blue. Twenty micrograms of lysates was loaded onto each lane in 12% SDS-PAGE Ready Gels (Bio-Rad) and transferred to nitrocellulose membrane.

Immunoblotting was performed as described previously (10). Topo I was detected using human and mouse antibodies to topo I (1:500). Lamin B was detected using human and goat antibodies to lamin B (1:500). Cathepsin L was detected using a goat polyclonal antibody to cathepsin L (1:100). Bound primary antibodies were detected using horseradish peroxidase–conjugated goat anti-human IgG, goat anti-mouse IgM, or rabbit anti-goat IgG secondary antibodies (Zymed, South San Francisco, CA; Santa Cruz Biotechnology) in combination with enhanced chemiluminescence (Perkin Elmer Life Sciences, Boston, MA).

In vitro cleavage of recombinant topo I. Two hundred nanograms of baculovirus-purified recombinant topo I, resuspended in phosphate buffered saline (PBS; pH 5.5 or 7.5), was incubated with individual cathepsins B, D, G, H, and L for 1 hour at 37°C. Reactions were stopped with lysis buffer and heated at 65°C for 15 minutes. In some experiments, cathepsin L was preincubated for 30 minutes with Z-FY-CHO (150 μM) prior to mixing with topo I. Samples were analyzed in NuPAGE 4–20% Bis-Tris gels (Invitrogen, Carlsbad, CA) or 12% SDS-PAGE Ready Gels, followed by immunoblotting.

Treatment of nuclei with individual cathepsins. Untreated L929 cells were rinsed with ice-cold PBS, trypsinized, centrifuged, and washed twice in cold PBS. Cells were resuspended in hypotonic buffer (20 mM HEPES [pH 7.5], 10 mM KCl, 1.5 mM MgCl₂, 1 mM dithiothreitol, 0.05 mM phenylmethylsulfonyl fluoride, 0.05 μg/ml aprotinin) for 30 minutes at 4°C and then disrupted with 40–50 strokes in a Dounce homogenizer in the presence of 0.03% Nonidet P40. Homogenates were transferred to a 1.5-ml tube layered with hypotonic buffer containing 35% sucrose and were then centrifuged at 800g for 15 minutes at 4°C. Pelleted nuclei were resuspended in PBS (pH 7.5) and used immediately for cleavage assays or stored in small aliquots at −80°C. Isolated nuclei were incubated with individual cathepsins for up to 6 hours at 37°C. In some experiments, cathepsin L was preincubated for 30 minutes with Z-FY-CHO (150 μM) prior to mixing with nuclei. To stop the cleavage reactions, samples were centrifuged, and the pelleted nuclei were resuspended in lysis buffer (100 mM Tris HCl [pH 6.8], 4% SDS, 10% glycerol) supplemented with the COMPLETE protease inhibitor cocktail to prevent further proteolysis. Nuclear lysates were passed through 21–30-gauge needles to shear the DNA, sonicated briefly, and processed for SDS-PAGE and immunoblotting.

Determination of lysosomal integrity, detection of intracellular cathepsin L activity, and visualization of intracellular topo I by fluorescence microscopy. L929 cells were exposed to 5 μg/ml acridine orange in complete DMEM for 15 minutes at 37°C, rinsed twice with medium, and directly examined under an Olympus BX50 epifluorescence microscope using a LUMPlanPl 60X/0.90W water immersion objective (Olympus). Images were acquired using a digital Spot camera system (Diagnostic Instruments, Sterling Heights, MI). Alternatively, cells were exposed to 1 μM LysoSensor Green DND-189 in complete medium for 2 hours at 37°C, rinsed twice in medium, and examined directly by fluorescence microscopy as indicated above.

Cathepsin L activity was detected using the fluorogenic substrate Magic Red MR-(FR)₂. Briefly, cultured L929 cells were exposed for 30 minutes to the cathepsin L substrate MR-(FR)₂, rinsed twice with medium, and directly examined under the fluorescence microscope. Cells were counterstained with Hoechst 33342 for nuclear visualization. CA-074 (20 μM), a specific inhibitor of cathepsin B (21), was added to cells to increase the specificity of the cleavage of the MR-(FR)₂ substrate by cathepsin L.

For intracellular visualization of topo I, L929 cells growing in glass coverslips were fixed and permeabilized with ice-cold methanol/acetone (3/1 [v/v]), rinsed with PBS, and incubated with a highly monospecific human anti–topo I autoimmune serum (10,11). After washing with PBS, cells were then incubated with a rhodamine-labeled goat anti-human IgG secondary antibody. Cells were counterstained with DAPI in PBS for chromatin visualization. Coverslips were mounted onto glass slides, and cells were examined by fluorescence microscopy.

Statistical analysis. All statistical analyses were done using GraphPad Prism, version 2.0 (GraphPad Software, San Diego, CA). The unpaired t-test was used in all cases. SDs were calculated based on at least 3 independent experiments performed in triplicate.

RESULTS

Induction of L929 cell death by TNFα. Treatment of L929 murine fibroblasts with TNFα induces a relatively slow death in which both apoptotic and necrotic cells can be observed (22–25). Blockade of caspase activation in these cells with Z-VAD-FMK dramatically potentiates TNFα-mediated necrosis (22), whereas inhibition of transcription or translation with actinomycin D or cycloheximide, respectively, in the presence of TNFα shifts cell death toward apoptosis (22–25). Incubation of L929 cells with TNFα (10 ng/ml) led to a gradual loss of cytoplasmic membrane integrity, characteristic of necrosis, as measured by the ability of cells to take up trypan blue (Figure 1A). Necrosis was dramatically enhanced in the presence of 100 μM Z-VAD-FMK, resulting in >90% of cells taking up trypan blue by 6 hours. Treatment with Z-VAD-FMK alone did not exert any cytotoxic effects (Figure 1A). This enhancement was reproduced with the broad caspase inhibitor Boc,D-FMK, the caspase 3 inhibitor Z-DEVD-FMK, and the caspase 8 inhibitor Z-IETD-FMK, all used at high concentrations (100 μM) to ensure caspase inhibition (data not shown). However, it was not observed with inhibitors of caspases 1, 2, 4, 6, 9, and 10 (data not shown).
To confirm that L929 cells treated with TNFα/H9251/Z-VAD-FMK died in a caspase-independent manner, we performed an immunoblotting analysis of PARP cleavage. Figure 1B shows that PARP underwent limited cleavage into its caspase 3–generated 85-kd fragment in cells treated with TNFα/H9251 alone, and extensive cleavage in cells treated with TNFα/H9251/actinomycin D. However, in the presence of Z-VAD-FMK, cleavage was minimal, suggesting that caspase 3 activity was significantly blocked. This was confirmed in a caspase activity assay using the colorimetric DEVD–paranitroanilide substrate, which revealed that while there was significant caspase 3 and caspase 7 activity in cells treated with TNFα alone or in combination with actinomycin D, this activity was reduced to background levels in cells treated with TNFα/Z-VAD-FMK (results not shown).

Cells treated with TNFα/Z-VAD-FMK displayed necrosis-like morphologic features such as condensed nuclei and swelled cytoplasms, with no cytoplasmic blebbing (Figure 1C). In contrast, cells treated with
TNFα/actinomycin D displayed the typical blebbing associated with apoptosis. Cultures treated with TNFα alone contained both apoptotic and necrotic cells, with the latter predominating after 24 hours (Figure 1C and results not shown).

Cleavage of topo I into 70-kd and 45-kd fragments during L929 necrosis mediated by TNFα. Our group showed previously that topo I is cleaved into a fragment of ~70 kd in both apoptotic and necrotic cells, and into a fragment of 45 kd in cells undergoing primary or secondary necrosis (10,11). To determine whether topo I was cleaved into these fragments during TNFα-mediated cell death, we analyzed its proteolysis in L929 cells treated with HgCl2, TNFα alone, TNFα/Z-VAD-FMK, TNFα/actinomycin D, or TNFα/actinomycin D/Z-VAD-FMK.

Lysates from L929 cells exposed to TNFα alone or to TNFα/actinomycin D for 6 hours showed the appearance of the 70-kd fragment, as detected by immunoblotting (Figure 2A). After 12 hours, the 45-kd fragment was already visible in lysates from cells treated with TNFα/actinomycin D, indicating that cells were making the transition from apoptosis to secondary necrosis (11) (Figures 2A and B). A 70-kd fragment, in addition to the 45-kd fragment, was also detected in lysates from cells treated with HgCl2, TNFα/Z-VAD-FMK, or TNFα/actinomycin D/Z-VAD-FMK (Figures 2A and B), consistent with the necrotic morphology of...
The 70-kd fragment was abundant by 6 hours (Figure 2A) and usually appeared by 3 hours after induction of necrosis (results not shown). The amount of the 45-kd fragment increased as the amount of the necrotic 70-kd fragment decreased (Figures 2A and B), suggesting that it is a late cleavage product of the 70-kd fragment. In control experiments, we used human and goat antibodies to lamin B, which is cleaved during apoptosis but not necrosis (10,11). Cleavage of lamin B into its signature 45-kd apoptotic fragment (10,11) was observed in cells treated with TNFα/H9251 (detected with the goat anti–lamin B antibody but not with the human antibody) or TNFα/actinomycin D, but not in cells treated with HgCl2, TNFα/Z-VAD-FMK, or TNFα/actinomycin D/Z-VAD-FMK (Figures 2A and B).

**Figure 3.** Immunoblots showing in vitro cleavage of topoisomerase I (topo I) by cathepsins (Cat). A, Approximately 200 ng of purified human topo I was incubated in phosphate buffered saline (pH 7.5) for 1 hour at 37°C with various milliunits of individual cathepsins. B, Cleavage of topo I by cathepsin L at pH 5.5. C, Inhibition of cathepsin L–mediated topo I cleavage at pH 7.5 after preincubation of cathepsin L with its inhibitor, Z-FY-CHO (150 μM), for 30 minutes. D, Nuclei from L929 cells were incubated with 5 milliunits of individual cathepsins for various periods of time (0–6 hours). Lysates from untreated cells and cells treated with TNFα/Z-VAD-FMK were included as controls for the detection of topo I cleavage in immunoblots. Lines indicate bands corresponding to intact proteins; arrows indicate bands corresponding to cleavage fragments. Bands were recognized using a human autoantibody to topo I. Blots are representative of at least 3 independent experiments. See Figure 1 for other definitions.

Generation by cathepsin L of signature necrotic cleavage fragments of topo I in vitro. We hypothesized that lysosomal cathepsins might be responsible for the cleavage of topo I into 70-kd and 45-kd fragments during TNFα/Z-VAD-FMK–induced necrosis in L929 cells. To determine whether cathepsins can generate the signature necrotic cleavage fragments of topo I, we incubated purified topo I or L929 nuclei at a neutral pH of 7.5 with various concentrations of cathepsins B, D, G, H, and L.

Figure 3A shows that only cathepsins L and H generated prominent bands of approximately 70 kd and 45 kd when incubated with purified topo I. Minor bands migrating below 45 kd were often observed after cathepsin treatment. Some of these have been observed previously in necrotic Jurkat T cells (10). The band generated
by cathepsins L and H around the 70-kd region appeared as a doublet or triplet of ~65–80 kd, suggesting that the protein might be cleaved at several different but closely spaced sites. These immunoblots were obtained using NuPAGE 4–20% Bis-Tris gels for optimal resolution of bands around this region. In contrast, immunoblots obtained using 12% SDS-PAGE Ready Gels showed only single bands of 70 kd and 45 kd (Figure 2 and results not shown). There was no difference in the cleavage profile of topo I produced by cathepsin L when the cleavage reactions were conducted at pH 5.5 (Figure 3D). The other cathepsins either did not cleave topo I or cleaved it without generating the necrotic 45-kd fragment. For instance, cathepsin B generated fragments of approximately 80 kd and 70 kd, whereas cathepsin G efficiently generated a single fragment of ~70 kd (Figure 3A).

In isolated L929 nuclei, only cathepsin L efficiently generated a prominent topo I band migrating in the 65–80-kd range, as well as the 45-kd cleavage product (Figure 3D). Given the prominence of the band around the 70-kd region (consistently observed in repeated experiments) (Figure 3D), it appears that cleavage of nuclear topo I by cathepsin L into fragments migrating around this region exposed epitopes that were highly reactive with the human anti–topo I autoantibody used for these experiments. The specific cathepsin L inhibitor Z-FY-CHO inhibited cathepsin L-mediated cleavage of purified and nuclear topo I (Figures 3C and D). The concentration ranges of cathepsins and the incubation conditions used in these experiments were based on reported studies (26,27). It should be noted that untreated topo I, either in purified form or in the isolated nuclei, underwent limited degradation into a 70-kd product during protein or nuclear preparation (Figure 3), consistent with previous reports (3–5). Nuclear preparations were done under limited protease inhibitory conditions to prevent inhibition of cathepsins.

**Intracellular relocalization of cathepsin L and topo I during L929 necrosis mediated by TNFα.** The rupture of lysosomes during necrosis causes extensive leakage of cathepsins into the cytoplasmic compartment (17). We hypothesized that if cathepsin L plays a role in the cleavage of topo I in L929 cells exposed to TNFα/Z-VAD-FMK, it should be possible to detect leakage of this enzyme into the cytosolic compartment and perhaps relocalization into the nucleus, and/or relocalization of topo I into the cytoplasm. To explore this, we first analyzed the integrity of lysosomes in L929 cells exposed to acridine orange in the presence and absence of TNFα/Z-VAD-FMK. Due to proton trapping, this vital dye accumulates mainly in the acidic vacuolar apparatus, preferentially in lysosomes (15). When excited by blue light, acridine orange emits red/orange fluorescence at high (lysosomal) concentrations and green fluorescence at low (nuclear and cytosolic) concentrations. Disruption of lysosomal integrity leading to leakage of acridine orange into the cytosol produces a bright yellow fluorescence.

Fluorescence microscopic analysis of lysosomes in untreated L929 cells showed intact lysosomal compartments as indicated by clusters of orange fluorescence separated from green fluorescence, whereas cells treated with TNFα/Z-VAD-FMK for 4 hours either lacked the orange clusters or displayed bright yellow fluorescence, indicative of acridine orange leakage into the cytosol (Figure 4A). This leakage was partially inhibited by preincubation with Z-FY-CHO. After 6 hours of treatment with TNFα/Z-VAD-FMK, however, Z-FY-CHO was no longer able to block acridine orange release (results not shown). Similar results (not shown) were obtained with LysoSensor Green DND-189, another specific probe for lysosome integrity.

To determine whether cathepsin L is released from lysosomes in L929 cells treated with TNFα/Z-VAD-FMK, we sought to examine changes in the intracellular localization of this enzyme by fluorescence microscopy. We used the fluorogenic substrate–based assay Magic Red MR-(FR)2, which allows the localization of cathepsin L activity in live cells. To augment the specificity of this localization, we simultaneously exposed the L929 cells to the cathepsin B inhibitor CA-074 to avoid nonspecific cleavage of the fluorogenic substrate by cathepsin B. Cells were counterstained with Hoechst 33342 to visualize nuclei. As expected, the red fluorescence was localized in untreated cells in cytoplasmic granules around the nuclei, corresponding to lysosomes, where cathepsin L is normally stored (Figure 4B). In cells treated with TNFα/Z-VAD-FMK, the red fluorescence was diffused in the cytosol and even appeared to colocalize with nuclei (Figure 4B). It should be cautioned, however, that since these experiments were done in live cells, it is not entirely clear whether cathepsin L actually penetrated the nuclear compartment. We also examined the localization of topo I in fixed L929 cells, using a highly specific human anti–topo I serum. In untreated cells, topo I was confined to the nucleus, whereas in cells treated with TNFα/Z-VAD-FMK, both nuclear and cytoplasmic staining were evident (Figure 4C).
Figure 4. Intracellular relocalization of cathepsin L activity and topoisomerase I (topo I) during TNFα/Z-VAD-FMK–induced necrosis in L929 cells. **A,** Determination of lysosomal integrity in L929 cells treated with TNFα/Z-VAD-FMK. Cells were exposed to acridine orange for lysosome visualization. Untreated cells showed localized granular orange fluorescence corresponding to lysosomal staining. In the cytoplasm of intact cells, acridine orange gives a green fluorescent color. In the TNFα/Z-VAD-FMK–treated cells, a decrease or absence of orange fluorescence, with increased yellow fluorescence (indicative of lysosomal leakage), was observed in some cells (**arrows**). Lack of orange or yellow fluorescence indicates leakage of lysosomal acridine orange into the extracellular milieu due to loss of cytoplasmic membrane integrity. Cells treated with TNFα after preincubation with Z-VAD-FMK and Z-FY-CHO for 1 hour displayed delayed loss of lysosomal integrity. Corresponding phase-contrast images are shown in the lower panel. **B,** Detection of intracellular cathepsin L activity in L929 cells, using the fluorogenic substrate MR-(FR)2. Cells treated with TNFα/Z-VAD-FMK show diffuse cathepsin L activity (red fluorescence, **white arrows**), whereas in untreated cells, the activity is localized mainly in cytoplasmic granules corresponding to lysosomes. The **yellow arrow** indicates a necrotic cell with very little cathepsin L activity that appeared to have lost cytoplasmic membrane integrity (see corresponding phase-contrast image at right). **C,** Intracellular visualization of topo I in L929 cells, using a highly specific human anti–topo I serum (10,11) as primary antibody and a rhodamine-conjugated goat anti-human IgG as secondary antibody. In untreated cells, the rhodamine fluorescence is confined to the nucleus, whereas in cells treated with TNFα/Z-VAD-FMK, the nuclei appear pyknotic and the fluorescence is localized both in the nucleus and cytoplasm. All representative images were acquired using an Olympus BX50 epifluorescence microscope 4–5 hours after exposure of cells to TNFα/Z-VAD-FMK. DAPI = 4′,6-diamidino-2-phenylindole (see Figure 1 for other definitions). (Original magnification × 40.)
Inhibition of cathepsin L activity delays necrosis and partially inhibits topo I cleavage. To determine whether cathepsin L activity is required for topo I fragmentation in necrotic L929 cells, we preincubated cells for 1 hour in the presence of the specific cathepsin L inhibitor Z-FY-CHO (150 μM) and Z-VAD-FMK (100 μM) prior to exposure to TNFα. We first examined whether cathepsin L is processed into the mature 29-kd form during TNFα/Z-VAD-FMK-induced necrosis. A time course study of cathepsin L processing revealed an increase in the appearance of the 29-kd mature form of this protease during necrosis (Figure 5A). This increase was not observed in cells preincubated with Z-FY-CHO, which instead consistently showed smaller amounts of the 39-kd proenzyme and a slight accumulation of an ~52-kd band. This band might correspond to an immature or complexed form of the enzyme that is not efficiently processed or released in the inhibited cells. These results suggested that Z-FY-CHO blocked the autocatalytic processing of cathepsin L into its mature 29-kd form (28). Preincubation of cells with Z-FY-CHO for 1 hour delayed necrosis during the first 6 hours of exposure to TNFα/Z-VAD-FMK (P < 0.05) (Figure 5B). However, the majority of cells eventually (after >12 hours) died by necrosis (data not shown). Consistent with these results, we detected partial inhibition of topo I cleavage into the 70-kd and 45-kd fragments in cells preincubated with Z-FY-CHO for 1 hour prior to exposure to TNFα/Z-VAD-FMK (Figure 5C).

SSc sera containing autoantibodies to topo I recognize the 70-kd and 45-kd cleavage fragments in necrotic L929 and endothelial cells. To determine whether the necrotic cleavage fragments of topo I are recognized by the majority of SSc sera containing anti-topo I autoantibodies, we used immunoblotting to analyze 38 SSc sera containing autoantibodies reactive with a band of ~100 kd and displaying an immunofluorescence staining pattern characteristic of anti–topo I autoantibodies. We assumed that this 100-kd band corresponded to topo I, although we could not rule out the possibility that some of the sera contained autoantibodies against the ~100-kd subunits of the SSc-associated autoantigens PM-Scl or RNA polymerase I.

Analysis of the reactivity of the 38 sera against whole cell lysates from L929 cells exposed to TNFα/Z-VAD-FMK revealed that 34 sera (89%) recognized the signature 70-kd and 45-kd necrotic cleavage fragments, indicating that these fragments display epitopes recognized by the majority of SSc sera containing anti–topo I autoantibodies. Representative results with sera reacting with the 70-kd and 45-kd necrotic cleavage fragments are shown in Figure 6A. In this particular experiment, the control lysates from L929 cells also showed moder-
ate reactivity against the 70-kd fragment (Figure 6A), a phenomenon that was occasionally observed when lysates were stored at –80°C for prolonged periods or when the viability of the untreated L929 cell cultures did not exceed 95% prior to lysate preparation for immunoblotting. Unlike previous reports on the U1–70-kd autoantigen (29), we did not observe preferential antibody reactivity against any of the cleavage fragments.

Since endothelial cell death has been implicated in the pathology of SSc (30), we next determined whether SSc sera also recognize the 70-kd and 45-kd cleavage fragments in endothelial cells undergoing necrosis. Figures 6B and C show representative results with SSc sera reacting with the 70-kd and 45-kd fragments in whole lysates from HDMECs and BCAECs, respectively, induced to die by necrosis with HgCl2. To confirm that the representative sera were recognizing topo I, we included control blots showing their reactivity against purified recombinant topo I (Figure 6D).

DISCUSSION

The results presented here provide evidence that cathepsins, particularly cathepsin L, are involved in the cleavage of topo I during necrotic cell death. Our previous analysis of topo I cleavage during nonapoptotic cell death revealed that this protein is cleaved into fragments of approximately 70 kd and 45 kd that are recognized by autoantibodies in Jurkat T cells dying by primary or secondary necrosis (10,11). In the present study, we detected these fragments in L929 fibroblasts undergoing caspase-independent death with necrotic morphology in response to treatment with TNFα/Z-VAD-FMK. We also detected these fragments in human and bovine endothelial cells undergoing necrosis induced with HgCl2. These observations suggest that the generation of the topo I 70-kd and 45-kd cleavage fragments is mediated by a conserved proteolytic mechanism that is activated in necrotic cell death. Hence, anti–topo I antibodies recognizing these fragments could be used to distinguish apoptosis from necrosis by immunoblotting. This distinction could be enhanced in combination with antibodies to lamin B, which is selectively cleaved in apoptotic cells but not in necrotic cells (10,11).

Cathepsins participate in apoptosis by cleaving a number of intracellular proteins (31,32), but knowledge of their substrates in necrosis is scarce. A previous report implicated cathepsins B and G in the necrotic cleavage of PARP, a lupus-associated autoantigen (26). Our results indicated that cathepsins B, G, H, and L can induce topo I fragmentation in vitro. However, only cathepsins L and H generated both 70-kd and 45-kd necrotic cleavage fragments. When incubated with purified nuclei, cathepsin L (but not cathepsin H) generated both fragments, suggesting that cathepsin L may access topo I more efficiently in the nucleus. It should be noted that the cleavage reactions were conducted at a neutral pH of 7.5. Cleavage reactions with cathepsin L conducted at an optimal pH of 5.5 yielded a cleavage profile identical to that observed at neutral pH. Although optimal enzymatic activity by cathepsins is achieved at pH 5.5, Goulet et al (27) demonstrated recently that cathepsin L is active and can process substrates at neutral pH. Those investigators suggested that the suboptimal pH of 7.5 should not be taken as an obstacle but as a key element that enables cathepsin L and perhaps other cathepsins to play an important role in the limited...
protein cleavage outside the lysosomes, particularly in the nucleus.

We detected cathepsin L activity outside the lysosomes and in the nucleus during TNFα/Z-VAD-FMK-mediated cell death. We also showed by immunoblotting a gradual increase in the levels of processed 29-kd cathepsin L during necrosis, most likely arising via autocatalytic processing (28). These results suggested that cathepsin L was active even in the presence of 100 μM Z-VAD-FMK, a high concentration that is known to inhibit cathepsins B and H in cultured cells (33). Interestingly, topo I was also detected in the cytoplasm of necrotic cells, suggesting that a portion of it may leak from the nucleus into the cytoplasm during necrosis. Thus, cathepsin L may encounter topo I both in the nucleus and in the cytoplasm.

While results of our studies suggest that cathepsin L is involved in topo I cleavage during necrosis, other cathepsins are also likely to generate topo I cleavage in necrotic L929 cells because it was not completely blocked in cells preincubated with the specific cathepsin L inhibitor Z-FY-CHO. However, Z-FY-CHO may block cathepsin L activity only when the protease leaks from the lysosomes into the cytosol, and, conceivably, it may not completely block all the released cathepsin L. Our data also suggest that cathepsin L plays a role in the progression of L929 necrosis mediated by TNFα/Z-VAD-FMK, because preincubation of cells with Z-FY-CHO delayed but did not inhibit cell death. This indicates that other cathepsins are involved, although late, in this necrosis process. Additional insights into the role of cathepsins in necrosis and in the necrotic cleavage of intracellular autoantigens could be obtained in future studies using cathepsin-deficient cell lines and mouse models. Cell lines have been described in which cathepsin L has been genetically knocked out (16), silenced via RNA inhibition (34), or rendered inactive by persistent viral infection (35). In preliminary experiments, we observed that the L929-derived mutant cell line LX, which was obtained by persistent reovirus infection and lacks activity of both cathepsin B and cathepsin L (35), showed a slight delay in TNFα/Z-VAD-FMK-mediated necrosis and topo I cleavage compared with L929 cells (Pacheco FJ, et al: unpublished observations). However, LX cells abundantly express cathepsin H (35), which could compensate for the absence of cathepsin L.

There is increasing interest in necrotic cells, particularly when they arise as the result of defective clearance of apoptotic cells, as reservoirs of altered autoantigens and danger signals that could contribute to the generation of autoantibody responses in rheumatic diseases (36–39). A current hypothesis is that impaired phagocytic removal of apoptotic cells may cause accumulation of secondary necrotic cells in germinai centers of secondary lymphoid organs, leading to exposure by the immune system to high concentrations of altered forms of autoantigens for which tolerance has not been previously achieved (36,37). Consistent with this hypothesis, our group demonstrated previously that the progression from apoptosis to secondary necrosis is associated with fragmentation of topo I and other intracellular autoantigens into forms not normally generated during apoptosis (11). In the presence of appropriate environmental adjuvants, danger signals, or inflammatory cytokines that can act as maturation factors for DCs, fragmented autoantigens revealing cryptic epitopes and released by dying cells may initiate autoantibody responses (40).

Cathepsins and caspases both produce an ~70-kd topo I cleavage fragment that is recognized by the SSc autoantibodies, which indicates strongly that this fragment corresponds to the immunogenic C-terminal domain of the protein. Because these enzymes have different amino acid specificities (aspartic acid for caspases and various nonaspartic amino acids for cathepsins), their cleavage sites in topo I must be different. However, it is possible that these sites could be present within a protease-sensitive region separating the nonimmunogenic N-terminus from the immunogenic C-terminus. Studies by Hu and colleagues have shown that the N-terminal domain of topo I comprising the first 200 amino acids of the protein is not recognized by anti–topo I–positive sera from SSc patients (1). Those investigators demonstrated that in all anti–topo I–positive SSc patients, the autoantibodies recognize both linear and conformational epitopes and have the same molecular recognition pattern, with the core central region of the protein primarily targeted. The nonantigenic N-terminal region of the protein appears to be irrelevant for the initiation of anti–topo I responses, and it might be removed from the whole molecule before topo I is taken up and processed by APCs (1). This N-terminal region is removed by caspases during apoptosis (13) and, most likely, by cathepsins during necrosis.

There is evidence that autoantibody responses to topo I in SSc are driven by cryptic determinants and that a very specific combination of fragmented forms of topo I and the type of APCs that process these forms may be involved in breaking tolerance to topo I in the early stages of development of this disease (1,2,7,41). It is conceivable that immunogenic fragmented forms of topo I could arise in vivo via caspase- or cathepsin-
mediated cleavage of the protein during apoptosis or secondary necrosis. Endothelial cell death appears to play an important role in the pathogenesis of SSc (30), and our observation that cleavage of topo I into 70-kd and 45-kd fragments can occur in cultured endothelial cells undergoing necrotic cell death suggests that dying endothelial cells (both apoptotic and necrotic) could serve as reservoirs of potentially immunogenic fragments of topo I in SSc patients.

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REFERENCES

Tumor Necrosis Factor α Blockade Treatment Down-Modulates the Increased Systemic and Local Expression of Toll-like Receptor 2 and Toll-like Receptor 4 in Spondylarthropathy

Leen De Rycke, Bernard Vandooren, Elli Kruithof, Filip De Keyser, Eric M. Veys, and Dominique Baeten

Objective. Abnormal host defense against pathogens has been implicated in the pathogenesis of spondylarthropathy (SpA), a disease characterized by abundant synovial infiltration with innate immune cells. Given the role of Toll-like receptors (TLRs) in activation of innate inflammation and the occurrence of TLR-dependent infections after tumor necrosis factor α (TNFα) blockade treatment, the present study was undertaken to analyze TLRs and their modulation by TNFα blockade in SpA.

Methods. Peripheral blood mononuclear cells (PBMCs) were obtained from SpA and rheumatoid arthritis (RA) patients during infliximab therapy, and from healthy controls. TLR-2 and TLR-4 expression and TNFα production upon lipopolysaccharide (LPS) stimulation were analyzed by flow cytometry on different monocyte subsets. Synovial biopsy specimens from 23 SpA patients before and after infliximab or etanercept treatment, from 15 RA patients, and from 18 osteoarthritis (OA) patients were analyzed by immunohistochemistry.

Results. Expression of TLR-4, but not TLR-2, was increased on PBMCs from patients with SpA, whereas both TLRs were increased in RA patients. TLR expression was particularly increased on the CD163+ macrophage subset. Infliximab reduced TLR-2 and TLR-4 expression on monocytes of SpA and RA patients, leading to lower levels than in controls and to impaired TNFα production upon LPS stimulation. In inflamed synovium, the expression of both TLRs and of CD163 was significantly higher in patients with SpA than in those with RA or OA. Paralleling the systemic effect, TLRs in synovium were down-regulated following treatment with infliximab as well as etanercept, indicating a class effect of TNFα blockers.

Conclusion. Inflammation in SpA is characterized by increased TLR expression, which is sharply reduced by TNFα blockade. These findings suggest a potential role of innate immunity-mediated inflammation in SpA and provide an additional clue regarding the mechanism of action as well as the potential side effects of TNFα blockade.

The spondylarthropathies (SpA) are a group of chronic inflammatory joint diseases characterized by axial involvement and peripheral arthritis. Although the pathogenesis of SpA is still unclear, a major clue is provided by findings in the reactive arthritis subtype, in which chronic joint inflammation is triggered by gastrointestinal or urogenital infection with bacteria (1). The absence of evidence of viable microbes in the joint (2–4), the frequent occurrence of gut inflammation in other SpA subtypes independent of gastrointestinal infections (5–8), and the influence of the germ-free state on the development of SpA-like gut and joint disease in HLA-B27-transgenic rats (9) suggest that bacterial triggering of the immune system, rather than infection itself, is important in SpA. Considering the strong genetic link
with HLA–B27, it was originally proposed that microbial products might be presented, in the context of this specific class I major histocompatibility complex molecule, to cytotoxic CD8+ lymphocytes, which in turn may cross-react with self peptides in the joint. Despite extensive investigation, however, the role of antibacterial CD8+ lymphocytes in the pathogenesis of the disease remains to be formally demonstrated (10).

Recently, we presented the hypothesis that cells of the innate immune system, such as polymorphonuclear cells and macrophages, may be more important than lymphocytes in SpA inflammation. First, both peripheral blood and gut lymphocytes had impaired cytokine production in SpA (11,12). Second, macrophages expressing the scavenger receptor CD163 were increased in both the gut and the joints of SpA patients, and local production of soluble CD163 inhibited T cell activation in the joint (13,14). Third, in addition to the increased levels of CD163+ macrophages, we observed a significant increase of polymorphonuclear cells in the synovium of SpA versus rheumatoid arthritis (RA) patients (15,16). Finally, levels of both CD163+ macrophages and polymorphonuclear cells, but not of CD3+ or CD20+ lymphocytes, correlated with global disease activity in SpA (17). Taken together, these data suggest that activation of innate immune cells in the gut as well as in the joints, by microbial products and/or cross-reactive self molecules, may be relevant to inflammation in SpA.

Recently, the activation of innate immune cells by pathogen-associated molecular pathways has been linked to the Toll-like receptors (TLRs) (18–21). The TLR family comprises 11 different members, of which membrane TLR-2 and TLR-4 are ligated by lipoproteins and peptidoglycans from gram-positive bacteria and by lipopolysaccharides (LPS) from gram-negative bacteria, respectively. Upon binding of their ligands, TLRs activate a complex signaling cascade which results ultimately in the production of mediators of inflammation. This pathway is crucial for host defense against a wide variety of pathogens including the invasive and intracellular bacteria involved in reactive arthritis (22–25), but can also lead to sterile inflammation in the absence of microbes (20). This may relate to the recognition by TLRs of self motifs such as heat-shock protein 70, fibronectin, hyaluronic acid, heparan sulfate, and fibrinogen (26–30). Given that these self ligands are present in abundance in the joint and that TLRs have been found in the synovial membrane (31–34), this pathway may be involved in the innate immune inflammation of the joint.

Indeed, there is evidence for a role of TLRs in murine arthritis models (35,36).

Based on these observations on microbial triggering and innate immune cells in SpA and on the involvement of TLRs in murine arthritis models, the present study was undertaken to investigate the expression and potential role of TLR-2 and TLR-4 in systemic and synovial inflammation in SpA in humans. Moreover, the impressive down-modulation of inflammation and the occurrence of serious infectious side effects related to TLR-dependent pathogens during tumor necrosis factor α (TNFα) blockade treatment (37–41) led us to investigate the effect of TNFα blockers on systemic and local expression of TLRs.

**PATIENTS AND METHODS**

**Patients and samples.** The study protocol was approved by the Ethics Committee of Ghent University Hospital. Eighty-two subjects were included. All subjects provided written informed consent before enrollment. All SpA and RA patients had active disease that fulfilled the European Spondylarthropathy Study Group classification criteria for SpA (42) or the American College of Rheumatology (formerly, the American Rheumatism Association) criteria for RA (43). All osteoarthritis (OA) patients had active synovitis affecting at least 1 knee joint. Baseline demographic and clinical characteristics of the patient groups are shown in Table 1, and response to treatment among the patients treated with TNFα blockade is shown in Table 2.

Peripheral blood samples from 8 SpA patients (all with ankylosing spondylitis [AS]), 9 RA patients, and 9 healthy controls (median age 28 years [range 23–35]) were obtained at baseline. The 8 SpA and 9 RA patients were treated with infliximab (5 mg/kg and 3 mg/kg, respectively), at weeks 0, 2, and 6. Additional blood samples were obtained from the SpA and RA patients at weeks 2 and 6 (prior to infusion). Peripheral blood mononuclear cells (PBMCs) were isolated by Ficoll-Paque gradient centrifugation (Pharmacia, Uppsala, Sweden) and used for phenotypic and functional analysis.

Synovial tissue samples (16 biopsy specimens from each patient) were obtained from clinically involved knee joints of 23 SpA patients (8 with AS, 7 with psoriatic arthritis, and 8 with undifferentiated SpA), 15 RA patients, and 18 OA patients, by needle arthroscopy as described previously (44). Eight of the SpA patients were treated with infliximab (5 mg/kg at weeks 0, 2, and 6) and 15 were treated with etanercept (25 mg twice weekly); in these SpA patients, paired biopsy samples were also obtained at week 12. Five-micrometer sections of these samples were used for histologic and immunohistochemistry analysis.

**Flow cytometry.** PBMCs were washed in phosphate buffered saline (PBS) and incubated for 30 minutes with the appropriate amount of the following fluorochrome-labeled monoclonal antibodies (mAb) for phenotypic characterization: fluorescein isothiocyanate (FITC)–conjugated anti–TLR-2 (Immunosource, Halle-Zoersel, Belgium), FITC-conjugated...
anti–TLR-4 (Serotec, Oxford, UK), phycoerythrin (PE)–conjugated anti-CD163 (BD Biosciences PharMingen, San Diego, CA), PE/Cy5-conjugated anti-CD33 (BD Biosciences PharMingen), and allophycocyanin (APC)–conjugated anti–HLA–DR (BD Biosciences PharMingen). The labeled PBMCs were washed, fixed in PBS/1% formaldehyde, and analyzed by 4-color flow cytometry (FACSCalibur; Becton Dickinson, San Diego, CA) using CellQuest software (Becton Dickinson). Monocytes were identified using a forward and side scatter gate in combination with a gate on CD33high cells (previous experiments showed that all of these cells were CD14+/H11001).

Within the global monocytic population identified by CD33, specific monocyte subpopulations were analyzed based on the expression of CD163 (13,14). Nonspecific staining and autofluorescence were determined using isotype-matched control mAb. Samples from different disease groups and samples obtained pre- and posttreatment were analyzed in a single run under blinded conditions, to avoid interassay variability. Results are expressed as mean fluorescence intensity (MFI). Differences in MFI for TLR-2 and TLR-4 were consistent in multiple experiments, and expression levels were comparable with reported data (33,34).

TNFα production. For analysis of TNFα production, PBMCs were resuspended in RPMI 1640 medium (Invitrogen, Merelbeke, Belgium) and stimulated for 6 hours with either 10 ng/ml LPS (Escherichia coli O26:B6; Sigma, St. Louis, MO) or 25 ng/ml phorbol 12-myristate 13-acetate (PMA), both in the presence of 10 g/ml brefeldin A (Sigma) for the last 5 hours in order to inhibit the secretion of produced cytokines. Unstimulated cells incubated for 6 hours in RPMI 1640 were used as controls. The cells were subsequently incubated for 30 minutes with PE/Cy5-conjugated anti-CD33 (BD Biosciences PharMingen). Next, 2 ml of lysing buffer (BD Biosciences PharMingen) was added for 10 minutes, cells were

Table 1. Demographic and clinical data on the SpA, RA, and OA patients included in the analysis of PBMCs or synovial tissue*

<table>
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<th>PBMCs</th>
<th>Synovium</th>
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<tbody>
<tr>
<td></td>
<td>SpA (n = 8)</td>
<td>RA (n = 9)</td>
</tr>
<tr>
<td></td>
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<tr>
<td></td>
<td>OA (n = 18)</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>6/17</td>
<td>13/2</td>
</tr>
<tr>
<td></td>
<td>11/7</td>
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</tr>
<tr>
<td>Disease duration, years</td>
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<td>11 (1–19)</td>
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<td></td>
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<tr>
<td></td>
<td>5 (0.17–35)</td>
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<tr>
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<tr>
<td></td>
<td>5 (3–15)</td>
<td>1 (1–7)</td>
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<td>Serum CRP, gm/liter</td>
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<td></td>
<td>23 (1–150)</td>
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<td>ESR, mm/hour</td>
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<td></td>
<td>7 MTX, 1 LEF,</td>
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<td></td>
<td>1 MTX + LEF</td>
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* Values for sex and treatment are the number of patients; other values are the median (range). SpA = spondylarthropathy; RA = rheumatoid arthritis; OA = osteoarthritis; PBMCs = peripheral blood mononuclear cells; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; NSAIDs = nonsteroidal antiinflammatory drugs; DMARDs = disease-modifying antirheumatic drugs; MTX = methotrexate; LEF = leflunomide; SSZ = sulfasalazine.

Table 2. Clinical response in the SpA and RA patients included in the analysis of PBMCs or synovial tissue*

<table>
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<td>6-week infliximab treatment, RA patients (n = 4)</td>
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<td>16 (0–9)†</td>
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<td>Pretreatment</td>
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<tr>
<td>Posttreatment</td>
<td>0 (0–3)†</td>
<td>1 (0–18)†</td>
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<tr>
<td>Serum CRP, gm/liter</td>
<td>13 (4–39)</td>
<td>16 (3–114)</td>
</tr>
<tr>
<td>Pretreatment</td>
<td>3 (1–10)†</td>
<td>6 (1–124)†</td>
</tr>
<tr>
<td>Posttreatment</td>
<td>31 (10–42)</td>
<td>30 (4–69)</td>
</tr>
<tr>
<td>ESR, mm/hour</td>
<td>2.5 (1–18)†</td>
<td>7 (2–68)†</td>
</tr>
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</table>

* Values are the median (range). See Table 1 for definitions.
† P < 0.05 versus pretreatment.
centrifuged, and 500 μl of permeabilization buffer (BD Biosciences PharMingen) was added for another 10 minutes. The cells were then incubated for 30 minutes with APC-conjugated anti-TNFα (BD Biosciences PharMingen). Isotype- and concentration-matched control mAb were again used to assess nonspecific binding. The labeled cells were analyzed by flow cytometry as described above.

**Immunohistochemistry analysis.** Synovial biopsy specimens were fixed, stained, and scored as described in detail previously (13–17,44–47). Briefly, 8 paraffin-embedded specimens from each patient were stained with hematoxylin and eosin for histologic evaluation of inflammatory infiltration. The remaining 8 samples were embedded in tissue freezing medium. Frozen sections were fixed in acetone and incubated with anti-CD68 and anti-CD163 mouse mAb (both from Dako, Glostrup, Denmark) for detection of macrophages (13,14) and with mAb against TLR-2 and TLR-4 (both from Abcam, Cambridge, UK). After rinsing of the specimens, endogeneous peroxidase was blocked with 1% hydrogen peroxide. The sections were subsequently incubated for 15 minutes with streptavidin–peroxidase complex (LSAB Kit; Dako). The color reaction was developed using 3-amino-9-ethylcarbazole substrate (Dako) as chromogen. Finally, the sections were counterstained with hematoxylin. The stained sections were masked with regard to diagnosis and time of sampling and scored by 2 independent observers (DB and LdR). The synovial lining layer and sublining layer were scored separately for each parameter, using a 4-point semiquantitative scale (0 = the lowest and 3 = the highest level of expression) that had been extensively validated previously (13–17,44–47).

Interobserver correlation was high for all parameters ($r > 0.90$, $P < 0.01$), with interobserver agreement reached in >85% of cases. In instances of discordant scores between the 2 observers (which never differed by >1 point), the mean of the 2 scores was used. Semiquantitative scoring was previously shown to generate results similar to those obtained with manual counting and digital image analysis (48,49).

**Statistical analysis.** The flow cytometric data were normally distributed and are expressed as the mean ± SD. The significance of the differences between groups was determined using Student’s $t$-test. Correlation coefficients were calculated with Pearson’s correlation test. The nonparametric immunohistochemical data are expressed as the median (range), and the significance of the differences between groups was determined using the Mann-Whitney U test for unpaired data and the Wilcoxon signed rank test for paired data. $P$ values less than 0.05 were considered significant.

**RESULTS**

### Increased expression of TLR-4, but not TLR-2, on monocytes from patients with SpA

The expression of TLR-2 and TLR-4 on PBMCs from SpA patients, as identified by their forward and side scatter and their bright expression of CD33, was measured by flow cytometry. As shown in Figure 1, the MFI of TLR-4 (mean ± SD 106 ± 33 versus 73 ± 7; $P = 0.005$), but not of TLR-2 (82 ± 24 versus 87 ± 9), was increased in SpA versus healthy controls. In order to assess whether these alterations were specific to SpA or were more generally related to chronic arthritis, patients with RA were analyzed as well. The RA cohort exhibited increased expression of both TLR-4 (MFI 111 ± 45) and TLR-2 (119 ± 44) ($P = 0.011$ and $P = 0.025$, respectively, compared with healthy controls), and there was a trend toward higher expression of TLR-2 in RA cells versus SpA cells ($P = 0.055$). Since this difference between the findings in SpA patients and those in RA patients suggested that the alterations of TLR-4 expression in SpA were not merely a reflection of global monocyte activation, we additionally investigated the expression of HLA–DR as an activation marker on PBMCs and found no significant difference between levels in SpA patients (mean ± SD MFI 303 ± 172) and healthy controls (278 ± 167).

Accordingly, TLR-4 expression correlated with neither HLA–DR nor TLR-2 expression in SpA, whereas there was a strong correlation between these latter 2 markers ($r = 0.85$, $P = 0.007$). Taken together, these data indicate a specific increase of TLR-4 expression on PBMCs from patients with SpA.

### Increased expression of TLR-2 and TLR-4 on the CD163+ monocyte subset

Since findings in previous studies suggested a role for CD163+ macrophages in SpA inflammation (13–17), we investigated in more detail the expression of TLRs on CD163+ versus CD163– cells within the overall peripheral blood CD33+ monocyte population. Compared with CD163– monocytes, CD163+ monocytes in healthy controls showed clearly increased expression of TLR-2 (mean ± SD MFI 118 ± 38 versus 85 ± 16; $P = 0.011$) and TLR-4 (95 ± 55 versus 72 ± 19; $P = 0.033$). Similar findings were obtained in monocytes from patients with SpA (TLR-2 102 ± 19 versus 79 ± 19; $P = 0.012$ and TLR-4 114 ± 17 versus 102 ± 22; $P = 0.046$) and patients with RA (TLR-2 147 ± 28 versus 107 ± 22; $P < 0.001$ and TLR-4 123 ± 20 versus 103 ± 20; $P = 0.016$) (Figure 1).

In contrast to findings in studies of target tissues such as synovium and gut (13,14), the percentage of CD163+ monocytes in peripheral blood tended to be lower in patients with SpA (mean ± SD 1.2 ± 0.2%) than in healthy controls (3.6 ± 3.6%) or patients with RA (7.6 ± 6.9%). Therefore, the increased expression of TLR-4 in SpA patients compared with healthy controls was not due to an increased percentage of CD163+...
monocytes in the peripheral circulation, but rather reflected an increase in TLR-4 levels on both CD163+ and CD163− monocytes in SpA.

**Increased expression of TLR-2 and TLR-4 in SpA synovium.** The intrinsic increase of TLR-4 on monocytes from patients with SpA, the increased TLR expression on CD163+ versus CD163− cells in all 3 study cohorts, and the previously reported increase in the CD163+ macrophage subset in SpA synovitis (13,14) raised the question of the local expression of TLR-2 and TLR-4 in SpA synovium. As seen in Figure 2, immunohistochemical analysis revealed abundant cellular staining for TLRs in SpA synovial tissue. Positively staining cells were observed in both the synovial lining and sublining layers, where they were predominantly located in the perivascular regions or in close proximity to lymphocytic infiltrates. The staining pattern was similar for TLR-2 and TLR-4, with a strong correlation between levels of the 2 TLRs in the lining (r = 0.63, P = 0.001) and sublining (r = 0.60, P < 0.001). Of interest, TLR-2 and TLR-4 expression in the lining also correlated with the number of CD163+ macrophages (r = 0.40, P = 0.07 for TLR-2; r = 0.50, P = 0.02 for TLR-4), but not with CD68+ cells.

Whereas the overall staining pattern was similar in RA and OA synovium, the expression level of both...
TLRs was significantly higher in SpA than in RA or OA synovium (Table 3). Compared with RA and OA synovium, the expression of TLR-2 in SpA synovium was higher in both the lining layer and the sublining layer (both $P \leq 0.001$). In contrast to findings in peripheral blood, the degree of expression of TLR-2 was also increased in SpA synovium compared with RA and OA synovium, in both the lining and sublining layers (both $P \leq 0.001$). Whereas overall inflammatory infiltration was lower in OA than in SpA samples ($P \leq 0.001$), the absence of a significant difference between SpA and RA indicates that the increased TLR expression in SpA synovitis was not merely inflammation-related, but was also disease-specific. Moreover, comparing SpA with RA, there was no difference in the number of CD68+ macrophages (median [range] score 2.5 [0.5–3] versus 1.5 [1–3] in the lining and 2 [0–3] versus 2 [1–3] in the sublining) but an increase in the number of CD163+ macrophages (2.5 [1–3]...
versus 0 [0–2.5]; \( P = 0.001 \) in the lining and 2 [0–3] versus 0 [0–3]; \( P = 0.007 \) in the sublining), thereby providing further evidence that TLR expression was related to the CD163+ macrophage subset rather than to the degree of synovial inflammation per se.

**Down-regulation of TLR-2 and TLR-4 expression on SpA monocytes by infliximab treatment.** Given the major effect of TNFα blockade on inflammation in SpA, we next investigated whether treatment with infliximab in vivo would modulate the expression of TLRs on PBMCs in SpA. As shown in Figure 3, the expression of TLR-4 decreased gradually over a 6-week period of infliximab treatment (mean ± SD MFI 106 ± 33 at baseline, 93 ± 14 at week 2, and 78 ± 12 at week 6; \( P = 0.011 \) at week 6). Although not increased at baseline, the expression of TLR-2 also gradually declined during TNFα blockade treatment (82 ± 24 at baseline, 73 ± 26 at week 2, and 53 ± 16 at week 6; \( P = 0.003 \) at week 6), reaching an expression level 40% lower than that in healthy controls (\( P < 0.001 \)). In RA patients, analyzed as a control group in these experiments, infliximab treatment had a similar effect on monocyte expression of TLR-4 (MFI 111 ± 45 at baseline, 64 ± 23 at week 2, and 69 ± 25 at week 6; \( P = 0.019 \) at week 6) and TLR-2 (119 ± 44 at baseline, 85 ± 36 at week 2, and 82 ± 26 at week 6; \( P = 0.065 \) at week 6). Underscoring the specificity of the decrease of TLR-2 and TLR-4 during TNFα blockade in vivo, the expression of HLA–DR on monocytes did not decrease in SpA patients (MFI 303 ± 172 at baseline versus 454 ± 326 at week 6).

**Functional impairment of the TLR-4 pathway after infliximab treatment.** Since we had previously demonstrated the ability of CD163+ monocytes to produce high amounts of TNFα upon LPS stimulation (13), which could potentially be related to their high TLR-4 expression, we next investigated whether down-
Figure 4. Tumor necrosis factor (TNF) production before and after treatment with infliximab, in SpA PBMCs stimulated with either lipopolysaccharide (LPS) or phorbol 12-myristate 13-acetate (PMA), as assessed by intracellular flow cytometry. * = P = 0.002 versus pretreatment. See Figure 1 for other definitions.

Figure 5. Expression of TLR-2 and TLR-4 in the synovial membrane of SpA patients before and after 12 weeks of treatment with either infliximab (n = 8) or etanercept (n = 15). Cells that are positive for TLR-2 or TLR-4 stain red (thick arrows in a). a, Baseline TLR-2 expression in SpA synovium (score of 1 in the lining and 2.5 in the sublining). b, TLR-2 expression in SpA synovium after infliximab therapy (score of 0 in the lining and 0.5 in the sublining). c, Baseline TLR-4 expression in SpA synovium (score of 1.5 in the lining and 3 in the sublining). d, TLR-4 expression in SpA synovium after infliximab therapy (score of 0 in the lining and sublining). e, Baseline TLR-2 expression in SpA synovium (score of 3 in the lining and sublining). f, TLR-2 expression in SpA synovium after etanercept therapy (score of 0 in the lining and sublining). g, Baseline TLR-4 expression in SpA synovium (score of 2 in the lining and sublining). h, TLR-4 expression in SpA synovium after etanercept therapy (score of 0 in the lining and 0.5 in the sublining). Thin arrows in a and e indicate the synovial lining layer. (Original magnification × 320.) Corresponding graphs of individual patient scores are shown to the right of the photomicrographs. See Figure 1 for definitions.
regulation of TLR expression on monocytes by TNFα blockade in vivo would result in a functional impairment of their capacity to produce proinflammatory cytokines such as TNFα. As shown in Figure 4, activation of SpA PBMCs through the TLR-4 pathway by LPS resulted in significantly reduced TNFα production after infliximab treatment (mean ± SD MFI 179 ± 84) compared with baseline (259 ± 70) (*P* = 0.002). In contrast, TNFα production upon PMA stimulation was not affected by infliximab treatment in vivo (MFI 272 ± 174 versus 269 ± 67).

**Down-regulation of synovial TLR-2 and TLR-4 expression in SpA by infliximab treatment.** Given the increased expression of TLR-2 and TLR-4 in SpA synovium and the down-regulation of the expression and function of these TLRs on peripheral blood monocytes with infliximab treatment, we next assessed whether this effect of infliximab also extended to the inflamed SpA synovium, by analyzing synovial biopsy samples obtained at baseline and week 12. As shown in Figure 5 and Table 4, TLR-2 expression in the synovial sublining layer (*P* = 0.023) and TLR-4 expression in the lining layer (*P* = 0.034) and sublining layer (*P* = 0.018) decreased significantly after infliximab treatment. A similar trend was observed for TLR-2 expression in the synovial lining layer (*P* = 0.074).

**Down-regulation of synovial TLR-2 and TLR-4 expression in SpA by etanercept treatment.** In order to assess whether this down-regulation of synovial TLR-2 and TLR-4 expression in SpA occurs only with infliximab or is a more general class effect of TNFα blockers, we additionally investigated synovial biopsy specimens obtained at baseline and week 12 of etanercept treatment. As was observed with infliximab, there was a pronounced down-regulation of both TLR-2 and TLR-4 expression in the synovial lining layer (*P* < 0.001 for both TLRs) and in the sublining layer (*P* < 0.001 and *P* = 0.005, respectively) (Figure 5 and Table 4). The median decrease in TLR-2 and TLR-4 expression was similar in the infliximab-treated and etanercept-treated groups, although the *P* values were smaller in the latter group due to the larger number of samples (15 etanercept-treated patients versus 8 infliximab-treated patients).

**DISCUSSION**

In view of the role of bacterial triggering in the pathogenesis of SpA and our recent observations indicating a relative increase of monocyte/macrophages and polymorphonuclear cells in SpA synovitis (13–17), the present study was undertaken to explore the hypothesis that alterations in the TLR pathway could be involved in an abnormal activation of innate immunity-mediated inflammation in SpA. Since TLR-2 (which associates with TLR-1 or TLR-6) and TLR-4 recognize products of gram-positive and gram-negative bacteria, respectively, and are expressed on the cell surface of monocyte/macrophages and neutrophils, the present study focused on the expression of these 2 members of the TLR family.

A new finding of our study was that PBMCs from SpA patients have a nearly 50% increase in TLR-4 expression compared with those of healthy controls. Of interest, neither TLR-2 nor HLA–DR expression was increased on these cells, indicating that this phenomenon is not just a reflection of systemic inflammation or nonspecific activation of phagocytes. This was further emphasized by the absence of a correlation of TLR-4 expression in SpA with parameters of systemic inflammation such as C-reactive protein level and erythrocyte sedimentation rate (data not shown). Moreover, RA patients with comparable systemic inflammation exhibited a different profile, with increases of both TLR-4 and TLR-2.
TLR-4 and TLR-2 expression levels were roughly comparable with those previously reported for RA PBMCs (33,34), and similar findings were obtained when the flow cytometric data were analyzed as the percentage positive cells rather than by MFI (data not shown).

Although it is unclear which stimuli and mechanisms are responsible for the up-regulation of TLRs in vivo (50–52), differential regulation of TLR-2 and TLR-4 was recently demonstrated in septic shock as well as in RA (33,53,54). In a recent study, it appeared that TLR-4 was increased on all monocytes whereas TLR-2 was specifically up-regulated on the CD16+ subset (33). These CD16+ monocytes produce high amounts of proinflammatory cytokines such as TNF and have been implicated in the pathogenesis of RA (55–57).

Transposing this observation to SpA, we investigated whether the increase in TLR-4 expression could be observed on all phagocytes or was due to a specific subset. We focused particularly on phagocytes expressing the scavenger receptor CD163, which are increased in SpA inflammation and are also able to produce high amounts of TNFα (13,14). Consistent with the notions of a proinflammatory role of these cells and a link between TLR and scavenger receptor expression (58), the present study showed that CD163+ phagocytes expressed higher levels of TLR-2, TLR-4, and HLA–DR than their CD163– counterparts. However, this difference was observed not only in patients with SpA, but also in RA patients and healthy controls, and the fraction of CD163+ monocytes in the peripheral circulation tended to be lower in SpA. Therefore, the increased systemic TLR-4 expression in SpA could not be explained solely by the CD163+ subset, as was confirmed by the fact that, as in RA (33), TLR-4 expression was increased on all monocyte subsets in SpA patients compared with healthy controls (data not shown).

In contrast to the situation in peripheral blood, the demonstration of higher TLR-2 and TLR-4 expression on CD163+ phagocytes may be of importance with regard to tissue inflammation in SpA, since we previously demonstrated a specific increase of this macrophage subset in the synovium, as well as the gut, in patients with SpA (13–17). In RA, recent studies demonstrated the expression of TLR-2 and TLR-4 on monocytes as well as fibroblasts in the synovial lining and sublining layers (31–34). Results of the present study extend these findings by indicating not only that TLR-2 and TLR-4 are expressed with a similar pattern in SpA synovitis, but also that the expression of both TLRs is significantly higher in SpA compared with RA synovium despite a similar degree of local inflammation. Given the similar TLR-4 expression and the higher TLR-2 expression on RA versus SpA PBMCs, it is likely that the specific increase of CD163+ macrophages, rather than systemic alteration, contributes to the increased synovial TLR expression in SpA. Alternatively, it should be considered that TLR expression might also be abnormal on other cell types, such as synovial fibroblasts.

Independent of these considerations, the elevated expression in SpA and the fact that both synovial phagocytes and fibroblasts produce inflammation mediators such as cytokines and chemokines upon TLR-2 or TLR-4 stimulation (13,31,33,34,59) indicate a need for further investigation of a potential role of the TLR pathway in inducing and/or perpetuating synovial tissue inflammation in SpA. Whereas it is difficult to provide direct functional evidence in human SpA and thereby to demonstrate the biologic significance of our findings, animal studies have indicated the importance of TLR-4 in the early inflammatory cytokine response of phagocytes upon infection with SpA-associated gram-negative bacteria such as Salmonella, Yersinia, and Chlamydia (22–25,60). Accordingly, the role of bacterial products such as streptococcal cell wall and LPS in the induction and/or perpetuation of experimental arthritis is critically dependent on TLR-2 and TLR-4, respectively, and the associated adaptor molecule, myeloid differentiation factor 88 (35,36). Moreover, self molecules such as proteoglycans, which are abundant in the joint and are involved in SpA synovitis, also signal through the TLR pathway and can induce inflammatory responses in vivo (26–30,61).

In an attempt to address the functional importance of the increased TLR expression in human SpA, we investigated whether anti-TNFα therapy, which is highly effective in SpA, was associated with modulation of the expression and/or function of TLRs. Analysis of PBMCs indicated that in both RA and SpA, TLR-4 expression was significantly down-modulated by infliximab treatment over a period of 6 weeks. Consistent with the decrease in inflammatory cytokine production after down-regulation of surface TLR-4 expression in endotoxin tolerance (62), the infliximab-induced down-regulation of TLR-4 was associated with impairment of TNFα production by monocytes upon LPS stimulation. The fact that TNFα production upon PMA stimulation was unaltered is compatible with the notion of specific interference with the TLR-4 pathway, rather than a generalized hyporesponsiveness of the phagocytes. Differential regulation of the 2 pathways has previously been demonstrated with other drugs, such as indomethacin and rosiglitazone (63,64). Although TLR-2 expression on SpA phagocytes was not increased at baseline, it...
was also significantly down-regulated by infliximab treatment, resulting in lower levels than in healthy controls.

Paralleling the findings in peripheral blood, infliximab also decreased the expression of TLR-2 and TLR-4 in inflamed synovial membrane, which is probably the combined result of the systemic effect of infliximab on TLR expression and the treatment-induced reduction of inflammatory phagocyte infiltration (46,47). Moreover, the similar down-regulation of synovial TLR-2 and TLR-4 expression with etanercept treatment indicates that this is a class effect of TNFα blockers rather than an infliximab-specific mechanism.

Taken together, our results suggest that TNFα blockade in vivo interferes with the innate immune system in SpA, although this remains to be proven by functional studies. Such interference could then lead to a reduction in the systemic and local inflammation. However, the down-regulation of TLRs, and especially TLR-2, which declines to levels below those found in healthy controls, might also interfere with normal host defense. In RA, the increased susceptibility to infections with essentially intracellular pathogens during TNFα blockade treatment has previously been related to decreased TLR-4 expression and interferon-γ production by myeloid cells (40,41). The present in vivo data and our recent finding of tuberculosis reactivation and abscesses with streptococci in infliximab-treated SpA patients (37–39) are compatible with the hypothesis that TNFα blockade in vivo interferes with the normal innate immune response to pathogens and suggest that this might even be more pronounced in SpA than in RA.

In conclusion, the present findings indicate that inflammation in SpA is characterized by strongly increased levels of TLR-2 and TLR-4 expression, which are sharply reduced by TNFα blockade. These data suggest a potential role of innate immunity–mediated inflammation in SpA and could provide additional clues regarding the efficacy as well as the potential side effects of TNFα blockade in this disease. The demonstration of altered TLR-2 and TLR-4 expression on phagocytes and in inflamed synovium in SpA suggests the need for further evaluation of TLR expression and function on other synovial cell types such as fibroblasts (31,32,59) and polymorphonuclear cells (65,66), assessment of other target tissues of SpA inflammation such as the gut mucosa (67–69), analysis of the relationship to genetic risk factors such as HLA-B27 and the innate immune system–related NOD2 polymorphisms (70,71), and investigation of other TLRs such as TLR-9 (72).

ACKNOWLEDGMENTS

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REFERENCES

13. Baeten D, Demetter P, Cuvelier CA, Kruithof E, van Damme N, de Vos M, et al. Macrophages expressing the scavenger receptor CD163 in inflamed synovial membrane (31,32,59) and polymorphonuclear cells (65,66), assessment of other target tissues of SpA inflammation such as the gut mucosa (67–69), analysis of the relationship to genetic risk factors such as HLA-B27 and the innate immune system–related NOD2 polymorphisms (70,71), and investigation of other TLRs such as TLR-9 (72).
et al. Synovial histopathology of psoriatic arthritis, oligo- and polyarticular, resembles more spondyloarthropathy than rheuma-


Angiography-Negative Primary Central Nervous System Vasculitis in Children

A Newly Recognized Inflammatory Central Nervous System Disease

Susanne M. Benseler,1 Gabrielle deVeber,1 Cynthia Hawkins,1 Rayfel Schneider,1 Pascal N. Tyrrell,1 Richard I. Aviv,1 Derek Armstrong,1 Ronald M. Laxer,1 and Earl D. Silverman2

Inflammatory central nervous system (CNS) diseases in childhood comprise a wide spectrum of heterogeneous conditions. We studied 4 children with primary CNS vasculitis in whom results of magnetic resonance imaging studies were abnormal but results of conventional angiography were normal. We determined that angiography-negative, biopsy-confirmed primary small-vessel CNS vasculitis is a previously unrecognized distinct disease entity in children. The diagnosis must be considered in a child with a progressive, acquired diffuse or focal neurologic deficit, even if the results of conventional angiography are normal. A lesional brain biopsy is required to confirm the diagnosis. Use of immunosuppressive therapy plus aspirin leads to an excellent neurologic outcome.

Inflammatory diseases of the central nervous system (CNS) in children are a challenging group of disorders with regard to diagnosis, classification, and therapy. CNS inflammation can be associated with infections, malignancies, metabolic diseases, and systemic collagen vascular disorders. The anatomic distribution of inflammation within the CNS can be primarily parenchymal, as is seen in multiple sclerosis. Less frequently, the inflammatory process predominantly attacks blood vessels, as seen in CNS vasculitis (1,2). In children, primary or isolated CNS vasculitis is rare, because it is more commonly related to a generalized or systemic illness.

In adults, the diagnostic criteria for primary angiitis of the CNS (PACNS) include the following: an unexplained acquired neurologic deficit, angiographic or histologic features of CNS vasculitis, and no evidence of a systemic condition associated with these CNS findings (3). In the majority of patients with PACNS, abnormalities, albeit nonspecific, are present on cerebral angiograms (4). Brain biopsies in adult patients with PACNS frequently show granulomatous vasculitis of parenchymal and leptomeningeal arterial vessels in the brain (5); however, reports of nongranulomatous, lymphocytic CNS vasculitis have been published (6–8).

Childhood PACNS (cPACNS) has been described in the literature, in case reports and small case series (9–11). Thus far, no diagnostic criteria, disease classification, treatment regimens, and/or long-term outcome data have been reported. The diagnosis of cPACNS is based primarily on “typical angiographic findings” (10). Elective brain biopsies have rarely been performed in cases of suspected cPACNS, and biopsy specimens have been obtained predominantly from children who required the insertion of a ventriculoperitoneal shunt due to increased intracranial pressure (9) or for whom a biopsy was performed in order to exclude a malignant or infectious CNS process.

The aim of this study was to describe a new disease entity of angiography-negative primary CNS vasculitis of childhood.
METHODS

Patients. Between January 1, 1990 and December 12, 2002, the diagnosis of primary CNS vasculitis was made in 66 patients attending The Hospital for Sick Children. After approval was obtained from the Research Ethics Board (file no. 002[020023), clinical and laboratory data for all patients were obtained and reviewed. Imaging studies were reanalyzed. None of the patients had an underlying systemic disease or a known cause of vasculopathy (e.g., collagen vascular disease, hemoglobinopathy, infection, dissection, thromboembolism, moyamoya disease, or metabolic disease).

In 62 patients the diagnosis of cPACNS was based on angiographic findings, while in 4 patients the results of conventional angiography were normal. In these 4 patients the diagnosis of cPACNS was confirmed by results of an elective brain biopsy. Additional patients with angiography-negative cPACNS were sought by reviewing the results of all brain biopsies (8) that had been performed to rule out CNS vasculitis during the study interval. The inception cohort therefore included all patients with angiography-negative, biopsy-confirmed primary CNS vasculitis of childhood who were seen at our institution during the study period.

Clinical data. Clinical data, including demographic information, the medical history of the patient and his or her family, the history of the patient’s current illness, and results of detailed neurologic and rheumatologic examinations were obtained from prospectively collected, standardized assessments and entered into a designated Microsoft Access database (Microsoft, Seattle, WA). Treatment protocols, dosing, and side effects of medications were documented. Followup visits were assigned for each patient at 0, 3, 6, 12, 18, and 24 months and then yearly following diagnosis, as per the research protocol. In addition, the time of the last followup visit was determined. Treatment was given at the discretion of the treating rheumatologist. Response to treatment was noted when no further clinical and radiographic disease progression occurred and the patient showed improvement, including clinical improvement and regression of radiographic findings. Neurologic deficits were classified in standardized assessments using the previously validated Pediatric Stroke Outcome Measure (12).

Laboratory data. Laboratory studies included the following: erythrocyte sedimentation rate (ESR); complete blood cell count (CBC), including the white blood cell (WBC) differential count; determination of the levels of C-reactive protein (CRP), serum immunoglobulin, alanine aminotransferase, aspartate aminotransferase, urea, creatinine, lupus anticoagulant, and C3 and C4 complement; and urinalysis. The cerebrospinal fluid (CSF) was analyzed for cell count, protein level, and opening pressure. Autoantibody testing included antinuclear antibody, rheumatoid factor, anti–double-stranded DNA, anti-Ro, anti-La, anti-Sm, anti-RNP, antineutrophil cytoplasmic antibodies, and anticardiolipin antibodies. Viral and bacterial cultures, serology, and viral polymerase chain reaction were performed using both peripheral blood and CSF, according to the standardized institutional encephalitis workup.

Neuroimaging. All patients underwent computed tomography (CT), magnetic resonance imaging (MRI), magnetic resonance angiography (MRA), and conventional angiography at the time of diagnosis. Conventional angiography was performed as follows: after cannulation and direct injection of contrast material into the anterior and posterior circulation, images were acquired and analyzed using digital subtraction software.

During followup, MRI and MRA were performed. MRI studies included standardized T1-weighted, T2-weighted, and fluid-attenuated inversion recovery (FLAIR)–weighted sequences, and diffusion-weighted images. Gadolinium-enhanced sequences were included when available. All imaging studies were blindly analyzed by 2 radiologists, and differences were resolved by consensus. Data from these analyses were entered into a designated radiology Microsoft Access database.

Brain biopsy. CNS lesions were identified by MRI. Lesional brain biopsies were performed through open craniotomy. Biopsy specimens included the leptomeninges, the cerebral cortex, and subjacent white matter. Brain biopsy specimens were fixed in paraffin and stained with hematoxylin and eosin and Movat’s pentachrome. All biopsy specimens were assessed by electron microscopy. Immunohistochemistry staining was performed using monoclonal mouse anti-human antibodies and peroxidase. The specific antibody panel included anti-CD20 (B cell marker), anti-CD3 and anti-CD43 (T cell markers), and anti-CD45 (panlymphocytic marker).

Statistical analysis. All data, including those from the radiographic analysis, were collected and entered into 2 designated Access databases using the double data entry verification technique. Data were described as frequencies, medians with ranges, and means with standard deviations. Statistical analyses, including the chi-square test, Fisher’s exact test, and Student’s t-test, were performed in SAS (SAS Institute, Cary, NC).

CASE REPORTS

Patient 1. The patient, a previously healthy 10-year-old girl, presented with a 2-week history of left-sided facial droop and a 2-day history of weakness in the left arm and leg, with difficulty walking. The results of her general physical examination were normal. Neurologic abnormalities included a left-sided hemiparesis with decreased ipsilateral muscle strength, sensory deficit, upgoing toe reflexes, and a facial droop. No constitutional symptoms, including fatigue and fever, were present. No intercurrent illness was reported. Laboratory measures of inflammation markers, including the CBC, the ESR, and the CRP level, were normal. MRI revealed T2-weighted bright abnormalities involving both basal ganglia and the right frontal lobe, in a nonarterial distribution. Results of both MRA and conventional angiography were normal. Clinically, the
child improved spontaneously and was discharged without medications. A lumbar puncture was not performed.

Four weeks later, the patient presented with the recurrence of left-sided weakness. In addition, significant concentration difficulties, a severely compromised short-term memory, and urinary incontinence had developed (Table 1). The WBC count was elevated (25.0 × 10⁹ cells/liter), with 76% neutrophils, no bands, and 21% lymphocytes (Table 2). The lumbar puncture revealed an increased level of CSF protein (0.47 gm/liter), 9 × 10⁶ WBCs/liter (mainly lymphocytes), and an increased opening pressure (87 cm H₂O) (Table 2). A repeat MRI demonstrated new parenchymal lesions and progression of the previously documented abnormalities. Results of MRA were normal. A lesional brain biopsy of an area identified by MRI was performed.

**Patient 2.** The patient, a previously healthy 9-year-old girl, presented with uncontrolled left arm movements and a decreased level of consciousness, with a score of 6 on the Glasgow coma scale (possible range 0–15). She was intubated, ventilated, and treated with intravenous antibiotics for meningitis. The ESR was elevated, at 45 mm/hour. The WBC count was 10.6 × 10⁹ cells/liter, with 71% neutrophils, no bands, and 21% lymphocytes. The lumbar puncture showed normal levels of CSF protein and no pleocytosis. All cultures were negative. An electroencephalogram demonstrated a right frontotemporal focus, and MRI

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<th>Table 1. Clinical presentation of patients with angiography-negative childhood primary angiitis of the central nervous system</th>
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<td><strong>Patient 1</strong></td>
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<td>Preceding systemic symptoms</td>
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<td>Preceding diffuse neurologic deficit</td>
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<td>Preceding focal deficit</td>
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<td>Duration of preceding symptoms</td>
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<td>Neurologic deficit at diagnosis</td>
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<th>Table 2. Inflammation markers in patients with angiography-negative childhood primary angiitis of the central nervous system*</th>
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<td><strong>Test</strong></td>
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<td>ESR, mm/hour (normal 0–10)</td>
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<td>CRP, mg/liter (normal 0–8)</td>
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<td>Normal</td>
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<td>WBC, × 10⁹/liter (normal 4–10)</td>
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<td>At diagnosis</td>
</tr>
<tr>
<td>C3/C4 at diagnosis</td>
</tr>
<tr>
<td>C3, gm/liter (normal 0.77–1.43)</td>
</tr>
<tr>
<td>C4, gm/liter (normal 0.07–0.4)</td>
</tr>
<tr>
<td>Anticardiolipin antibodies</td>
</tr>
<tr>
<td>Lupus anticoagulant</td>
</tr>
<tr>
<td>CSF analysis at diagnosis</td>
</tr>
<tr>
<td>WBC, × 10⁹/liter (normal 0–3)</td>
</tr>
<tr>
<td>Protein, gm/liter (normal 0.15–0.4)</td>
</tr>
<tr>
<td>Opening pressure</td>
</tr>
</tbody>
</table>

* ESR = erythrocyte sedimentation rate; CRP = C-reactive protein; WBC = white blood cell differential count; CSF = cerebrospinal fluid.
revealed a right parietal parenchymal lesion. Treatment with carbamazepine was started, and the patient was discharged but continued to have focal seizures.

One month later, severe toxic epidermolysis developed, and she was treated with intravenous immunoglobulin. The patient's anticonvulsive therapy was switched to clonazepam. After another month, she was readmitted because of the increased frequency and severity of seizures plus the new onset of rightsided weakness, headache, significant concentration difficulties with decreased school performance, and fever of 38.2°C. The ESR was now elevated at 95 mm/hour, and the WBC count was raised at 20.2 × 10⁶ cells/liter, with 81% neutrophils, 1% bands, and 13% lymphocytes (Tables 1 and 2). The repeat lumbar puncture again showed a normal CSF protein level, but this time pleocytosis was present, with a WBC count of 51 × 10⁶ cells/liter (49% neutrophils, 43% lymphocytes, and 8% monocytes) (Table 2). Cultures remained negative. A repeat MRI demonstrated progression of the right parietal and temporal lesions, with evidence of a new right frontal lesion. The results of MRA and conventional angiography remained normal. A brain biopsy of the parietal lesion was performed.

Patient 3. The patient, a 16-year-old girl, presented with a 1-month history of headaches, flu-like symptoms without documented fever, significant behavior changes, and severe emotional instability. Depression had previously been diagnosed in this patient, and treatment with fluoxetine had been started. She reported poor, worsening school performance. At presentation, she showed an ataxic gait on the right side, a right gaze–evoked nystagmus, and a rightsided facial droop. Levels of inflammation markers were mildly elevated, with an ESR of 20 mm/hour, a CRP level of 45 mg/dl, and a raised WBC count of 16.3 × 10⁹ cells/liter, with 87% neutrophils, no bands, and 26% lymphocytes (Table 2). The lumbar puncture revealed significant pleocytosis (WBC count 747 × 10⁶ cells/liter, with 2% neutrophils, 74% lymphocytes, and 18% monocytes), increased CSF protein (0.75 gm/liter), and a high opening pressure (69 cm H₂O) (Table 2). The MRI demonstrated multiple focal parenchymal lesions. Results of angiography were normal. A lesional biopsy of the parietal lobe was performed.

Patient 4. The patient, a previously healthy 5-year-old girl, presented with the inability to walk due to generalized weakness, abdominal pain, and a recent generalized tonic-clonic seizure. She was afebrile. The only abnormal marker of inflammation was an elevated ESR (108 mm/hour). The WBC count was 10.4 × 10⁹ cells/liter, with 74% neutrophils, no bands, and 26% lymphocytes (Table 2). Results of a CSF examination, including cultures, were completely normal. Over the next several days, the patient's condition deteriorated rapidly, and fever, focal seizures, choreiform movements, and a progressively decreasing level of consciousness developed (Table 1). A repeat examination of the CSF showed an increased protein level (0.6 gm/liter), no pleocytosis, but a high opening pressure (69 cm H₂O) (Table 2). The MRI demonstrated multiple focal parenchymal lesions. Results of angiography were normal. A lesional biopsy of the parietal lobe was performed.

RESULTS

Clinical presentation. All 4 patients were female, with a mean age at the time of diagnosis of 10.2 years (range 5.1–16.4 years). Symptoms that occurred prior to the diagnosis, clinical features at presentation, and the duration of symptoms prior to biopsy, are shown in Table 1. Three children had nonspecific systemic symptoms (i.e., flu-like illness and low-grade fever). The fourth child was completely well until the time of her first visit. In all patients disease progressed within weeks, with worsening or acquisition of neurologic deficits. The most frequent neurologic signs and symptoms were neurocognitive dysfunction, headache, hemiparesis, and focal seizures (see Table 1).

Laboratory findings. All patients had laboratory evidence of systemic inflammation, as shown in Table 2. Repeated measurements showed that the levels of inflammation markers steadily increased during disease progression. An elevated ESR and mildly raised WBC count was seen in all of the patients. Elevated C3 levels were observed in 3 of the 4 children. Autoantibody testing was not helpful. One patient had positive anticardiolipin antibodies; no other autoantibodies were detected. Lupus anticoagulant was negative and the thrombophilia workup was normal in all patients tested. A CSF abnormality was present in all 4 patients, with an elevated level of CSF protein in 3 of 4 patients and CSF pleocytosis in 3 of 4 patients. However, results of the initial CSF examination were frequently normal. High opening pressure was seen in all 3 patients in whom it was measured.
Neuroimaging results. CT. In all 4 patients, CT was performed at the time of diagnosis. In one patient (patient 3) a focal lesion plus evidence of raised intracranial pressure were noted. The other 3 CT scans obtained at the time of diagnosis did not show any abnormalities.

MRI. In all patients, MRI revealed evidence of multifocal lesions of both gray and white matter (Table 3). Unilateral and bilateral lesions were each seen in 50% of patients each. MRI lesions were best viewed on T2-weighted images and FLAIR sequences in all patients. Abnormalities were not detected on diffusion-weighted images. Gadolinium-enhanced studies were done in only 2 patients. In one of 2 children, lesions that were positive on T2-weighted and FLAIR MRI sequences showed gadolinium enhancement. Gadolinium-enhanced studies were done in only 2 patients. In one of 2 children, lesions that were positive on T2-weighted and FLAIR MRI sequences showed gadolinium enhancement. In all patients, repeat MRI studies were performed prior to brain biopsy. MRI lesions rapidly progressed, reflecting progression of the clinical disease (Figures 1A and B). Followup MRI studies, which monitored immunosuppressive therapy, demonstrated regression of the size and/or complete resolution of CNS lesions closely correlating with resolution of clinical findings (Figure 1C). During followup, CNS lesions were consistently best viewed on T2-weighted images and FLAIR sequences. Gadolinium enhancement was no longer detectable.

Angiography. In all 4 patients, results of conventional angiography were normal. In one patient MRA had demonstrated possible CNS vessel stenosis, which was not confirmed on conventional angiography.

Findings on brain biopsy. Lesional brain biopsy specimens from all patients demonstrated histologic features similar to those of lymphocytic CNS vasculitis. A transmural lymphocytic inflammation of small and medium-sized vessels of both white and gray matter was present (Figure 2). There was no evidence of viral inclusions in neurons or glial cells (on light or electron microscopy), endothelial tubuloreticular inclusions, or white matter demyelinization. Reactive changes of the brain parenchyma, including mild to moderate gliosis, and areas of vessel obliteration were seen. Immunohistochemical staining characterized the transmural infiltrate as consisting of mainly T cells with the memory phenotype. In addition, varying numbers of macrophages, B cells, and eosinophils were present. All patients had evidence of vasculitis in the leptomeninges in addition to the brain parenchyma. Vessel wall inflammation with various degrees of perivascular mononuclear infiltrates was observed. No granulomas were seen in any of the patients.

Treatment. All patients received therapy with oral prednisone (2 mg/kg) and low-dose aspirin (3–5 mg/kg). In 2 patients, a monthly intravenous pulse of cyclophosphamide (500–750 mg/m²) was added. Another patient was treated with oral azathioprine (2 mg/kg). The treatment regimens and responses are summarized in Table 4. The dose of prednisone was tapered slowly over a minimum of 6 months. Steroid side effects observed included significant weight gain (>10% of the baseline weight), hypertrichosis, and striae distensae. Transient lymphopenia was seen in children treated

### Table 3. MRI characteristics in patients with angiography-negative childhood primary angiitis of the central nervous system

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient 1: frontotemporal lobe, basal ganglia</th>
<th>Patient 2: frontal, parietal, temporal lobes</th>
<th>Patient 3: cerebellum</th>
<th>Patient 4: parietal, temporal lobes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Multifocal</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Unilateral</td>
<td>Absent</td>
<td>Absent</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Bilateral</td>
<td>Present</td>
<td>Present</td>
<td>Absent</td>
<td>Absent</td>
</tr>
<tr>
<td>Gray matter</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>White matter</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>T1 sequences</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>T2 sequences</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>FLAIR</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal</td>
</tr>
<tr>
<td>DWI</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>Gadolinium enhancement</td>
<td>Not done</td>
<td>Present</td>
<td>Absent</td>
<td>Not done</td>
</tr>
</tbody>
</table>

* MRI = magnetic resonance imaging; T1 = T1-weighted MRI; T2 = T2-weighted MRI; FLAIR = fluid-attenuated inversion recovery imaging; DWI = diffusion-weighted images.
with intravenous cyclophosphamide pulses. No significant adverse events were noted.

Followup and outcome. Patients were followed up for a mean period of 33 months (range 14–59 months). Although in the majority of patients disease progression occurred prior to diagnosis, all patients responded to therapy. No disease relapse was seen during the study interval. Complete neurologic recovery was seen in all 4 children. The average time until recovery was 14 months (range 10–18 months).

DISCUSSION

This study describes a newly recognized distinct disease entity in the spectrum of inflammatory CNS diseases of childhood: angiography-negative, small-
vessel primary CNS vasculitis (cPACNS). A cohort of 4 patients was identified. Because cPACNS has previously been considered a disease of medium-to-large vessels, conventional angiography was the preferred diagnostic modality or gold standard for cPACNS (10). All 4 patients had clinical features suggestive of CNS vasculitis and consistent MRI findings but normal results of conventional angiography. A diagnostic lesional brain biopsy was performed, showing lymphocytic, small-vessel PACNS, a new distinct disease entity in children.

Clinical findings at the time of diagnosis of adult CNS vasculitis have been reported to include a wide spectrum of focal neurologic deficits (13). This clinical variability likely reflects the differing localization, size, and pathology of the affected CNS vessels (10). Our experience of rapidly progressing neurologic deficits including the addition of new and/or worsening of preexisting deficits is supported by the pediatric literature and is in contrast to a more indolent course reported in biopsy-proven granulomatous vasculitis as reported in the adult literature (4,9). In the pediatric cases of CNS vasculitis reported in the literature, disease progression was most commonly rapid and led to a fatal outcome (9,14). Our cohort was small and therefore may not capture the entire spectrum of clinical features at the time of diagnosis of small-vessel cPACNS. However, the data suggest that the development of newly acquired focal and, more importantly, diffuse neurologic deficits with rapid progression in a previously healthy child without evidence of any infection or malignancy should lead to the clinical suspicion of small-vessel cPACNS.

The clinical presentation of rapidly progressing acquired neurologic deficits suggests an inflammatory CNS lesion. All patients in our series had elevated levels

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**Table 4.** Treatment and outcome of patients with angiography-negative childhood primary angitis of the central nervous system*

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Patient 1</th>
<th>Patient 2</th>
<th>Patient 3</th>
<th>Patient 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Oral steroids</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>Immunosuppressive agent</td>
<td>IV pulse cyclophosphamide</td>
<td>Ongoing</td>
<td>Oral azathioprine</td>
<td>None</td>
</tr>
<tr>
<td>Duration of immunosuppression</td>
<td>Ongoing</td>
<td>None</td>
<td>None</td>
<td>6 months</td>
</tr>
<tr>
<td>Time of last followup</td>
<td>14 months</td>
<td>22 months</td>
<td>32 months</td>
<td>59 months</td>
</tr>
<tr>
<td>Relapse</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Neurologic deficit</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
</tbody>
</table>

* IV = intravenous.
of nonspecific inflammation markers at the time of brain biopsy. However, it is important to note that initially the results of these tests were frequently normal. CSF testing was an excellent diagnostic tool. At the time of diagnosis, 100% of the patients had abnormal results of CSF testing. However, as was seen with the inflammation markers in the peripheral blood, results of the CSF examination were frequently normal at the initial presentation. Opening pressure measurements during lumbar puncture were abnormal in all 3 patients tested. The diagnostic value, sensitivity, and specificity of the opening pressure measurement have to be determined in future studies.

Abnormal levels of systemic inflammation markers are infrequently reported in adult PACNS (3,15) and in the limited literature of pediatric PACNS (10,16). However, CSF abnormalities are common in adult PACNS (13,17), whereas CSF data from previous pediatric case reports are controversial (14,18,19).

Investigations in patients with the new onset of significant cognitive dysfunction include CT, MRI, and angiography. CT failed to demonstrate any abnormality in 75% of the patients. CT has been shown to be less sensitive than MRI in adult PACNS (17). Calabrese demonstrated a sensitivity of MRI approaching 100% in biopsy-confirmed cases of PACNS (20,21). All study patients showed multifocal parenchymal lesions involving both gray and white matter. Both unilateral and bilateral CNS involvement was seen. New lesions were primarily identified on T2 sequences but also demonstrated enhancement on FLAIR images, supporting their hyperemic, inflammatory character (22). In addition, in sequential studies of individual patients, FLAIR sequences closely correlated with clinical disease activity. Clinical disease progression was associated with the extension of preexisting lesions and/or development of new lesions on FLAIR sequences. In addition, lesions demonstrated on FLAIR sequences vanished rapidly with clinical improvement and response to treatment.

Two distinct histologic types of CNS vasculitis have been described in adults: lymphocytic and, more frequently, granulomatous vasculitis. The histologic characteristics of granulomatous CNS vasculitis include a transmural infiltrate with well-defined granulomas, evidence of giant cells, and areas of vessel wall necrosis (23,24). Lymphocytic, nongranulomatous vasculitis is characterized by a transmural, predominantly T cell and B cell infiltrate, without evidence of granulomas (14,19). However, most patients have positive results of angiography, and few case reports of angiography-negative CNS lymphocytic vasculitis have been published in the adult literature (6,7).

All patients in our cohort demonstrated similar histologic features of lymphocytic vasculitis with intramural mononuclear lymphocytic infiltration of the small muscular arterial vessels. Both brain parenchymal vessels and leptomeningeal vessels were affected. The intramural infiltrate consisted of predominantly T cells and B cells, with various amounts of macrophages and some eosinophils. The degree of perivascular infiltration as well as reactive gliosis varied between patients. No granulomatous vasculitis was seen. It is possible that if it is left untreated, the lesion in patients with angiography-negative CNS vasculitis may progress to involve larger vessels that may be detected by angiography. However, there is no evidence in the literature to support this hypothesis, and we would suggest that a brain biopsy is indicated at the earliest time possible in order to confirm the suspected diagnosis of cPACNS.

The published literature on the treatment response in and outcome of cPACNS is limited. Earlier reports suggested a poor prognosis (14,18,24–26). More recent case reports have described a good response to steroids plus cyclophosphamide (9,10). In a recent cohort study of 41 patients with adult PACNS, a relapse rate of 29% and an overall favorable outcome in 80% of patients were reported (27). In our study, all patients were treated with high-dose prednisone and low-dose aspirin. Three children received additional immunosuppressive agents (cyclophosphamide, azathioprine). Treatment led to rapid clinical improvement. Diffuse neurologic deficits improved within weeks to months, levels of inflammation markers normalized rapidly, and MRI FLAIR lesions vanished. None of the patients had persistently elevated levels of inflammation markers at the 3-month followup visit. The response of focal neurologic deficits to therapy was slower. This delayed recovery likely reflects ischemic CNS damage as opposed to persistent inflammatory lesions.

Angiography-negative PACNS is a new distinct disease entity in childhood. The diagnostic algorithm includes 1) clinical evidence of acquired focal and/or diffuse neurologic deficits in a previously healthy child, with rapid worsening over weeks; 2) elevated levels of inflammation markers in the blood and CSF, potentially requiring repetitive testing; 3) multifocal lesions on MRI with enhancement on T2 and FLAIR sequences; 4) normal results of conventional angiography; and 5) confirmation of the suspected diagnosis by results of brain and leptomeningeal biopsy demonstrating intramural CNS vessel infiltrates.
We suggest that angiography-negative small-vessel cPACNS should be included in the differential diagnosis of inflammatory diseases of the CNS. Early recognition of this new disease entity may be important, because in our study, treatment was associated with a favorable outcome.

REFERENCES

Damage Caused by Wegener’s Granulomatosis and Its Treatment

Prospective Data From the Wegener’s Granulomatosis Etanercept Trial (WGET)

Philip Seo,1 Yuan-I. Min,1 Janet T. Holbrook,1 Gary S. Hoffman,2 Peter A. Merkel,3 Robert Spiera,4 John C. Davis,5 Steven R. Ytterberg,6 E. William St.Clair,7 W. Joseph McCune,8 Ulrich Specks,6 Nancy B. Allen,7 Raashid A. Luqmani,9 and John H. Stone,1 for the WGET Research Group

Objective. To analyze damage occurring in patients with Wegener’s granulomatosis (WG) enrolled in the WG Etanercept Trial (WGET) and to correlate that damage with disease activity, adverse events, and quality of life.

Methods. The Vasculitis Damage Index (VDI) was applied to all 180 patients at trial entry and every 6 months throughout the trial. Items of damage were analyzed by presumed etiology (i.e., secondary to WG, to therapy, or both) and time of occurrence. Spearman’s rank correlation coefficients were calculated between VDI scores and the Birmingham Vasculitis Activity Score for WG (BVAS/WG), frequency of flares, number of adverse events, and the patients’ quality-of-life assessments.

Results. The mean VDI score was 1.3 at the study enrollment and 1.8 at the end of the trial. This increase was due to damage that occurred despite (or because of) therapy, including visual impairment, hearing loss, nasal blockade, pulmonary fibrosis, hypertension, renal insufficiency, peripheral neuropathy, gonadal failure, and diabetes mellitus. Only 11% of the enrolled patients had not sustained a single VDI item after 1 year of enrollment. When adjusted for baseline VDI, the baseline BVAS/WG correlated moderately well with the VDI score at 1 year ($r$ = 0.20, $P$ = 0.015). Increases in adjusted VDI scores also correlated with the number of adverse events, particularly among patients with limited WG ($P$ = 0.06).

Conclusion. Damage from both active disease and its treatment remain important problems for patients with WG. Despite the dramatic improvements in patient survival achieved over the last several decades, only a few patients with WG emerge from a period of active disease without sustaining some damage from the disease itself, its treatment, or both. An important measure.

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of future therapeutic approaches will be their ability to reduce the damage accrued over time.

Until the introduction of cyclophosphamide-based regimens, generalized Wegener’s granulomatosis (WG) was nearly always fatal within 1 year of diagnosis (1,2). With the prompt institution of current standard therapies, patients now rarely die of uncontrolled disease, and the majority achieve disease remissions (3,4). Unfortunately, relapse remains the rule for WG, and only a few patients have durable remissions (3,4). Moreover, disease remission is frequently accompanied by substantial treatment-associated morbidity (3,4).

In the longitudinal WG cohort from the National Institutes of Health, 86% of patients experienced permanent damage as a consequence of the disease, including end-stage renal disease, chronic pulmonary dysfunction, diminished hearing, destructive sinus disease, saddle nose deformities, proptosis, and blindness (3). The side effects of standard therapies for WG were also substantial in that cohort: 42% of patients experienced permanent treatment-related morbidity, including chemical (drug-induced) cystitis, osteoporotic fracture, bladder cancer, myelodysplasia, and avascular necrosis (3).

Because death from overwhelming disease is no longer the inevitable result of WG, damage from both the disease and its treatment is an important measure by which to assess the effects of new therapies. In 1997, Exley and colleagues (5) developed the Vasculitis Damage Index (VDI), the first instrument designed to standardize the clinical assessment of damage related to systemic vasculitis. Intended for use in all forms of systemic vasculitis, the index consists of 64 items chosen by the Birmingham Vasculitis Group as being representative of the damage inflicted by vasculitis or its therapy. Since its development, the VDI has been used in several multicenter clinical investigations in vasculitis (including randomized clinical trials in both Europe and the US), but there has been little reexamination of the instrument.

The Wegener’s Granulomatosis Etanercept Trial (WGET) is a multicenter double-blind trial of 180 patients with WG randomized to receive either etanercept or placebo in addition to standard-of-care therapies (6–8). The results of this trial do not support the use of etanercept for the treatment of WG (8). There were no differences between the etanercept and control groups in the percentages of patients achieving either sustained remissions or sustained periods of low disease activity. Moreover, there were no statistically significant differences in the time required to achieve these measures. Because etanercept does not alter the expression of this disease, the WGET cohort provides an excellent opportunity to study the progression of damage in a large, well-defined population of patients with WG.

The WGET cohort is the largest reported group of WG patients ever assembled for a prospective research protocol. In the WGET, a modified VDI was performed at baseline and prospectively every 6 months to record the damage accrued by the trial subjects. We report data related to the use of the VDI in the WGET, examining the relationship of the VDI to disease activity, adverse events, and quality of life in several WGET patient subsets. We also propose ways in which damage assessment could be modified for use in future clinical trials of WG and related diseases.

PATIENTS AND METHODS

Trial design. The design of the WGET, clinical characteristics of the patients at baseline, and primary trial results have been described in detail elsewhere (6–8). All 180 patients in the WGET cohort met at least 2 of the 5 modified American College of Rheumatology criteria for the classification of WG (6) and had a minimum Birmingham Vasculitis Activity Score for Wegener’s Granulomatosis (BVAS/WG) of 3 (9). For the purpose of assigning standard therapies, patients were classified as having either severe or limited disease. Severe WG, denoting disease that threatened a patient’s life or the function of a vital organ at the time of trial entry, required treatment with cyclophosphamide and glucocorticoids. Patients with limited WG, denoting disease that did not threaten the patient’s life or the function of a vital organ, were treated with methotrexate and glucocorticoids. Trial visits for the purpose of data collection occurred at baseline, 6 weeks after randomization, 3 months after randomization, and then every 3 months until the common closeout date (September 30, 2003, which was 1 year after randomization of the last patient).

Disease assessments. Disease activity was scored with the BVAS/WG (9). Patients’ quality of life was self-reported on the Short-Form 36 version 2 (SF-36 v. 2) health survey (10). Adverse events were graded by the investigators according to a modified National Cancer Institute Toxicity Grading Scale (11). Data relating to the VDI were collected at baseline and every 6 months thereafter.

The Vasculitis Damage Index. In its original conception, 4 major principles guided the use of the VDI (5). First, the index was intended to reflect all medical events that occurred after the appearance of the first symptoms attributable to the underlying vasculitis, whether or not the event was clearly attributable to the underlying vasculitis or its treatment. Second, damage must be scored independently of disease activity on the VDI, even though damage is often the result of previously active disease. Third, damage was defined as a scar (i.e., not active disease) that had been present for a minimum of 3 months. Finally, damage is, by definition, irreversible.
**Figure 1.** The Vasculitis Damage Index data collection form used in the Wegener's Granulomatosis Etanercept Trial (WGET). ENT = ear, nose, and throat; BP = blood pressure; GFR = glomerular filtration rate (≤50% of premorbid baseline).
Thus, the VDI does not decrease with time, even if evidence of a previously scored item of damage becomes difficult to detect.

Damage items attributable directly to vasculitis (rather than consequences of therapy) comprise the majority of items in the VDI and are found in all 11 organ system subcategories. The subcategories of ear/nose/throat (ENT), renal, gastrointestinal, and peripheral vascular disease are comprised entirely of items attributable to the underlying vasculitis. Items of damage that are likely to be related to complications of treatment (e.g., alopecia, cataracts, gonadal failure, and chemical cystitis) are interspersed throughout the VDI in the appropriate organ system subcategories.

Differences in application from the original VDI.

There were 3 differences in the rules by which the instrument was applied to the WGET cohort. First, the WGET investigators chose to score as damage only items that were clearly related either to WG or to its treatment (and not to intercurrent events). This approach prevented the scoring of items that were not related even indirectly to WG. Second, because some potential damage items reverse within several weeks, the WGET Research Group required that an item of damage be present for at least 6 months before it was scored. Finally, the items “absent peripheral pulses in 2 or more limbs” was omitted, leaving a total of 64 items of damage (Figure 1). The clinic research coordinators and study physicians were trained in the use of the VDI. A complete item glossary, which is included in the WGET Manual of Operations (version 4.0) and is available on the Internet at http://www.vasculitis.med.jhu.edu, is essentially identical to that published by Exley et al (6).

Statistical analysis. We analyzed the frequencies with which each item in the VDI were scored by the investigators, both in aggregate and when classified by presumptive etiology (i.e., the vasculitis itself versus therapy), which was determined by consensus of the Steering Committee. Items of damage believed to be caused by some combination of WG and its therapy were placed in a third category for analysis. We also evaluated the number of damage items that were attributable directly to WG, those that were assigned more appropriately to an effect of treatment, and those that likely resulted from a combination of these 2 factors. Because most patients (153 patients [85% of the overall cohort]) were followed up for at least 1 year, VDI scores at 1 year were used for comparisons with other disease assessments. The other disease assessments included disease activity at study entry, the number of disease flares during the trial, the number of adverse events, the number of adverse events categorized by the investigators as being either severe or life-threatening (grades 3 or 4 [11]), and patients’ assessments of their quality of life (evaluated at 1 year using the physical and mental component scores of the SF-36 v. 2 health survey [10]). Trends in VDI scores were evaluated in the subgroup of patients with a minimum of 2 years of followup (97 patients [54% of the overall cohort]).

Spearman’s correlation coefficients were used to assess linear associations between VDI scores at 1 year, baseline disease activity, and other outcome variables. Spearman’s correlation coefficients were calculated for 3 subsets of the WGET cohort: patients with severe versus limited disease, patients newly diagnosed versus those previously diagnosed (i.e., those who had been diagnosed and treated for at least 1 disease flare prior to entry into the trial), and experimental treatment group assignment (etanercept versus placebo). None of the correlation coefficients calculated were significantly influenced by treatment group assignment. Mean numbers of severe adverse events, mean numbers of flares, and the odds of having at least 1 severe flare of disease during the first year of followup were compared by VDI score (i.e., 0, 1, 2, 3, ≥4) at 1 year using either analysis of variance or logistic regression models, as appropriate. All analyses were adjusted for baseline VDI scores. Baseline BVAS/WG was also adjusted when evaluating the association of VDI scores at 1 year with other variables.

RESULTS

Characteristics of the WGET cohort. Between June 9, 2000 and September 30, 2002, 180 patients with WG were enrolled in the WGET. The baseline features of the WGET cohort are shown in Table 1. Of the 180

<table>
<thead>
<tr>
<th>Feature</th>
</tr>
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<tbody>
<tr>
<td>Result</td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Demographic characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age at onset of symptoms, mean ± SD years</td>
</tr>
<tr>
<td>Sex, %</td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td>Ethnicity, %</td>
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<td>Hispanic</td>
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<td>Time since diagnosis, %</td>
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<td>Median no. of months</td>
</tr>
<tr>
<td>Total patient-years</td>
</tr>
<tr>
<td>Time since diagnosis, %</td>
</tr>
<tr>
<td>Total patient-years</td>
</tr>
<tr>
<td>BVAS for WG, mean ± SD score*</td>
</tr>
<tr>
<td>Physician’s global assessment, mean ± SD</td>
</tr>
<tr>
<td>Patient’s global assessment, mean ± SD</td>
</tr>
<tr>
<td>Vasculitis Damage Index</td>
</tr>
<tr>
<td>Mean ± SD</td>
</tr>
<tr>
<td>Median (quartile 1, quartile 3)</td>
</tr>
<tr>
<td>Minimum/maximum</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Damage at baseline, by organ system, % of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Musculoskeletal</td>
</tr>
<tr>
<td>Skin/mucous membranes</td>
</tr>
<tr>
<td>Ocular</td>
</tr>
<tr>
<td>Ear, nose, throat</td>
</tr>
<tr>
<td>Pulmonary</td>
</tr>
<tr>
<td>Cardiovascular</td>
</tr>
<tr>
<td>Peripheral vascular disease</td>
</tr>
<tr>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>Renal</td>
</tr>
<tr>
<td>Neuropsychiatric</td>
</tr>
<tr>
<td>Other</td>
</tr>
</tbody>
</table>

* BVAS for WG = Birmingham Vasculitis Activity Score for Wegener’s granulomatosis.

Table 1. Baseline characteristics of the study population
Table 2. Damage in the Wegener’s Granulomatosis Etanercept Trial cohort

<table>
<thead>
<tr>
<th>Items of damage</th>
<th>%</th>
<th>“Other” items</th>
<th>%</th>
<th>Unused Items</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hearing loss</td>
<td>25.6</td>
<td>Lung nodule</td>
<td>3.3</td>
<td>Easy bruising</td>
</tr>
<tr>
<td>Proteinuria ≥0.5 gm/24 hours</td>
<td>18.9</td>
<td>Striae</td>
<td>3.3</td>
<td>Glaucoma</td>
</tr>
<tr>
<td>Nasal blockade/chronic discharge/crusting</td>
<td>17.8</td>
<td>Anxiety</td>
<td>2.2</td>
<td>Glottic stenosis</td>
</tr>
<tr>
<td>Nasal bridge collapse/septal perforation</td>
<td>17.8</td>
<td>Weight gain</td>
<td>2.2</td>
<td>Hypopituitarism</td>
</tr>
<tr>
<td>GFR ≤50% of premorbid baseline</td>
<td>17.8</td>
<td>Depression</td>
<td>2.2</td>
<td>Hypothyroidism</td>
</tr>
<tr>
<td>Subglottic stenosis</td>
<td>17.8</td>
<td>Bilateral tympanic membrane scarring</td>
<td>2.2</td>
<td>Ageusia</td>
</tr>
<tr>
<td>Chronic sinusitis/radiologic damage</td>
<td>12.2</td>
<td>Fibromyalgia</td>
<td>2.2</td>
<td>Palse defect</td>
</tr>
<tr>
<td>Diastolic hypertension</td>
<td>9.4</td>
<td>Nasolacrical duct obstruction</td>
<td>2.2</td>
<td>Vasculitic neuropathy</td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>7.2</td>
<td>Proposis</td>
<td>2.2</td>
<td>Corneal scarring</td>
</tr>
<tr>
<td>Diabetes mellitus</td>
<td>7.2</td>
<td>Scleral scarring/thinning</td>
<td>2.2</td>
<td>Overwhelming fatigue</td>
</tr>
<tr>
<td>Significant muscle atrophy/weakness</td>
<td>7.2</td>
<td>Bone marrow hypoplasia</td>
<td>0.5</td>
<td>Pulmonary artery stenosis</td>
</tr>
<tr>
<td>Impaired lung function</td>
<td>7.2</td>
<td>Breast deformity from WG</td>
<td>0.5</td>
<td>Pulmonary infiltrate</td>
</tr>
<tr>
<td>Chronic breathlessness</td>
<td>6.7</td>
<td>Carcinoma in situ, vulva</td>
<td>0.5</td>
<td>Renal transplantation</td>
</tr>
<tr>
<td>End-stage renal disease</td>
<td>6.7</td>
<td>Chronic episcleritis</td>
<td>0.5</td>
<td>Right ventricular hypertrophy</td>
</tr>
<tr>
<td>Cataract</td>
<td>6.1</td>
<td>Chronic endobronchial dysfunction</td>
<td>0.5</td>
<td>Right ventricular hypertension</td>
</tr>
<tr>
<td>Osteoporosis/vertebral collapse</td>
<td>5.0</td>
<td>Eustachian tube dysfunction</td>
<td>0.5</td>
<td>Rotator cuff tear</td>
</tr>
<tr>
<td>Gonadal failure</td>
<td>5.0</td>
<td>Testicular atrophy</td>
<td>0.5</td>
<td>Searring on chest radiograph</td>
</tr>
<tr>
<td>Alopecia</td>
<td>4.4</td>
<td>Chronic rhinitis</td>
<td>0.5</td>
<td>Tinnitus</td>
</tr>
<tr>
<td>Visual impairment/diplopia</td>
<td>3.9</td>
<td>Coronary artery bypass</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Blindness in 1 eye</td>
<td>3.9</td>
<td>Cold sensitivity</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Malignancy</td>
<td>3.3</td>
<td>Systolic hypertension</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Cranial nerve lesion</td>
<td>2.8</td>
<td>Diabetes insipidus</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Chemical cystitis</td>
<td>2.2</td>
<td>Erectile dysfunction</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Pleural fibrosis</td>
<td>2.2</td>
<td>Deep venous thrombosis</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>Complicated venous thrombosis</td>
<td>2.2</td>
<td>Cutaneous ileostoma</td>
<td>0.5</td>
<td></td>
</tr>
</tbody>
</table>

* Diastolic hypertension is defined as ≥95 mm Hg or hypertension requiring antihypertensive agents. GFR = glomerular filtration rate; WG = Wegener’s granulomatosis.
patients, 60.0% were male and 92.2% were white. The mean age at the time of first symptom onset in the WGET cohort was 47 years. One hundred twenty-eight patients (71.1%) had severe disease at the time of enrollment and 52 (28.9%) had limited disease. Eighty of the patients (44.4%) were newly diagnosed with WG at the time of trial entry; 100 (55.6%) had been treated before trial entry for at least 1 episode of active WG.

**Baseline and final VDI scores.** The mean baseline VDI score, reflecting damage already present at enrollment, was 1.3. The ENT (40.2%) and the kidneys (15.5%) were the most frequently damaged organ systems at the time of enrollment (Table 1). Twenty of the 180 patients (11%) had VDI scores of 0 both at baseline and at 1 year of followup. The baseline VDI score among patients with limited disease was 1.5, compared with 1.3 among those with severe disease ($P = 0.21$). As reported previously (7), patients with limited disease tended to have longer pre-WGET disease durations and a higher likelihood of pretreatment courses for active disease. The mean VDI score increased to 1.8 by the time of the 1-year followup visit. At the end of the trial (median followup 25 months; range 5–38 months), the average VDI score was 2.0.

**Most frequently recorded damage items.** The 25 most commonly scored cumulative damage items in the WGET are shown in Table 2. The first 15 items were reported by more than 5% of patients in the WGET cohort. The most frequently recorded item was hearing loss, which was reported by 46 patients (25.6%). The VDI does not distinguish among conductive, sensorineural, and mixed causes of hearing loss. Proteinuria ($\geq 0.5$ gm/24 hours), the second most commonly observed damage item, was found in 34 patients (18.9%). Nasal blockage/chronic discharge/crusting, nasal bridge collapse/septal perforation, glomerular filtration rate $\leq 50\%$ of the patient’s premorbid baseline level, and subglottic stenosis (with or without surgical intervention) were each seen in 32 patients (17.8%). Chronic sinusitis/radiologic damage, hypertension ($\geq 95$ mm Hg diastolic or requiring antihypertensive agents), pulmonary fibrosis, diabetes mellitus, significant muscle atrophy or weakness, impaired lung function, chronic breathlessness, end-stage renal disease, cataract, osteoporosis/vertebral collapse, and gonadal failure were all reported in 5–10% of patients. The remaining 43 items were seen infrequently; 11 were seen in only 1 patient each. Sixteen of the 64 items (25%) were not recorded for any patient at any visit (Table 2).

**Additional items of damage.** The category “other,” which included items seen in a total of 42 patients, was comprised of a total of 43 different damage items (Table 2). Ten such items were seen in 2 or more patients, including psychiatric conditions (i.e., anxiety and depression), the direct consequences of disease (i.e., bilateral tympanic membrane scarring, lung nodule, nasolacrimal duct obstruction, proptosis, and scleral scarring or thinning), the consequences of therapy (i.e., weight gain and striae); and fibromyalgia. A complete list of additional items of damage found among patients in the WGET is shown in Table 2.

**Damage items attributable to WG.** Complications of WG itself accounted for 362 (73%) of the 496 items scored. Items of damage directly attributable to the effects of WG also accounted for 30 of 73 (41%) of the items reported in the “other” category in Figure 1. The most numerous contributions to the VDI score for patients in the WGET cohort were made by the ENT and renal subcategories, which accounted for 149 of 496 (30%) and 78 of 496 (16%) of the items scored, respectively.

**Damage items attributable to treatment.** Items of damage caused by treatment were responsible for 72 of 496 (15%) of all items recorded in the VDI. Items attributable to a combination of effects of both treatment and the disease accounted for 13% of all items. The most common treatment-related items were diabetes mellitus and gonadal failure, which were found in 6.7% and 5.0% of the patients enrolled in the WGET cohort, respectively. A number of “other” items were also related to treatment: weight gain, striae, easy bruising, glaucoma, erectile dysfunction, and testicular atrophy (Table 3).

**Association between damage and disease activity.** When adjusted for the baseline VDI score, the correlation between the baseline BVAS/WG and the VDI score at 1 year was moderate ($r = 0.20$, $P = 0.015$). This correlation was not significantly different across any of the 3 cohort subsets evaluated: severe versus limited disease at enrollment ($r = 0.079$ versus $r = 0.12$; $P = 0.82$), newly diagnosed versus previously diagnosed disease ($r = 0.23$ versus $r = 0.06$; $P = 0.28$), or etanercept versus placebo ($r = 0.018$ versus $r = 0.19$; $P = 0.90$).

**Association between damage and disease flares.** The VDI score at 1 year was associated positively with the number of flares recorded during the first year of followup in the WGET. When adjusted for baseline VDI and baseline BVAS/WG scores, patients with higher VDI scores at 1 year had more flares during the first year.
Table 3. Items of damage, classified by presumptive etiology*

<table>
<thead>
<tr>
<th>Damage due to vasculitis</th>
<th>Damage due to therapy</th>
<th>Damage due to vasculitis and/or therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deforming/erosive arthritis</td>
<td>Esophageal stricture/surgery</td>
<td>Angina/angioplasty</td>
</tr>
<tr>
<td>Cutaneous ulcers</td>
<td>GFR ≥50% of premorbid baseline</td>
<td>Myocardial infarction</td>
</tr>
<tr>
<td>Mouth ulcers</td>
<td>Proteinuria ≥0.5 gm/24 hours</td>
<td>Subsequent myocardial infarction</td>
</tr>
<tr>
<td>Retinal change</td>
<td>End-stage renal disease</td>
<td>Cardiomyopathy</td>
</tr>
<tr>
<td>Optic atrophy</td>
<td>Seizures</td>
<td>Valvular disease</td>
</tr>
<tr>
<td>Visual impairment/diplopia</td>
<td>Cranial nerve lesion</td>
<td>Pericarditis ≥3 months/pericardiectomy</td>
</tr>
<tr>
<td>Blindness in 1 eye</td>
<td>Peripheral neuropathy</td>
<td>Diastolic hypertension</td>
</tr>
<tr>
<td>Blindness in second eye</td>
<td>Bilateral tympanic membrane scarring†</td>
<td>Cognitive impairment</td>
</tr>
<tr>
<td>Orbital wall destruction</td>
<td>Breast deformity from WG†</td>
<td>Major psychosis</td>
</tr>
<tr>
<td>Hearing loss</td>
<td>Chronic endobronchial dysfunction†</td>
<td>Cerebrovascular accident</td>
</tr>
<tr>
<td>Nasal blockade/chronic discharge/crusting</td>
<td>Chronic episcleritis†</td>
<td>Second cerebrovascular accident</td>
</tr>
<tr>
<td>Nasal bridge collapse/septal perforation</td>
<td></td>
<td>Transverse myelitis</td>
</tr>
<tr>
<td>Chronic sinusitis/radiologic damage</td>
<td>Diabetes insipidus†</td>
<td>Osteoporosis/vertebral collapse</td>
</tr>
<tr>
<td>Subglottic stenosis</td>
<td>Eustachian tube dysfunction†</td>
<td>Cataract</td>
</tr>
<tr>
<td>Pulmonary hypertension</td>
<td>Glottic stenosis†</td>
<td>Anxiety†</td>
</tr>
<tr>
<td>Pulmonary fibrosis</td>
<td>Ageusia†</td>
<td>Carcinoma in situ, vulva†</td>
</tr>
<tr>
<td>Pulmonary infarction</td>
<td>Lung nodule†</td>
<td>Coronary artery bypass†</td>
</tr>
<tr>
<td>Pleural fibrosis</td>
<td>Nasolacrimal duct obstruction†</td>
<td>Cutaneous ileostoma†</td>
</tr>
<tr>
<td>Chronic asthma</td>
<td>Scarring on chest radiograph†</td>
<td>Depression†</td>
</tr>
<tr>
<td>Chronic breathlessness</td>
<td>Corneal scarring†</td>
<td>Fibromyalgia†</td>
</tr>
<tr>
<td>Impaired lung function</td>
<td>Propotis†</td>
<td>Glaucoma†</td>
</tr>
<tr>
<td>Absent pulses in 1 limb</td>
<td>Pulmonary artery stenosis†</td>
<td>Hypopituitarism†</td>
</tr>
<tr>
<td>Second episode of absent pulses in 1 limb</td>
<td>Pulmonary infiltrate†</td>
<td>Hypothyroidism†</td>
</tr>
<tr>
<td>Major vessel stenosis</td>
<td>Renal transplantation†</td>
<td>Palate defect†</td>
</tr>
<tr>
<td>Claudication ≥3 months</td>
<td>Scleral scarring/thinning†</td>
<td>Right ventricular hypertension†</td>
</tr>
<tr>
<td>Minor tissue loss</td>
<td>Tinnitus†</td>
<td>Right ventricular hypertrophy†</td>
</tr>
<tr>
<td>Major tissue loss</td>
<td>Vascular neuropathy†</td>
<td>Rotator cuff tear†</td>
</tr>
<tr>
<td>Subsequent major tissue loss</td>
<td>Deep venous thrombosis†</td>
<td>Cold sensitivity†</td>
</tr>
<tr>
<td>Complicated venous thrombosis</td>
<td></td>
<td>Systolic hypertension†</td>
</tr>
<tr>
<td>Gut infection/resection</td>
<td></td>
<td>Weight gain†</td>
</tr>
<tr>
<td>Mesenteric insufficiency/pancreatitis</td>
<td></td>
<td>Easy bruisingability†</td>
</tr>
<tr>
<td>Chronic peritonitis</td>
<td></td>
<td>Overwhelming fatigue†</td>
</tr>
</tbody>
</table>

* Diastolic hypertension is defined as ≥95 mm Hg or hypertension requiring antihypertensive agents. GFR = glomerular filtration rate; WG = Wegener's granulomatosis.
† Item of damage was scored by investigators under the “other” category (item 17f on the Wegener’s Granulomatosis Etanercept Trial Vasculitis Damage Index form).
of followup (Table 4). Patients with a 1-year VDI score of 0 had a mean ± SD of 0.47 ± 0.17 flares during the first year. In contrast, those with a 1-year VDI score of 2 had a mean of 1.27 ± 0.19 flares. A higher VDI score at 1 year was associated with greater odds of having at least 1 severe flare during the previous year (Table 5). Compared with patients with a VDI score of 0, the adjusted odds ratio associated with having a severe flare in patients with a VDI score of 1 was 1.32 (95% confidence interval [95% CI] 0.37–4.69). In contrast, the adjusted odds ratio of having a severe flare in patients with a VDI score of ≥4 at 1 year had a mean of 3.24 severe adverse events (P < 0.001).

Association between damage and adverse events. When adjusted for both the VDI and BVAS/WG scores at baseline, the VDI at 1 year correlated moderately with the total number of adverse events that occurred during the first year of followup among patients with limited disease, but not among patients with severe disease (r = 0.24 versus r = −0.095; P = 0.06). The correlation between the VDI scores at 1 year and the number of adverse events by new diagnosis of WG versus previous diagnosis of WG was not significantly different (r = −0.048 versus r = 0.13; P = 0.28). The number of severe adverse events recorded during the first year of followup was associated positively with the VDI scores at 1 year. After adjustments for baseline VDI and BVAS/WG scores, patients with a VDI score of 0 at 1 year had a mean of 0.57 severe adverse events. In comparison, patients with a VDI score of ≥4 at 1 year had a mean of 3.24 severe adverse events (P < 0.001).

Association between damage and quality of life. The VDI at 1 year correlated inversely with the SF-36 v. 2 physical component score at 1 year (r = −0.31, P < 0.0001). This association was not significantly different when analyzed either by time of diagnosis (i.e., new diagnosis versus previous diagnosis r = −0.23 versus r = 0.31; respectively; P = 0.45) or by disease severity (i.e., limited versus severe disease r = −0.48 versus r = −0.24; P = 0.12). The overall correlation between VDI scores at 1 year and the SF-36 v. 2 mental component score at 1 year was more pronounced among patients with limited disease than among those with severe disease (r = −0.45 versus r = 0.059; P < 0.01).

**DISCUSSION**

Because survival rates in patients with WG have improved, our ability to document the impact of disease-

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**Table 4.** Mean number of serious adverse events and flares during the first year of followup, as determined by the VDI score at 1 year

<table>
<thead>
<tr>
<th>VDI score</th>
<th>No. of patients</th>
<th>Serious adverse events</th>
<th>Flares</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD</td>
<td>Adjusted mean ± SD*</td>
<td>P†</td>
</tr>
<tr>
<td>0</td>
<td>43</td>
<td>0.81 ± 0.29</td>
<td>0.57 ± 0.35</td>
</tr>
<tr>
<td>1</td>
<td>42</td>
<td>1.00 ± 0.29</td>
<td>0.89 ± 0.30</td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>0.80 ± 0.41</td>
<td>0.85 ± 0.38</td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>2.67 ± 0.41</td>
<td>2.91 ± 0.43</td>
</tr>
<tr>
<td>≥4</td>
<td>25</td>
<td>2.76 ± 0.38</td>
<td>3.24 ± 0.52</td>
</tr>
</tbody>
</table>

* Adjusted for baseline scores on both the Vasculitis Damage Index (VDI) and the Birmingham Vasculitis Activity Score.
† For adjusted means among VDI subgroups (P value estimated based on ranked values of flares).

**Table 5.** Odds ratios of having at least 1 severe flare during up to 1 year of followup, as determined by the VDI score at 1 year

<table>
<thead>
<tr>
<th>VDI score</th>
<th>No. of patients</th>
<th>% with at least 1 severe flare</th>
<th>Odds ratio</th>
<th>Adjusted (95% CI)*</th>
<th>P†</th>
<th>P for trend‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>43</td>
<td>14.0</td>
<td>1.00</td>
<td>1.00</td>
<td>0.30</td>
<td>0.047</td>
</tr>
<tr>
<td>1</td>
<td>42</td>
<td>14.3</td>
<td>1.03</td>
<td>1.32 (0.37–4.69)</td>
<td>0.047</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>24</td>
<td>16</td>
<td>1.18</td>
<td>1.70 (0.39–7.34)</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>20</td>
<td>19</td>
<td>1.45</td>
<td>2.71 (0.56–13.2)</td>
<td>0.12</td>
<td></td>
</tr>
<tr>
<td>≥4</td>
<td>25</td>
<td>28</td>
<td>2.40</td>
<td>7.45 (1.12–49.6)</td>
<td>0.047</td>
<td></td>
</tr>
</tbody>
</table>

* Adjusted for baseline scores on both the Vasculitis Damage Index (VDI) and the Birmingham Vasculitis Activity Score. 95% CI = 95% confidence interval.
† For adjusted odds ratio among VDI subgroups.
and treatment-related damage has become a critical outcome measure. This study, the largest evaluation of WG-associated damage reported to date, offers several important findings. First, damage remains common among patients with WG, even when they receive what is currently considered optimal therapy at centers that specialize in the treatment of this disease. Among the 180 patients enrolled in the WGET, only 11% had not sustained a single VDI item after 1 year of enrollment. Second, the 1-year VDI score correlated with the number of disease flares experienced during that period, which is consistent with the concept that disease activity leads ultimately to damage. Third, although WG itself accounted for nearly three-fourths of the total number of damage items scored, VDI scores also correlated with treatment-related adverse events. Fourth, WG-associated damage (encompassing that related either to the disease itself or to treatment) translated into a measurable decline in patients’ quality of life, as confirmed by the inverse correlation between VDI scores and the physical component of the SF-36 v.2. Fifth, patients with limited disease are not at lower risk of WG-related damage. In fact, some of our analyses indicate that this group is at higher risk of damage than are those with severe disease.

The increases in many damage items over the course of the trial highlight the inadequacy of current treatment approaches to halting the accrual of damage in patients with WG. A large European trial of 158 patients with WG and microscopic polyangiitis also demonstrated the inexorable progression of damage among patients with these diagnoses (12). In that trial, the mean score at trial entry was 1.3, but increased to 2.5 after 18 months of followup (12), similar to what was observed in the WGET cohort. These findings echo those of a Dutch study (13) in which a mean increase of ~1 mg/dl in the serum creatinine level was observed for each recurrence of glomerulonephritis.

Other studies have also demonstrated the importance of baseline damage in the evaluation of patients with WG. In a prospective study of 23 patients with WG with a median of 37 months of followup, Kamali et al (14) found that patients who died had a higher baseline VDI score than did those who survived (1.33 versus 0.42, \( P = 0.002 \)). In a retrospective study of 52 patients with WG, Koldingsnes and Nossent (15) noted that a baseline VDI score \( >1 \) was a predictor of mortality (hazard ratio 6.10; 95% CI 1.68–22.17). The results of that study also indicated that existing damage heralds future damage: a baseline VDI score \( >1 \) was predictive of a future VDI score of \( >4 \) (hazard ratio 1.92, 95% CI 0.98–3.78) (15).

Another retrospective study demonstrated that resistance to therapy is associated with the presence of organ damage at baseline (16).

The results of our study are not completely concordant with those of previous studies of damage in vasculitis. For example, in a retrospective study of 32 patients with WG or microscopic polyangiitis, the VDI score was not associated with the number of relapses (17). In addition, no correlation between the VDI score and quality of life was observed in another study of 51 patients with primary systemic vasculitis (including WG, microscopic polyangiitis, and Churg-Strauss syndrome) (18). The reasons for the discrepancies between those studies and our own are not completely clear, but may reflect a bias introduced by studying smaller numbers of patients or a more heterogeneous population.

Because the VDI was designed to reflect the clinical sequelae of all forms of vasculitis, it includes a number of items that are of limited utility for the study of WG. For example, large-vessel inflammation (such as that which occurs in Takayasu arteritis and giant cell arteritis) is not characteristic of WG. Not surprisingly, therefore, items such as “absent pulses” and “major vessel stenosis” were reported very rarely in the WGET. (In fact, the only instance of loss of pulses in the WGET was the result of a failed dialysis-access procedure.) Similarly, deform ing/erosive arthritis, chronic peritonitis, and transverse myelitis occur rarely in WG and were not scored at all during the WGET. In contrast, some items listed on the VDI that do occur in WG (e.g., oral ulcers, which are part of 1 of the ACR criteria for the classification of this disease [19]) were not scored because they usually do not lead to permanent scars.

The original VDI validation study, based on the use of the instrument in a group of 100 patients with various forms of vasculitis (and an additional 20 patients with systemic vasculitis who had died), noted that an optional open category failed to reveal significant items of damage that were not already included in the VDI. Although 19 additional features were noted, each was reported only once (5). Application of a modified VDI to the WGET cohort, however, revealed 38 features of WG not included in the VDI, 10 of which were reported by 2 or more patients. The observation of these potentially important items of damage may be the result of examining a larger number of WG patients over a longer period of time (>500 observations). Many of the additional items of damage reported in the “other” category (such as proptosis, scleral scarring/thinning, bone marrow hypoplasia, eustachian tube dysfunction, and glaucoma) are widely recognized to be associated with WG,
but are not listed in the VDI. Because these forms of damage are not easily recorded using the VDI, they may be overlooked or recorded inconsistently from center to center (depending on local experience and expertise), leading to an incomplete depiction of the progression of damage in WG over time.

Certain items of damage reported by the WGET cohort highlight the difficulties inherent in attributing certain forms of damage to a single etiology. For example, some items of damage may not be the direct consequence of WG, but rather, represent a process that cosegregates with it (e.g., hypothyroidism), perhaps through common risk factors. Other items of damage may be the result of both the underlying vasculitis and its therapy, such as mood disorders, hypertension, or cardiac disease (which may result from both the vasculitis itself and the atherogenic effects of glucocorticoids).

The value of some items may be apparent only after a prolonged period of study. Although myocardial infarction and angina were noted only infrequently among patients in this study, studies of other rheumatic illnesses (20,21) indicate that the incidence of accelerated coronary artery disease is an important consequence of chronic inflammatory diseases. Longer follow-up of our cohort may lead to the identification of more damage events related to atherosclerosis. Conversely, items such as pulmonary nodules may not be “irreversible,” even if they are present on radiographic studies over 6 months. The most appropriate means of classifying such phenomena may become apparent with time, especially as their correlations with outcomes such as quality of life and mortality become clearer.

Finally, it should be acknowledged that the definitions for disease states used in this trial are different from those used in other clinical trials of WG. In particular, the use of the term “limited WG” differs from that introduced by the European Vasculitis Study Group, which uses the phrase “limited” to denote patients with localized or early systemic WG (22). This difference in nomenclature must be kept in mind when reviewing the results of this study.

In conclusion, we have reported the most comprehensive analysis of WG-associated damage to date. Our results indicate that damage from both active disease and its treatment remain important problems for patients with WG. Despite the dramatic improvements in patient survival achieved over the last several decades, only a few patients with WG emerge from a period of active disease without sustaining some damage from the disease itself, from its treatment, or both. An important measure of future therapeutic approaches will be their ability to reduce the damage occurring in patients over time.

REFERENCES


APPENDIX A: THE WEGENER’S GRANULOMATOSIS ETANERCEPT TRIAL (WGET) RESEARCH GROUP

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NF-κB Protects Behçet’s Disease T Cells Against CD95-Induced Apoptosis Up-Regulating Antiapoptotic Proteins

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Objective. To determine whether prolongation of the inflammatory reaction in patients with Behçet’s disease (BD) is related to apoptosis resistance and is associated with the up-regulation of antiapoptotic factors.

Methods. The percentage of cell death was evaluated by flow cytometry in peripheral blood mononuclear cells from 35 patients with BD and 30 healthy volunteers. The expression levels of antiapoptotic factors and NF-κB regulatory proteins were measured using Western blotting and immunohistochemical analyses. To down-regulate NF-κB nuclear translocation, BD T lymphocytes were exposed in vitro to thalidomide and subjected to transfection with NF-κB small interfering RNA.

Results. Although CD95 is highly expressed in BD T cells, the absence of sensitivity to CD95-induced apoptosis observed may be attributable to the inhibitory action of antiapoptotic genes. Immunoblot analysis for major antiapoptotic proteins showed considerable up-regulation of the short form of cellular FLIP (cFLIP) and Bcl-xL in BD activated T cells, while levels of Bcl-2, caspase 3, and caspase 8 in activated T cells from patients with BD were comparable with those in activated T cells from normal donors. Moreover, expression of IKK and IκB was up-regulated, whereas NF-κB translocated to the nucleus in BD T cells, suggesting that NF-κB activation may modulate the expression of antiapoptotic genes. Interestingly, thalidomide and NF-κB small interfering RNA down-regulated cFLIP and Bcl-xL expression levels and sensitized BD activated T cells to CD95-induced apoptosis.

Conclusion. Taken together, these results indicate that NF-κB plays a crucial role in the pathogenesis of BD, and that its pharmacologic control could represent a key strategy in modulating specific immune-mediated disease.

Behçet’s disease (BD) is a chronic inflammatory disorder affecting various organ systems. Originally described as a triad of ocular inflammation, oral ulcerations, and genital ulcerations, BD is better understood as a multisystem disease involving skin, joints, the central nervous system (1,2), the large bowel, and peripheral veins (3). Patients with BD may manifest all or only some of these clinical features. BD has a chronic course, with periodic exacerbations and progressive deterioration, sometimes leading to death within the first few years of the disease (4). The etiology and pathogenesis of BD are unknown, but considerable data indicate that immunologic abnormalities are important. Several humoral and cellular abnormalities in patients with BD have been described, and high serum levels of interleukin-2 (IL-2), interferon-γ (IFNγ), IL-1β, IL-6, tumor necrosis factor α (TNFα), and IL-8 have been
reported (5,6). The detection of these cytokines in patients with active disease points to a polarized Th1 immune response, as suggested by in vitro and in vivo studies of experimental autoimmune uveoretinitis (7).

According to a previous report, a strong Th1 immune response occurs in active BD, and IL-12 may play a substantial part in the pathogenesis of BD. IL-12 prevents spontaneous and CD95-induced cell death, resulting in abnormal growth of autoreactive Th1 lymphocytes, which could be responsible for the prolonged state of inflammation observed in patients with BD (8).

These data indicate that BD T cells are resistant to activation-induced cell death (AICD), which results in sustained abnormal growth of activated T lymphocytes and progression of the pathogenic response. Elimination of T cells during the termination phase of an immune response is called AICD and occurs by apoptosis (9,10). In particular, apoptosis is fundamental for inducing the suicide of supernumerary or damaged cells with high specificity and efficiency and for maintaining T cell homeostasis (11). Activated T cells that have escaped this control can initiate an inappropriate immune response against their target tissue (11,12). Two major mechanisms mediate the death of peripheral T cells: cytokine withdrawal and apoptosis induced by engagement of death receptors (9). Among the death receptors, CD95 (Fas/APO-1) plays the most important role in the AICD mechanism (13,14).

The susceptibility of peripheral T cells to CD95-induced apoptosis depends on the reactivity of T cells. In fact, resting and activated T cells that are resistant to CD95-induced apoptosis during the early response maintain the ability to determine an immune response (13,15). T cells become sensitive to AICD during the late phase of the immune response. Apoptosis resistance of T cells has been described in different autoimmune diseases, such as rheumatoid arthritis (RA) and multiple sclerosis. T cells from patients with RA express high levels of Bcl-2 and FLIP, and the level of Bcl-xL increases in apoptosis-resistant chronically activated RA T cells (16).

Triggering of the CD95 death receptor requires recruitment and activation of initiator and effector caspases, which can be antagonized by antiapoptotic molecules such as cellular FLIP (cFLIP; also known as FLAME-1, I-FLICE, CASH, Casper, CLARP, MACH-related inducer of toxicity [MRIT], and Usurpin) (17).

On the protein level, cFLIP exists as 2 endogenous forms: the long form (cFLIPL) and the short form (cFLIPS). Both forms of cFLIP were found to be recruited to the death-inducing signaling complex (DISC), and both contain 2 death effector domains. It has been demonstrated that up-regulation of cFLIPS correlates with apoptosis resistance of T cell receptor/CD3-restimulated T cells (15). Bcl-2 and Bcl-xL antagonize CD95-mediated apoptosis by preventing the propagation of apoptotic signals by mitochondria.

Cellular FLIP and Bcl-xL can also be regulated through several pathways, such as MAPK, phosphatidylinositol 3-kinase/Akt, or NF-κB (18,19). NF-κB transcription factor is ubiquitous and is sequestered in the cytoplasm in an inactive form associated with inhibitory proteins called IκB (20). IκB is regulated by 2 kinases, IκKa and IκKB, both of which are able to phosphorylate IκB. Phosphorylated IκB is ubiquitinated and degraded in the proteasome. Degradation of IκB frees NF-κB, permitting it to translocate to the nucleus and activate transcription of its target genes, involving mediators of the immune and inflammatory response such as IL-8, IL-12, and TNFα, antiapoptotic proteins, and growth factors. NF-κB can be activated by several cytokines, apoptosis-inducer factors, and necrosis-inducer factors (18–22).

Thalidomide has been identified as an antiinflammatory, immunomodulatory, and antiangiogenic compound. In particular, thalidomide selectively (but not completely) inhibits production of the inflammatory cytokine TNFα in human monocytes (23), thereby reducing the half-life of its messenger RNA (mRNA) (24). Given the key function of TNFα in immune and inflammatory responses, the ability of thalidomide to accelerate TNFα degradation may explain its clinical success in a variety of clinical conditions caused by high serum levels of TNFα, such as erythema nodosum leprosum, chronic cutaneous lupus erythematosus, prurigo nodularis, Crohn’s disease (25–28), and BD (29,30). Thalidomide has been used with clinical success in some autoimmune diseases such as multiple myeloma and RA, in which it was found to have proapoptotic properties, down-regulating NF-κB and consequently cFLIP (31). Additionally, thalidomide was demonstrated to inhibit NF-κB DNA binding, suppressing the IKK activity that leads to a decrease in IL-8 mRNA expression in Jurkat cells (32,33).

We therefore hypothesized that NF-κB activation has a key function in regulating expression of antiapoptotic proteins such as FLIP and Bcl-xL, preventing the death of T lymphocytes in BD cells. These new findings could be useful for better understanding the pathogenic mechanisms leading to the apoptotic resistance of activated T cells in patients with BD and establishing
pharmacologic control of aberrant expansion of T lymphocytes.

**PATIENTS AND METHODS**

**Patients.** The study group comprised 20 men and 15 women (mean ± SD age 40 ± 15 years) with a diagnosis of BD according to the criteria of the International Study Group for Behcet’s Disease (4), and 30 healthy volunteers (12 men and 18 women within the same age range). In addition, 5 women (mean ± SD age 26.2 ± 6.5 years) with systemic lupus erythematosus (SLE) were studied as disease controls. At the time of venipuncture, almost all of the patients with BD had at least 2 of the major manifestations (arthritis/arthralgia and oral and/or genital ulcerations and/or eye lesions, including uveitis, choriorretinitis, iridocyclitis, and photophobia) in the active stage. BD activity was assessed by the 1994 criteria for disease activity of BD, proposed by the Behcet’s Disease Research Committee of Japan (34). Thirty milliliters of peripheral blood was obtained from patients who were receiving no therapy at the time of venipuncture. Approval from the human studies committee and informed consent from each patient were obtained.

**Cell preparation.** Peripheral blood mononuclear cells (PBMCs) from patients with BD, patients with SLE, and healthy volunteers were isolated from heparinized blood by Ficoll-Hypaque gradient centrifugation. For stimulation, resting PBMCs (2 × 10⁶ cells/ml) (day 0) were exposed to 1 µg/ml of phytohemagglutinin (Sigma, St. Louis, MO) for 16 hours (day 1) in RPMI 1640 (EuroClone, Paington, UK). Following washes with RPMI 1640, day 1 cells were cultured for an additional 4 days with 25 IU/ml of IL-2 (Sigma) (day 5). The medium was changed every 2 days until day 5 and replaced with fresh medium containing 25 IU/ml of IL-2 (Sigma) (day 5). To evaluate the susceptibility of resting T cells (T day 0) and day 5 preactivated T cells (T day 5) to spontaneous and CD95-induced apoptosis, CD3+ T cells were purified from PBMCs, at day 0 and after activation (day 5), using MACS Cell Isolation Kits (Miltenyi Biotec, Bergisch Gladbach, Germany) and then incubated in the presence of the agonist anti-CD95 monoclonal antibody (200 ng/ml) (CH-11, IgM; Upstate Biotechnology), caspase 3 (rabbit polyclonal IgG; PharMingen), caspase 8 (5F7, mouse IgG2b; Upstate Biotechnology), NF-κB (3034, rabbit polyclonal IgG; Cell Signaling Technology, Beverly, MA), phospho-IKKα/β (Ser32, 9241 rabbit polyclonal IgG; Cell Signaling Technology), and phospho-IκBα (Ser24, 9241 rabbit polyclonal IgG; Cell Signaling Technology). The blots were then incubated for 1 hour with horseradish peroxidase–conjugated anti-rabbit or antimouse antibodies (Amersham Biosciences, Piscataway, NJ) and were visualized using an enhanced chemiluminescence detection system (SuperSignal ULTRA Chemiluminescent Substrate; Pierce, Rockford, IL). Cellular FLIP-transduced HUT-78 cells, transgenic mouse hearts expressing Bcl-xL (provided by Prof. G. Condorelli, Rome, Italy), and HeLa cells treated with TNFa for 30 minutes were used as positive controls.

The intensity of the band signals in exposed film was determined by densitometric scanning and analyzed using NIH Image version 1.62 software (by Wayne Rasband, National Institutes of Health, Research Services Branch, National Institute of Mental Health). FLIPα and IKK analyses were carried out by summing the band density at 55 kd and 43 kd

**Flow cytometry analysis of PBMCs.** Resting (day 0) and preactivated (day 5) PBMCs from control subjects and patients with BD were incubated for 30 minutes at 4°C with a combination of phycoerythrin (PE)–conjugated anti-CD3 (SK7, IgG1; Becton Dickinson, San Jose, CA) and the following fluorescein isothiocyanate (FITC)–labeled monoclonal antibodies: anti-CD69 (L78, IgG1; Becton Dickinson) or anti-CD95 (DX2, IgG1; PharMingen, San Diego, CA) or anti-CD71 (L01.1, IgG2a; Becton Dickinson) or anti-CD25 (2A3, IgG1; Becton Dickinson) or control IgG1 (Becton Dickinson). Cells were then washed and analyzed by 2-color flow cytometry on a FACScan (Becton Dickinson). The percentage of positive cells was determined on electronically gated CD3+ cells for each sample by comparing negative- and positive-fluorescence histograms, using a CellQuest program (Becton Dickinson), as previously described (35,36).

**Cell lysis and Western blot analysis.** To analyze protein expression levels in T lymphocytes prior to lysing, CD3+ T cells were purified from resting and activated PBMCs, as described above, and resuspended on ice-cold lysis buffer containing 50 mM Tris HCl (pH 7.4), 1% Nonidet P40 (NP40), 150 mM NaCl, 1 mM EGTA, 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM phenylmethylsulfonyl fluoride (PMSF). After 30 minutes on ice, lysates were centrifuged at 14,000g for 10 minutes at 4°C to remove insoluble material.

To prepare cytoplasmic and nuclear extracts, T cells (1 × 10⁶) were washed with ice-cold phosphate buffered saline (PBS) and lysed in 50 µl of lysis buffer containing 10 mmoles/liter HEPES (pH 7.9), 10 mmoles/liter KCl, 0.1 mmmole/liter EGTA, 0.1 mmmole/liter EDTA, 1 mmmole/dithiothreitol (DTT), 1 µg/ml aprotinin, 1 µg/ml leupeptin, 1 µg/ml pepstatin, and 1 mM PMSF. After 15 minutes of incubation, 0.6% NP40 solution was added to the lysates, which were centrifuged at 20,000g for 10 minutes at 4°C. Supernatants (cytoplasmic extracts) and pelleted nuclei were recovered. Nuclei were resuspended in 50 µl of buffer containing 20 mmoles/liter HEPES (pH 7.9), 0.4M NaCl, 1 mmmole/liter EGTA, 1 mmmole/liter EDTA, 1 mmmole/diter DTT, and 1 mM PMSF and centrifuged at 25,000g at 4°C to remove nuclear membranes.

Proteins were separated on a 12% sodium dodecyl sulfate–polyacrylamide gel and transferred to nitrocellulose. Nitrocellulose membranes were blocked for 1 hour with nonfat dry milk in Tris buffered saline–Tween 20 and successively blotted overnight at 4°C with specific antibodies to actin (Ab-1, mouse IgM; Calbiochem, San Diego, CA), FLIP (NF6, mouse IgG2A; Alexis, Lausen, Switzerland), Bcl-2 (H-5, mouse IgG1; Santa Cruz Biotechnology, Santa Cruz, CA), Bcl-2 (124, mouse IgG1; Upstate Biotechnology), caspase 3 (rabbit polyclonal IgG; PharMingen), caspase 8 (5F7, mouse IgG2b; Upstate Biotechnology), NF-κB (3034, rabbit polyclonal IgG; Cell Signaling Technology, Beverly, MA), phospho-IKKα/β (2681, rabbit polyclonal IgG; Cell Signaling Technology), and phospho-IκBα (Ser32, 9241 rabbit polyclonal IgG; Cell Signaling Technology). The blots were then incubated for 1 hour with horseradish peroxidase–conjugated anti-rabbit or antimouse antibodies (Amersham Biosciences, Piscataway, NJ) and were visualized using an enhanced chemiluminescence detection system (SuperSignal ULTRA Chemiluminescent Substrate; Pierce, Rockford, IL). Cellular FLIP-transduced HUT-78 cells, transgenic mouse hearts expressing Bcl-2 (provided by Prof. G. Condorelli, Rome, Italy), and HeLa cells treated with TNFa for 30 minutes were used as positive controls.

The intensity of the band signals in exposed film was determined by densitometric scanning and analyzed using NIH Image version 1.62 software (by Wayne Rasband, National Institutes of Health, Research Services Branch, National Institute of Mental Health). FLIPα and IKK analyses were carried out by summing the band density at 55 kd and 43 kd...
(for FLIP) and at 85 kd and 87 kd (for IKK) and then dividing by the band density of β-actin. Results are expressed as the gene:β-actin optical density (OD) ratio.

**Immunostaining procedure.** Immunohistochemical stains were done on cytopsin of purified resting or activated T cells. Slides were immersed in 4% paraformaldehyde for 10 minutes at 4°C and then washed with Tris buffered saline (TBS) at room temperature. Cells were permeabilized with 0.1% Triton X-100 for 5 minutes at room temperature. After washing with TBS, the slides were incubated for 30 minutes at room temperature with TBS containing 1% bovine serum albumin to block the nonspecific staining. Following elimination of excess serum, the slides were exposed to specific antibodies against NF-κB (3034, polyclonal antibody; Cell Signaling Technology) overnight at 4°C or isotype-matched control at appropriate dilutions. After 2 washes with TBS/Triton X-100, the slides were treated with biotinylated anti-rabbit immunoglobulins, washed in TBS/Triton X-100, and incubated with streptavidin peroxidase (Dako LSAB 2 kit; Dako, Carpinteria, CA). Staining was detected using 3-amino-9-ethyl-carbazole as colorimetric substrate. Counterstaining of the cells was assessed using aqueous hematoxylin.

**Analysis of apoptosis.** The susceptibility of T cells to spontaneous and CD95-induced apoptosis was detected by cytofluorimetric analysis of propidium iodide (PI) or annexin V–FITC cell staining on CD3+ cells, as described above. For PI staining, after washing in PBS, cell pellets were resuspended in hypotonic fluorochrome solution containing PI (50 μg/ml) in 0.1% sodium citrate and 0.1% Triton X-100 and kept in the dark overnight at 4°C until flow cytometry analysis was performed. The percentage of apoptotic cells was quantified using a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), evaluating the number of hypodiploid nuclei (15,37). For annexin V–FITC staining, washed CD4+ or CD8+ cells (SK3 and SK1, respectively; IgG1, phycoerythrin-conjugated; Becton Dickinson) that were already stained with anti-CD4 and anti-CD8 antibodies were resuspended in 100 μl of staining solution containing 20 μl annexin V–FITC and 20 μl PI in 1 ml of HEPES buffer, according to the manufacturer’s instructions (Boehringer, Mannheim, Germany), and analyzed by flow cytometry.

Alternatively, cell viability was determined using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI), according to the manufacturer’s instructions. This assay is based on bioreduction of a novel tetrazolium compound, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS), by cells into a colored formazan product that is soluble in tissue culture medium. Dye absorbance was measured at 490 nm.

**NF-κB small interfering RNA (siRNA) construction.** Small interfering RNAs with 2 thymidine residues at the 3’ end of the sequence were designed for the p65 gene (sense 5’-GCCGCGGCGCATCCCAGCGG-3’, 38) and the scrambled gene (sense 5’-CAGUCGCGUUUGCGACUGG-3’), along with their corresponding antisense RNA oligonucleotides (Dharmacon Research, Lafayette, CO), as previously described (39). These RNAs were dissolved in 10 mM Tris HCl (pH 8.0) and 1 mM EDTA as a 200-μM solution and annealed at room temperature following heating to 95°C in buffer (30 mM HEPES KOH [pH 7.9], 100 mM potassium acetate, 2 mM magnesium acetate). BD cells were transfected by Oligofectamine Reagent (Life Technologies, Rockville, MD). NF-κB siRNA (50 nM) or scrambled oligonucleotide was complexed in serum-free medium with 3 μM Oligofectamine. After 20 minutes, the complexes were added to BD PBMCs, and transfected cells were harvested. Transfection was monitored by immunoblot analysis and reverse transcription–polymerase chain reaction (RT-PCR) in order to obtain a decrease in NF-κB expression in purified T cells. Total cytoplasmic mRNA was prepared from scrambled and siRNA NF-κB–transfected cells from patients with BD, with Jurkat cells serving as the positive control. RNA was extracted using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. RT and PCR amplification for each preparation, with 150 ng of total RNA, was performed using the OneStep RT-PCR Kit (Qiagen). Two primers specific for the NF-κB coding sequence, 5’-GCGGGGCGCATCCCAGCGG-3’, nucleotides 198–217 (exon 3), and 5’-TGTTGGGGCCACGATTGTCA-3’, complementary to nucleotides 643–624 (exon 6) (GenBank accession number NM_021975), were selected to specifically amplify NF-κB complementary DNA. The GAPD gene was amplified from the same RNA preparations as housekeeping control (coding sequence 5’-TGACATCAAGAAGGTGGTGA-3’, nucleotides 843–863, and 5’-GCCACCCCCGTTGCTGTA-3’, complementary to nucleotides 1033–1053 [GenBank accession number NM_002046]). Transfected cells were stimulated with phytohemagglutinin/IL-2 as described above, and cFLIP and Bcl-xL expression levels were monitored by Western blotting. After CD3 purification, transfected cells were exposed to anti-CD95 monoclonal antibody (CH-11) for functional analysis.

**Statistical analysis.** The percentage of apoptotic events was calculated from the values obtained from the MTS assay, annexin V staining, and PI staining. Data were expressed as the total mean (±SD) of the mean calculated from each method used for apoptosis measurement. Analysis of variance (one-way or two-way) with Bonferroni adjustment was used to analyze the statistical significance of the results, and the analysis was performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA). P values less than 0.05 were considered significant.

**RESULTS**

Resistance of BD activated T cells to CD95-induced apoptosis. Human T cells require previous activation with phytohemagglutinin and IL-2 to be rendered sensitive to CD95-mediated AICD (9,40). To investigate whether resting or activated T cells from BD patients are resistant to CD95-induced apoptosis, we evaluated cell death using either cytofluorimetric analysis of PI or annexin V cell staining or MTS assay. We found that resting T cells (T day 0) from both normal donors and BD patients were protected from spontaneous apoptosis (mean ± SD 3 ± 1.4% and 2.2 ± 1%, respectively) and CD95-induced apoptosis (mean ± SD 5 ± 1.3% and 4 ± 1%, respectively) (Figures 1a and c). In contrast, chronically activated T cells (T day 5) from normal donors were significantly sensitized to CD95-
Figure 1. Resistance to CD95-induced apoptosis in activated T cells from patients with Behcet’s disease (BD). a and b, Cell death in resting (T day 0) and activated (T day 5) T cells from normal donors, patients with BD, and patients with systemic lupus erythematosus (SLE). Cells were treated with control IgM (Control) or CD95 agonistic monoclonal antibody (200 ng/ml) for 24 hours. Values are the mean and SD of 35 patients with BD, 5 patients with SLE, and 30 normal donors. c, Flow cytometry profiles of fluorescein isothiocyanate–annexin V staining of purified resting and activated T cells from patients with BD and normal donors. d, Apoptosis, as measured by annexin V staining of CD4+ and CD8+ cells in resting (T day 0) and activated (T day 5) T cells from normal donors (ND) and patients with BD. Values are the mean and SD of triplicate determinations in 8 normal donors and 8 patients with BD. e and f, Percentage of positive CD95, CD25, CD69, and CD71 cells in CD3-gated T cells from normal donors and patients with BD, which were activated for up to 5 days and analyzed by flow cytometry. Values are the mean ± SD of 18 patients with BD and 20 normal donors. * = P < 0.05; ** = P < 0.01; *** = P < 0.001, versus normal donors.
Figure 2. Expression of antiapoptotic molecules in T lymphocytes from patients with Behcet's disease (BD). a, Immunoblot analysis of the long and short forms of cellular FLIP (cFLIP<sup>L</sup> and cFLIP<sup>S</sup>, respectively), and their relative band densities, in resting (T day 0) and activated (T day 5) T cells from normal donors (ND) and patients with BD. Cellular FLIP–transduced HUT-78 cells (HuT78 FLIP) were used as positive control. b, Western blot analysis of Bcl-x<sub>L</sub> in resting (T day 0), short-term–activated (T day 1), and long-term–activated (T day 5) T cells from normal donors and patients with BD, and their relative band densities. Transgenic Bcl-x<sub>L</sub> (Tg Bcl-x<sub>L</sub>) was loaded as positive control. Loading control was assessed by β-actin staining. Results are representative of 10 experiments performed with cells from different donors. ** = P < 0.01; *** = P < 0.001, versus normal donors. c, Immunoblot analysis of Bcl-2, and its relative band densities. Results are representative of 6 experiments performed. * = P < 0.05 versus normal donors. d, Western blot analysis of cFLIP and Bcl-x<sub>L</sub>, and their relative band densities, in resting T cells from normal donors and patients with systemic lupus erythematosus (SLE). Results are representative of 6 experiments performed. Numbers above each lane indicate the number of patients or normal donors analyzed. Bars show the mean and SD optical density (OD) ratio.
induced apoptosis compared with T cells from BD patients (mean ± SD 85 ± 7% and 11 ± 5%, respectively) (Figures 1b and c). Decreased CD95-induced apoptosis was observed in both CD4+ and CD8+ activated T cells from patients with BD, suggesting that impaired AICD is not confined to a single T cell subset (Figure 1d). Thus, the resistance to CD95-induced apoptosis in BD activated T cells may be important for T cell permanence that is then involved in the BD pathogenic mechanism. In accordance with other reports (41,42), we found that resting (T day 0) and activated (T day 5) T cells from SLE patients, which were used as disease control, were refractory to both spontaneous apoptosis (mean ± SD 2.8 ± 1.6% and 3.4 ± 2, respectively) and CD95-induced apoptosis (mean ± SD 4.2 ± 1.3 and 5.8 ± 2.4, respectively) (Figures 1a and b).

To additionally determine whether impaired apoptosis in BD T cells is attributable to different expression of CD95 or different kinetics of activation, we used flow cytometry to evaluate the expression of CD95, CD25, CD69, and CD71 in electronically gated CD3+ cells from patients with BD and normal donors, for up to 5 days of activation. In accordance with previous findings, CD95 was expressed at a high percentage in both normal and BD T cells at day 0 (mean ± SD 71 ± 3% and 80 ± 4%, respectively; P < 0.001), and this expression continued to increase slightly, but significantly, throughout the 5 days of observation (Figures 1e and f).

Expression of the IL-2 receptor (CD25) was up-regulated on BD T cells compared with normal T cells at day 3 (mean ± SD 75 ± 4% and 55 ± 4%, respectively; P < 0.001) and at day 5 (mean ± SD 81 ± 3.5% and 68 ± 3.2%, respectively; P < 0.001) (Figures 1e and f). Moreover, expression of the early activation antigen CD69 was higher in BD T cells at days 1 and 3 (mean ± SD 71% ± 4.5 and 50% ± 2.7, respectively; P < 0.001) than in control cells (mean ± SD 57 ± 3% and 40 ± 3.4%, respectively) (Figures 1e and f). In contrast, expression of the transferring receptor (CD71) was comparable in both BD and normal T cells during the different times at which they were examined (Figures 1e and f). These data suggest that the T cell response in BD T cells was higher than that in normal T cells.

High-level expression of cFLIPS and Bcl-xL in BD T cells. To determine whether the absence of sensitivity to CD95-induced apoptosis observed in activated T cells from patients with BD was attributable to potent inhibitors of the apoptotic pathway, we evaluated the expression of several important antiapoptotic and proapoptotic proteins in resting (T day 0) and activated (T day 5) T cells from normal donors and patients with BD. Based on the results of 14 independent experiments, immunoblot analyses for antiapoptotic proteins showed that expression of cFLIPS was up-regulated in resting T lymphocytes from patients with BD compared with T cells from normal donors (mean ± SD 84 ± 20 versus 8 ± 5 OD; P < 0.001) and in activated T lymphocytes from BD patients compared with T lymphocytes from normal donors (mean ± SD 34 ± 10 versus 1 ± 1 OD; P < 0.01) (Figure 2a). In contrast, the expression of cFLIP_L was similar in both resting and activated T cells from both patients with BD and normal donors (Figure 2a). Activated T cells from patients with BD exhibited higher levels of Bcl-xL (mean ± SD 143 ± 17 OD) compared with those from normal donors (mean ± SD 5.7 ± 3 OD) (Figure 2b). Bcl-2 levels in T cells from both normal donors and patients with BD were not significantly affected by T cell activation. However, resting (day 0) T cells from patients with BD expressed significantly higher Bcl-2 levels compared with resting T cells from normal donors, as shown by immunoblot analysis (Figure 2c). In contrast, the expression levels of Bcl-xL, cFLIP_L, and cFLIPS protein were comparable in resting T cells from both normal donors and patients with SLE (Figure 2d). Cellular FLIP–transduced HUT-78 cells, transgenic Bcl-xL, and HL-60 cells were used as positive controls. The expression levels of caspase 3 and caspase 8 were comparable in T cells from patients with BD and T cells from normal donors (data not shown). Thus, cFLIPS and Bcl-xL should represent major candidates for mediating resistance of BD T cells to CD95-induced apoptosis.

T cell activation–induced NF-κB nuclear translocation in BD. To investigate the role of NF-κB as a transcription factor in a number of genes, including c-FLIP and Bcl-xL, cytoplasmic and nuclear extracts from resting T cells from normal donors and patients with BD were analyzed for NF-κB activity. Immunoblot analysis showed that NF-κB was highly expressed in nuclear extracts from BD T cells, whereas it was scarcely expressed or was not detectable in nuclear extracts from normal donor T lymphocytes (mean ± SD 281 ± 12 versus 1 ± 1 OD) (Figures 3a and b).

Immunohistochemical analysis of purified resting T cells (T day 0) from patients with BD confirmed that NF-κB was translocated to the nucleus (Figure 3c). Moreover, phosphorylation of the upstream steps of NF-κB activation, IKK and IκBα, resulted in high expression in activated (T day 5) T cells from patients with BD (mean ± SD 90 ± 7 and 171 ± 16 OD, respectively) compared with that in activated T cells from normal donors (mean ± SD 35 ± 5 and 100 ± 11 OD, respectively) (Figures 3d, e, and f). These data suggest that CD95-induced apoptosis resistance in BD T
cells is dependent on NF-κB and upstream IκBα activation, contributing to an abnormal growth of aberrant lymphocytes in patients with BD.

**Thalidomide- and NF-κB–induced down-regulation of cFLIP and Bcl-xL in BD T cells.** Previous reports have provided evidence that thalidomide can inhibit the DNA binding activity of NF-κB through a mechanism that involves suppression of IKK activity (33). To determine whether thalidomide could inhibit the activation of resting T cells from patients with BD, and specifically whether thalidomide could inhibit the nuclear translocation of NF-κB in these cells, we pre-treated PBMCs with thalidomide and then performed immunohistochemical analysis in CD3+ cells to evaluate...
the expression levels of nuclear NF-κB. Interestingly, after exposure to thalidomide, the expression levels of nuclear NF-κB were markedly decreased in BD T cells (T day 0) (Figure 4a).

We next determined whether thalidomide, in activated T cells from patients with BD, altered the expression of cFLIP and Bcl-xL. The levels of these proteins in BD activated T cells (T day 5) were significantly decreased after exposure to thalidomide (Figure 4b). In fact, as shown in Figure 4c, expression of cFLIP<sub>L</sub> and cFLIP<sub>S</sub> was down-regulated after exposure to thalidomide (mean ± SD 144 ± 15 versus 62 ± 10 OD [P < 0.05] and 203 ± 18 versus 4 ± 3 OD [P < 0.001], respectively), and the expression of Bcl-x<sub>L</sub> was markedly decreased following treatment with thalidomide (47 ± 10 versus 5 ± 3 OD; P < 0.01). These data confirm the hypothesis that Bcl-x<sub>L</sub> and cFLIP are regulated by NF-κB and represent 2 candidates for mediating resistance to CD95-induced apoptosis in T cell from patients with BD.

Thalidomide- and NF-κB siRNA–induced sensitization of BD T cells to CD95-mediated apoptosis. To establish whether the regulation of NF-κB could modulate sensitization to CD95-induced apoptosis in activated T cells from patients with BD, we transfected BD PBMCs with NF-κB siRNA or exposed them to thalidomide. Scrambled and NF-κB siRNA transfections were monitored by immunoblotting and RT-PCR analysis (Figures 5a, b, and c). Similarly to thalidomide, NF-κB siRNA transduction massively decreased expression of both cFLIP<sub>S</sub> and Bcl-x<sub>L</sub> protein in activated T cells from patients with BD (Figure 5d). A considerable percentage of BD activated T cells (T day 5) underwent CD95-induced apoptosis upon exposure to thalidomide (mean ± SD 82 ± 6%; P < 0.001) or NF-κB siRNA transduction (93 ± 7%; P < 0.001), indicating that down-regulation of NF-κB is capable of promoting cell death in apoptosis-refractory cells (Figures 5e and f). In cells pretreated with thalidomide, the percentage of BD activated T cells (T day 5) undergoing apoptosis was comparable with that observed in NF-κB siRNA–transfected cells.

DISCUSSION

The basic mechanisms governing the pathogenic process of BD are still unknown. However, it is becoming increasingly clear that prolongation of the inflammatory reaction in BD is caused by the apoptosis-refractory nature of activated T cells.

Our data show that T cells from patients with BD are resistant to spontaneous and CD95-induced apoptosis and express high levels of cFLIP<sub>S</sub> and Bcl-x<sub>L</sub>. Activated NF-κB was detected in purified T cells from patients with BD, and thalidomide, an immunomodulator agent, directly contributed to the down-regulation of cFLIP<sub>S</sub> and Bcl-x<sub>L</sub> by inhibiting the nuclear translocation of NF-κB. Similarly, NF-κB siRNA transduction directly down-regulated cFLIP<sub>S</sub> and Bcl-x<sub>L</sub> in BD activated T cells (T day 5). Moreover, upstream steps of NF-κB activation, such as IKK and IκB phosphorylation, were found to be expressed in resting BD cells and up-regulated in phytohemagglutinin-positive IL-2–activated BD cells.

Bcl-x<sub>L</sub> is a Bcl-2 family member that prevents apoptosis generated by the intrinsic pathway and partly
neutralizes cell death induced by death receptors. In contrast, cFLIP is a potent inhibitor of CD95-mediated apoptosis, by directly targeting caspase 8 processing at the DISC (9,17,43). Immune system homeostasis is tightly regulated by apoptosis, to eliminate self-reactive lymphocytes and avoid autoimmune reactions (10,11). In the early phase of the immune response, T cells acquire effector functions. Resting T cells are protected from CD95-mediated apoptosis, whereas prolonged T cell activation confers sensitivity to AICD (44). CD95-sensitive activated T cells from normal donors express undetectable levels of cFLIP<sub>S</sub>, which is considerably

**Figure 5.** Quantification of cell death in activated T cells from patients with BD following exposure to thalidomide and NF-κB small interfering RNA (siRNA). a and b, Immunoblot analysis of the expression of NF-κB, and its relative band density, on scrambled (Scr) and siRNA NF-κB (siNF-κB)–transfected peripheral blood mononuclear cells from patients with BD. Loading control was assessed by β-actin staining. Jurkat cell lysates were used as positive control. c, Reverse transcription–polymerase chain reaction analysis for NF-κB and GAPD genes. M = marker (100-bp DNA ladder). d, Immunoblot analysis of the expression of cFLIP<sub>L</sub>, cFLIP<sub>S</sub>, and Bcl-x<sub>L</sub> protein on activated (T day 5) BD T cells, as described above. Loading control was assessed by β-actin staining. e and f, Percentage of cell death in scrambled and siRNA NF-κB–transfected T cells from patients with BD. Cells were pretreated with thalidomide (e) and exposed to IgM (control) or anti-CD95 (f). Values are the mean and SD of 4 independent experiments. * = P < 0.05; *** = P < 0.001. See Figure 4 for other definitions.
expressed in resting and activated BD T cells and likely contributes to the impaired deletion of autoreactive lymphocytes in the active phases of this disease. Although the increased levels of Bcl-2 in activated BD T cells are not significant, high Bcl-xL expression correlates with resistance to apoptosis in the activated T lymphocytes of patients with BD. Because Bcl-xL does not exert a direct inhibitory effect on the CD95 DISC, Bcl-xL may contribute to shutting down the residual apoptosis signals that escaped DISC blockade, resulting in high cFLIP levels (45,46).

It has already been reported that lymphocytes from patients with BD are resistant to CD95-induced apoptosis (47). In the last few years, several studies have investigated the expression and function of CD95 and its soluble form in patients with BD (48–50). The results of these studies suggest a potential involvement of CD95 in the chronic inflammation of BD, associated with the clinical stage and clinical manifestations. The pattern of CD95 expression in CD4+ and CD8+ T cell subsets in patients with BD is controversial. Whereas some investigators have reported insufficient expression of CD95 on BD CD4+ T cells (49), other investigators observed the opposite (48). In contrast, there is general agreement that CD95 is up-regulated on CD8+ T cells from patients with BD (48,49). We found that CD95 expression was slightly, but significantly, higher in CD3+ cells from patients with BD. Additionally, our results indicate that the expression of early-activation molecules is up-regulated in activated T cells from patients with BD, while expression of the late activation marker CD71 was comparable in both normal and BD cells.

Both CD4+ and CD8+ T cells displayed decreased AICD in BD resting and activated T cells. Defective apoptosis, due to the lack of a decrease in cFLIPs and the increase in Bcl-xL in chronically activated BD T cells, may result in an abnormal expansion of autoreactive T cells, which allows persistence of the autoimmune response in BD. Thus, it is likely that defective CD95 signaling in effector/memory T cells contributes to the persistence of activated T cells that escape induction of tolerance.

Here, we demonstrated that inhibition of NF-κB by siRNA or thalidomide is able to restore spontaneous and CD95-induced cell death in BD T cells. NF-κB regulates the genes involved in the inflammatory response as well as those associated with the inhibition of apoptosis, such as Bcl-xL and cFLIP. Inhibition of NF-κB nuclear translocation increases the susceptibility of cells to undergo apoptosis induced by several stimuli, including TNFα, ionizing radiation, and chemotherapeutic drugs (51). Thalidomide directly induces apoptosis or growth arrest of multiple myeloma cells by down-regulating NF-κB activity and cFLIP expression (19). Moreover, thalidomide can impair NF-κB activation induced by different cytokines by suppressing IKK activity (32,33). Our data provide evidence that IKK and NF-κB activity are considerably increased in BD activated T cells.

The ability of thalidomide to restore AICD in the setting of BD may have considerable therapeutic implications. Sustained up-regulation of cyclooxygenase 2 protects activated SLE T cells from induction of anergy and apoptosis (42). Differently from BD cells, SLE T lymphocytes do not constitutively express high levels of FLIPs and Bcl-xL, which is consistent with the reported decrease in NF-κB activity in SLE T cells (52). Thus, although some inhibitory mediators of the death receptor pathway may be commonly exploited by autoimmune T cells in different systemic diseases, constitutive NF-κB activation may be regarded as a specific mechanism responsible for AICD resistance in BD T cells.

The bax:bel-2 ratio is potently increased by thalidomide, suggesting that both of the apoptotic genes involved in the intrinsic pathway are affected. Moreover, thalidomide stimulates natural killer cell cytotoxicity and inhibits the production of IL-6, TNFα, and vascular endothelial growth factor. In fact, thalidomide has been found to be a successful treatment in multiple myeloma (53) and in various immune and inflammatory diseases such as RA, chronic cutaneous lupus erythematosus, erythema nodosum leprosum, Crohn’s disease, and BD (25–28,54,55). The effects of thalidomide on the immune response are still poorly understood. However, the immunomodulatory and antiinflammatory properties of this drug have been reported. IL-12 was demonstrated to be implicated in the pathogenesis of BD (6), preventing spontaneous and CD95-induced cell death in the peripheral blood leukocytes of patients with BD (8). Because IL-12 production in lipopolysaccharide-stimulated human monocytic cells is directly regulated by JNK, activator protein 1, and NF-κB transcription factors (56), it is possible that thalidomide may also effectively decrease the production of IL-12 in patients with BD. Therefore, the down-regulation of cFLIP and Bcl-xL may allow the activation of caspase 8 activity at the DISC and the subsequent amplification of CD95 apoptotic signaling by the mitochondrial pathway.

In this study, we provide evidence that NF-κB activity may play a fundamental role in the pathogenesis of BD, and that its down-regulation by thalidomide could outline an alternative therapeutic approach for the
cure of BD. Our findings contribute to the delineation of the intracellular mechanism whereby BD T lymphocytes are resistant to cell death. Although the mechanisms responsible for the initiation of BD remain to be clarified, the pathogenetic machinery that rules activated T cell survival and subsequent prolongation of inflammation is becoming increasingly clear.

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Infliximab, but Not Etanercept, Induces IgM Anti–Double-Stranded DNA Autoantibodies as Main Antinuclear Reactivity

Biologic and Clinical Implications in Autoimmune Arthritis

Leen De Rycke, Dominique Baeten, Elli Kruithof, Filip Van den Bosch, Eric M. Veys, and Filip De Keyser

Objective. To analyze the clinical and biologic correlates of autoantibody induction during longer-term tumor necrosis factor α (TNFα) blockade with either the monoclonal antibody infliximab or the soluble receptor etanercept.

Methods. Thirty-four patients with spondylarthropathy (SpA) and 59 patients with rheumatoid arthritis (RA) were treated with infliximab for 2 years. Additionally, 20 patients with SpA were treated with etanercept for 1 year. Sera were blindly analyzed for antinuclear antibodies (ANAs), anti–double-stranded DNA (anti-dsDNA) antibodies, anti–extractable nuclear antigen (anti-ENA) antibodies, and antihistone, antinucleosome, and anticardiolipin antibodies (aCL). The anti-dsDNA antibodies were isotypet.

Results. High numbers of infliximab-treated patients with SpA or RA had newly induced ANAs (61.8% and 40.7%, respectively) and anti-dsDNA antibodies (70.6% and 49.2%, respectively) after 1 year, but no further increase between year 1 and year 2 was observed. In contrast, induction of ANAs and anti-dsDNA antibodies was observed only occasionally in the etanercept-treated patients with SpA (10% of patients each). Isotyping revealed almost exclusively IgM or IgM/IgA anti-dsDNA antibodies, which disappeared upon interruption of treatment. Neither infliximab nor etanercept induced other lupus-related reactivities such as anti-ENA antibodies, antihistone antibodies, or antinucleosome antibodies, and no clinically relevant lupus-like symptoms were observed. Similarly, infliximab but not etanercept selectively increased IgM but not IgG aCL titers.

Conclusion. The prominent ANA and anti-dsDNA autoantibody response is not a pure class effect of TNFα blockers, is largely restricted to short-term IgM responses, and is not associated with other serologic or clinical signs of lupus. Similar findings with aCL suggest that modulation of humoral immunity may be a more general feature of infliximab treatment.

Since the first proof of efficacy of tumor necrosis factor α (TNFα) blockade, both the number of patients treated worldwide and the number of indications for treatment have grown steadily (1–3). Surprisingly, the profound immunomodulation induced by TNFα blockers is associated with a relatively low incidence of immune-related complications such as demyelinating disease and lupus-like syndromes (3–5). This contrasts sharply with the prominent induction of autoantibodies such as antinuclear antibodies (ANAs) and anti–double-stranded DNA (anti-dsDNA) antibodies by TNFα blockers (6–14). Although this phenomenon has been recognized for several years, the clinical and biologic correlates of this antibody induction in autoimmune arthritis are not yet fully understood. Recent studies showed that induced anti-dsDNA antibodies belong to
the IgM and IgA subclasses but not to the IgG subclass (7,8,12). Moreover, it became clear that ANA and anti-dsDNA antibody induction was observed not only in patients with rheumatoid arthritis (RA) but also in patients with spondylarthropathy (SpA) and Crohn’s disease (8,12–17). However, large systematic studies comparing different TNFα blockers, different disease groups, different autoantibodies, and longer-term clinical and serologic outcomes are not yet available, which precluded better understanding of the clinical and biologic relevance of the changes in the autoantibody profiles that occur during TNFα blockade.

The aim of the present study was to assess the above-mentioned issues in further detail by systematically analyzing the serologic, biologic, and clinical parameters in a large cohort of patients who were treated with either the monoclonal anti-TNFα antibody infliximab or the soluble TNFα receptor etanercept for up to 2 years. Because baseline humoral autoimmunity is uncommon in SpA, we used this disease as our primary human model.

**PATIENTS AND METHODS**

**Patients and samples.** As shown in Table 1, a total of 113 patients with either SpA or RA were included in the study after giving informed consent. This study was approved by the Ethics Committee of the University Hospital Ghent. All patients with SpA fulfilled the European Spondyloarthropathy Study Group classification criteria for SpA (18); all patients with RA fulfilled the American College of Rheumatology (ACR; formerly the American Rheumatism Association) classification criteria for RA (19). Cohort 1 comprised 34 patients with SpA who were treated with a loading dose of infliximab (5 mg/kg) at weeks 0, 2, and 6, followed by a dose of 10 mg/kg every 14 weeks until week 48. In the second year of treatment, patients in cohort 1 received 5 mg/kg of infliximab every 8 weeks until week 104. Cohort 2 comprised 59 patients with RA who received 3 mg/kg of infliximab at weeks 0, 2, and 6, and every 8 weeks thereafter for up to 2 years (week 102). All patients with RA received concomitant treatment with methotrexate (≥7.5 mg/week). In 2 of the patients with RA, infliximab treatment was stopped between year 1 and year 2 due to pneumonia (n = 1) or fever/esophagitis/cystitis (n = 1). Cohort 3 comprised 20 patients with SpA who were treated with etanercept (25 mg twice weekly) for a 1-year period.

Additionally, we analyzed a control group of 15 patients with systemic lupus erythematosus (SLE) fulfilling the ACR classification criteria for SLE (20), who were not treated with TNFα blockers. Finally, 7 other patients (5 with RA and 2 with SpA) who were treated with infliximab for at least 5 months, and in whom treatment had to be interrupted, were tested at baseline, at the moment that infliximab was stopped, and after withdrawal from treatment in order to analyze the persistence over time of the induced autoimmune profiles.

In order to rule out inter-test variability and exclude possible technical biases, all serologic analyses were performed in a single test run, and investigators were blinded to the diagnosis, treatment, and time point of treatment.

**Detection of ANAs.** For reasons of sensitivity, serum was diluted 1:40 in phosphate buffered saline (PBS). Serum samples were tested for ANA reactivity using fixed HEp-2000 cells (Immunoconcepts, Sacramento, CA) and a fluorescein isothiocyanate (FITC)–labeled conjugate (anti-human IgG heavy and light chain specific; Immunoconcepts). The fluorescence intensity was scored semiquantitatively from 0 to 5+, relative to the intensity of a negative control and a positive (4+) control (21). A sample was considered positive for ANA if a score of at least 2+ was obtained, which corresponds with positivity using a serum dilution between 1:80 and 1:160 (data not shown). A significant increase in ANA intensity was defined as an increase of at least 2 points on a scale of 0 to 5+.

**Table 1.** Baseline characteristics of patients who were treated with TNFα blockers*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Cohort 1, SpA (n = 34)</th>
<th>Cohort 2, RA (n = 59)</th>
<th>Cohort 3, SpA (n = 20)</th>
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<td>TNFα blocker</td>
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<td>Infliximab</td>
<td>Etanercept</td>
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<tr>
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<td>Baseline, week 48, week 104</td>
<td>Baseline, week 46, week 102</td>
<td>Baseline, week 48</td>
</tr>
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</table>

* Except where indicated otherwise, values are the number of patients. TNFα = tumor necrosis factor α; SpA = spondylarthropathy; RA = rheumatoid arthritis; CRP = C-reactive protein; ESR = erythrocyte sedimentation rate; MTX = methotrexate; NSAIDs = nonsteroidal antiinflammatory drugs.
Detection of anti-dsDNA antibodies. Serum samples diluted 1:20 in PBS were tested for anti-dsDNA antibodies, using *Crithidia luciliae*-coated slides (Immunocore) as substrate and an FITC-labeled conjugate (anti-human IgG heavy and light chain specific; Immunocore). Samples were scored as being positive or negative, according to comparison with a negative control and a positive control. Similarly, isotyping of the anti-dsDNA antibodies was performed by indirect immunofluorescence on *C. luciliae*, using specific FITC-labeled conjugates against human IgG heavy (γ) chain (Production d’Anticorps Réactifs Immunologiques & Services, Compiègne, France), human IgM heavy (μ) chain (Dako, Glostrup, Denmark), and human IgA heavy (α) chain (Dako). The specificity of the conjugates was validated by the manufacturer as well as in our own enzyme-linked immunosorbent assay (ELISA) experiments, using purified human IgG, IgM, and IgA as substrate (data not shown). We and other investigators previously demonstrated that, in the context of TNFα blockade, indirect immunofluorescence on *C. luciliae* is a more sensitive and reliable technique than ELISA for the detection of anti-dsDNA antibodies (8,11).

Detection of anti–extractable nuclear antigen (ENA), antihistone, and antinucleosome antibodies. Sera were analyzed for anti-ENA and antihistone antibodies by line immunoblot assay (LIA) (INNO-LIA ANA update K1090; Innogenetics, Zwijnaarde, Belgium). This multiparameter assay contains the following antigens: SmB, SmD, RNP 70, RNP-A, RNP-C, SSA/Ro 52, SSA/Ro 60, SSB/La, CENP-B, ScI-70, Jo-1, ribosomal P, and histones (H1, H2a, H2b, H3, and H4). For detection of antinucleosome antibodies, sera were analyzed by ELISA (Anti-Nucleo; GA Generic Assays, Dahlewitz, Germany). In a diagnostic setting, the cutoff for positivity is 50 units/ml. Both tests were performed according to the manufacturer's instructions.

Detection of lupus-like characteristics. Because all of the patients were followed up in the context of clinical trials, lupus-related manifestations such as oral ulcers, serositis, neurologic disorder, and skin symptoms (rash, photosensitivity), as well as any other adverse events were reported systematically in the medical charts at every visit. Biologic evaluation included the peripheral blood cell count to test for hematologic disorders and urinalysis to test for proteinuria (i.e., kidney disease).

Detection of anticytotoxic antibodies (aCL). IgG aCL were detected by an in-house ELISA coated with cardiolin (Sigma, St. Louis, MO). Diluted sera (1:1,000) were tested using anti-human IgG Fc-specific conjugate coupled with alkaline phosphatase (Sigma) and *p*-nitrophenyl-phosphate substrate in diethanolamine buffer (Bio-Rad, Hercules, CA). For the detection of IgM aCL, we used a commercially available kit (Orgentec Diagnostika, Mainz, Germany). The ELISA was performed according to the manufacturer's instructions.

Statistical analysis. All values are presented as the median (range). For dichotomous data, we used the paired McNemar's test. For continuous data, we used the nonparametric paired Wilcoxon’s signed rank test. For comparison of proportions, we applied a chi-square test. *P* values less than or equal to 0.05 were considered significant.

RESULTS

Induction of ANAs and anti-dsDNA antibodies by infliximab. We investigated whether the induction of ANAs and anti-dsDNA autoantibodies that was observed after 6 months of infliximab treatment in patients with SpA (8) was further increased at 1 and 2 years. As shown in Figure 1A, the year 1 data confirmed the significant ANA induction, as reflected by the high number of patients with newly induced ANAs (21 of 34 patients [61.8%]) and those with a significant increase in ANA intensity (21 of 34 patients [61.8%]) compared with baseline. Accordingly, there was a significant induction of anti-dsDNA antibodies during the first year of infliximab treatment (24 of 34 patients [70.6%]). These data indicate that there is no additional increase of ANA and anti-dsDNA antibody positivity after 6 months (8), which was confirmed by the absence of further induction of ANA or anti-dsDNA antibodies between year 1 and year 2 (Figure 1). In addition, there was a trend toward a decrease in anti-dsDNA antibody positivity in the infliximab-treated SpA cohort between year 1 (73.5%) and year 2 (55.9%; *P* = 0.07).

Confirming these findings in an independent disease background, we found that among 59 infliximab-treated patients with RA, 24 (40.7%) had newly positive ANAs and 24 (40.7%) had a significant increase in ANA intensity between baseline and year 1. Anti-dsDNA antibodies were induced in 29 of 59 patients (49.2%) (Figure 1). As in patients with SpA, these values closely resemble the data at 6 months (8), which were further confirmed by the stable profiles between years 1 and 2 (Figure 1). Of interest, the induction of anti-dsDNA antibodies tended to be more pronounced in SpA than in RA at year 1 (*P* = 0.039).

Induction of ANAs and anti-dsDNA antibodies by infliximab and etanercept, and comparison with infliximab. In order to assess potential differences in autoantibody induction between different TNFα blockers, we next analyzed 20 patients with SpA who were treated with etanercept and who fulfilled the same inclusion criteria as those fulfilled by the previously reported infliximab-treated SpA cohort. There was no significant ANA induction after 1 year of etanercept treatment: only 3 patients (15%) developed newly positive ANAs, and only 1 patient (5%) had a significant increase in ANA intensity. These values were significantly lower than those observed in the infliximab group (61.8% of infliximab-treated patients had newly positive ANAs [*P* < 0.001], and 61.8% had a significant increase in ANA intensity [*P* < 0.001]). Similarly, the induction
of anti-dsDNA antibodies during 1 year of anti-TNFα treatment was significantly lower in the etanercept-treated SpA patients (3 of 20 patients [15%]) than in the infliximab-treated SpA patients (24 of 34 patients [70.6%]; \( P < 0.001 \)).

**Induction of IgM and IgA anti-dsDNA antibodies by TNFα blockade.** In order to confirm that the anti-dsDNA antibodies induced by infliximab treatment in RA as well as in SpA were exclusively of the IgM and/or IgA isotypes (8), and that isotype switching to IgG did not occur at later time points, we isotyped all induced anti-dsDNA antibodies. As shown in Figure 1, infliximab-induced anti-dsDNA antibodies in SpA as well as RA were predominantly of the IgM isotype at both 1 and 2 years. Among patients with SpA who were anti-dsDNA antibody positive, a combination of IgM and IgA isotypes was found in 44% at year 1 and in 11% at year 2. Among anti-dsDNA antibody-positive patients with RA, this combination of isotypes was found in 10% at year 1 and in 25% at year 2. Anti-dsDNA antibodies of the IgA class alone were not detected. Of major importance, no IgG anti-dsDNA antibodies were detected in either of the infliximab-treated cohorts over 2 years, with the exception of a single RA patient who was characterized by IgM anti-dsDNA antibodies at year 1 and a combination of IgM, IgA, and IgG anti-dsDNA antibodies at year 2. Interestingly, the anti-dsDNA antibodies observed in 3 etanercept-treated patients were also of the IgM isotype, and neither IgA nor IgG anti-dsDNA antibodies were detected. Because we detected high frequencies of IgM and virtually no IgG anti-dsDNA antibodies in the TNFα blockade-treated patients, we analyzed sera from 15 patients with SLE as an additional control. Nine of these SLE patients demonstrated anti-dsDNA reactivity, which was of the IgG isotype in all 9 patients, with associated IgM and/or IgA anti-dsDNA antibodies in 4 patients. Taken together, these data indicate that the pronounced induction of anti-dsDNA antibodies in SpA and RA is not associated with the occurrence of lupus-specific IgG anti-dsDNA antibodies.

**Disappearance of infliximab-induced ANAs and anti-dsDNA antibodies after treatment withdrawal.** Because short-term humoral immunity was suggested by the fact that anti-dsDNA antibodies were mainly of the IgM isotype, and that anti-dsDNA antibody levels fluctuated over time in individual patients and tended to decrease in patients with SpA after 1 year of treatment, we tested the persistence of induction over time in an additional 7 patients who were treated with infliximab for at least 5 months and in whom treatment had to be interrupted. As shown in Table 2, 4 patients had newly
Table 2. Persistence of infliximab-induced autoantibodies over time in 7 patients in whom treatment with infliximab was interrupted*

<table>
<thead>
<tr>
<th>Patient</th>
<th>No. of months receiving infliximab</th>
<th>Reason for stopping infliximab</th>
<th>No. of months after stopping infliximab</th>
<th>ANA intensity score</th>
<th>No. of patients with anti-dsDNA</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Baseline</td>
<td>Upon withdrawal</td>
<td>~1–3 years after withdrawal</td>
</tr>
<tr>
<td>RA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>12.5</td>
<td>Pneumonia</td>
<td>24</td>
<td>1+</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>14</td>
<td>Withdrawal of consent</td>
<td>33</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>3</td>
<td>12.5</td>
<td>Fever, esophagitis, cystitis</td>
<td>10.5</td>
<td>0</td>
<td>4+</td>
</tr>
<tr>
<td>4</td>
<td>5</td>
<td>Agranuloctysis</td>
<td>31</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>5</td>
<td>12.5</td>
<td>Herpes zoster</td>
<td>33</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>SpA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.5</td>
<td>Withdrawal of consent</td>
<td>34.5</td>
<td>0</td>
<td>3+</td>
</tr>
<tr>
<td>2</td>
<td>22.5</td>
<td>Pneumonia/abscess</td>
<td>16</td>
<td>0</td>
<td>3+</td>
</tr>
</tbody>
</table>

* Patients were tested at baseline, at the time infliximab was stopped, and ~1–3 years after withdrawal from treatment. ANA = antinuclear antibody; anti-dsDNA = anti–double-stranded DNA. ANAs were considered positive if the ANA intensity score was ≥2+ on a scale of 0 to 5+. RA = rheumatoid arthritis; SpA = spondylarthropathy.
positive ANAs and 5 patients had newly induced anti-dsDNA antibodies at the time when infliximab therapy was stopped. Isotyping revealed that the anti-dsDNA antibodies were predominantly of the IgM class. One patient also had IgG and IgA anti-dsDNA antibodies. A new analysis that was performed several months after infliximab treatment was stopped revealed no isotype switching to IgG anti-dsDNA antibodies in the other patients. In contrast, the ANA intensities decreased and the anti-dsDNA antibodies disappeared in all 5 patients.

**TNFα blockade and anti-ENA, antihistone, and antinucleosome antibodies.** In consideration of the induction of ANAs and anti-dsDNA antibodies, we investigated other lupus-related autoantibodies including anti-ENA, antihistone, and antinucleosome antibodies (22,23). As shown in Figure 1, the prevalence of anti-ENA and antihistone antibodies did not increase during TNFα therapy, but these levels fluctuated somewhat in individual patients. Similarly, antinucleosome antibody positivity developed in only 2 infliximab-treated patients (1 with SpA and 1 with RA) and in no etanercept-treated patients. In contrast, the control group of 15 patients with SLE who were not treated with TNFα blocker was characterized by multiple anti-ENA, antihistone, and antinucleosome antibody reactivities (15 anti-SSA, 5 anti-SSB, 6 anti-Sm, 3 anti-RNP, 1 anti-ribosomal P, 3 antihistone, 2 antinucleosome), confirming the different autoantibody profiles in patients treated with TNFα blockade compared with the profiles in untreated patients with SLE.

**Association of TNFα blockade–induced ANAs and anti-dsDNA antibodies with lupus-like characteristics.** Because the described biologic characteristics of the induced autoantibody profiles bring into question their clinical relevance in SLE, we systematically assessed other possible nonserologic lupus-like characteristics in all patients during longer-term followup. As shown in Table 3, no lupus-like features were observed in the etanercept-treated patients with SpA. Among the 34 patients with SpA who were treated with infliximab, mild leukopenia (>3,200 cells/μL) developed in 4 patients, and mild lymphopenia (>1,200 cells/μL) developed in 4 patients. However, these hematologic abnormalities were transient, occurred both in patients with (n = 6) and those without (n = 2) induction of anti-dsDNA antibodies, and were not associated with other lupus-like features. Lymphopenia was observed in nearly half of the infliximab-treated RA patients (n = 24) and was more pronounced in RA than in SpA. TNFα blockade was associated with mild leukopenia (>2,800 cells/μL) in 4 patients and with mild proteinuria in 4 patients. Again, however, these biologic abnormalities were not associated with clinical symptoms, were transient in most patients, and lymphopenia occurred both in patients with (n = 16) and those without (n = 8) induction of anti-dsDNA antibodies. None of the patients had to discontinue infliximab treatment due to development of lupus-like symptoms. The only patient with anti-dsDNA IgG antibodies at year 2 also had lymphopenia but no other associated clinical or biologic abnormalities. Because 3 RA patients and 5 SpA patients withdrew from the original infliximab studies before year 1 and were thus not included in the present serologic study, we also assessed the clinical followup files of these patients to exclude a possible selection bias; none of these patients experienced development of a lupus-like syndrome.

**TNFα blockade and aCL concentrations.** In order to investigate whether the induction of IgM but not IgG responses might also apply to other (auto)antibody systems, we analyzed another lupus-related reactivity, induction of aCL. In the infliximab-treated patients with SpA, the IgG aCL concentrations did not change significantly (5.46 units/mL at baseline, 5.21 units/mL at year 1, and 4.91 units/mL at year 2), whereas the IgM aCL concentrations increased significantly (1.69 units/mL at baseline, 2.14 units/mL at year 1, and 2.69 units/mL at year 2) (for baseline versus year 1, P = 0.043; for year

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**Table 3. Nonserologic lupus-like characteristics that occurred in the SpA and RA patients during TNFα blockade**

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>SpA (n = 34)</th>
<th>RA (n = 59)</th>
<th>SpA (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TNFα blocker</td>
<td>Infliximab</td>
<td>Infliximab</td>
<td>Etanercept</td>
</tr>
<tr>
<td>Followup period</td>
<td>2 years</td>
<td>2 years</td>
<td>1 year</td>
</tr>
<tr>
<td>Malar rash</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Discoid rash</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Photosensitivity</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Oral ulcers</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Serositis (pleurisy/</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>pericarditis)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurologic disorder</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(seizures/psychosis)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hematologic disorder†</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anemia</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Leukopenia</td>
<td>4</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Lymphopenia</td>
<td>4</td>
<td>24</td>
<td>0</td>
</tr>
<tr>
<td>Thrombocytopenia</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Renal disorder (proteinuria)</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
</tbody>
</table>

* Values are the number of patients. SpA = spondylarthropathy; RA = rheumatoid arthritis; TNFα = tumor necrosis factor α.
† Anemia was defined as hemolytic anemia with reticulocytosis; leukopenia was defined as a white blood cell count of <4,000/μL on at least 2 occasions; lymphopenia was defined as a lymphocyte count of <1,500/μL on at least 2 occasions; thrombocytopenia was defined as a thrombocyte count of <100,000/μL; proteinuria was defined as a protein concentration of >0.2 gm/liter.
1 versus year 2, \( P = 0.007 \) (Figure 2A). We confirmed these results in an independent experiment using commercially available ELISA kits (Orgentec) (data not shown).

Similarly, in the infliximab-treated patients with RA, the IgG aCL concentrations did not change (5.50 units/ml at baseline versus 5.40 units/ml at year 1), whereas the IgM aCL concentrations increased significantly (1.62 units/ml at baseline versus 2.59 units/ml at year 1; \( P < 0.001 \)) (Figure 2B). In the etanercept-treated patients with SpA, the IgG and IgM aCL concentrations did not change significantly (Figure 2C).

Figure 2. Effect of tumor necrosis factor \( \alpha \) blockade on IgG and IgM anticardiolipin antibodies in A, infliximab-treated patients with spondylarthropathy (SpA), B, infliximab-treated patients with rheumatoid arthritis, and C, etanercept-treated patients with SpA. Data are shown as box plots. Each box represents the 25th to 75th percentiles. Lines inside the boxes represent the medians. Lines outside the boxes represent the 10th and the 90th percentiles. Outliers are not displayed. ns = not significant. * = baseline versus year 1; † = year 1 versus year 2.
patients with SpA, the IgG aCL concentrations decreased between baseline and year 1 (4.53 units/ml and 3.47 units/ml, respectively; \( P = 0.028 \)), with a similar trend for IgM aCL concentrations (1.69 units/ml versus 1.42 units/ml; \( P = 0.056 \)) (Figure 2C). In terms of the aCL system, these data confirm that infliximab but not etanercept increases selectively the IgM autoantibody response without an isotype switch to IgG. However, the data also bring into question the clinical relevance, because the levels remained clearly below the diagnostic cutoff value associated with a phospholipid syndrome.

**DISCUSSION**

Although the fact that ANAs are induced during infliximab treatment is well known (1,7–12), the clinical and biologic correlates in autoimmune arthritis are not yet fully understood. The present study confirms and extends results from our previous short-term study (8) by indicating that the infliximab-induced autoantibody profiles are not critically dependent on the disease background and remain stable over a 2-year treatment period in both SpA and RA. Of interest, the induction of anti-dsDNA antibodies tended to be more pronounced in patients with SpA than in patients with RA. This effect could be related to the use of concomitant methotrexate therapy in patients with RA, because methotrexate can lead to a decrease in circulating autoantibodies in cutaneous lupus erythematosus (24). However, this influence of methotrexate could not be confirmed during infliximab treatment (7,9,11,12). Alternatively, the observed differences could be explained by the higher dosage of infliximab in patients with SpA, although the influence of the total dose of infliximab on new autoantibody formation remains controversial (7,9).

In the present study, 15 patients with RA received an extra (100-mg) vial of infliximab at each infusion, beginning at week 30, but this had no significant influence on autoantibody induction (data not shown).

The first major new finding of the present study is that etanercept did not lead to similar induction of ANAs and anti-dsDNA antibodies. Whereas the occasional appearance of ANAs and anti-dsDNA antibodies in a few etanercept-treated SpA patients is consistent with previous data in etanercept-treated patients with RA (4,14,25), this first prospective head-to-head comparison of both TNFα blockers indicates clearly that the induction of both ANAs and anti-dsDNA antibodies is far more pronounced during infliximab therapy than during etanercept treatment. Several explanations for this difference could be envisaged. First, the difference might be related to a general, nonspecific B cell activation by infliximab (26). However, neither infliximab-treated nor etanercept-treated patients with SpA showed significant changes in serum IgG, IgA, or IgM levels (data not shown). Second, the induction of ANAs has been suggested to be linked to alterations of apoptosis and the release of nuclear antigens (7,27). Although infliximab but not etanercept induces apoptosis of monocytes and T lymphocytes in Crohn's disease (28), both drugs induced apoptosis in RA synovium (29). Third, because the clearance of nuclear debris is also of crucial importance for ANA induction (30–32), down-regulation of the C-reactive protein (CRP) level could potentiate autoimmunity by reducing this clearance (7). However, the CRP level was profoundly decreased in both the infliximab-treated and etanercept-treated groups and was comparable in patients with and those without autoantibody induction (data not shown).

Besides the difference in autoantibody induction by infliximab and etanercept, a second biologic phenomenon of major importance is that the induction of autoantibodies was largely restricted to anti-dsDNA antibodies of the IgM or associated IgM–IgA subtype. Based on published data in human SLE and animal models of lupus, as well as our own SLE control group, one would expect the induction of antibodies of different isotypes, including IgG, against a variety of nuclear antigens released during apoptosis (22,23,33). Further studies have been undertaken to investigate whether the intriguing absence of other antinuclear reactivities and autoantibodies of the IgG subtype could be related to the T cell–dependent or independent nature of the nuclear antigen (34–39). This could also be related to modulation by TNFα blockade of specific B cell populations, especially the so-called IgM memory B cells, which are prone to differentiation in plasma cells producing predominantly high-affinity IgM and IgA against T cell–independent antigens (40). Independently of the exact mechanisms underlying the restriction of the induced responses to IgM and IgM/IgA anti-dsDNA antibodies, this profile suggests short-term, nonpathogenic humoral autoimmunity rather than a genuine SLE-associated signature (7,22,23,41–43). This is confirmed by 2 clinically important observations, as follows.

First, the level of induced ANAs decreased after therapy was withdrawn, with complete disappearance of the anti-dsDNA antibodies (including IgG anti-dsDNA antibodies in 1 patient). Second, we observed no lupus-like syndromes over a 2-year followup period, even in the rare case involving induction of IgG anti-dsDNA antibodies. The observed leukopenia and lymphopenia
were mostly mild and transient, occurred specifically in the RA patients who received concomitant methotrexate therapy, and were not associated with the induced autoantibodies. Although more than 40 cases of TNFα blockade–related lupus-like syndromes have been reported, the present data provide evidence that ANAs and anti-dsDNA antibodies as well as mild hematologic abnormalities can hardly be considered as lupus criteria in this context, and confirm that clinically relevant lupus erythematosus induced by TNFα blockade is rare (4,20,44,45).

A last important observation is that the differential effect on IgM and IgG antibody induction may be a more generalized effect of infliximab. Indeed, infliximab treatment induced an increase in IgM aCL concentrations in both SpA and RA but failed to increase IgG aCL, even during longer-term followup. In contrast, etanercept treatment decreased the aCL concentrations, indicating that infliximab and etanercept have not only quantitatively but also qualitatively different effects on the humoral autoimmune responses. Although the effect of TNFα blockade on aCL is controversial (10–12,46), the restricted increase of IgM aCL concentrations during therapy with infliximab but not etanercept parallels our findings for anti-dsDNA antibodies. Although these data have no direct clinical implication, because the aCL concentrations remained below the diagnostic cutoff, they indicate that the modulation of the humoral immune response by TNFα blockade is not restricted to the ANA/anti-dsDNA antibody system.

Taken together, the results of this study indicate that the prominent ANA and anti-dsDNA autoantibody response is not a pure class effect of TNFα blockers, is independent of the disease background, and is not associated with clinically relevant lupus-like symptoms. Furthermore, the restricted induction of anti-dsDNA antibodies of the IgM isotype and the similar findings for aCL suggest a broader biologic effect of TNFα blockade on humoral immunity.

ACKNOWLEDGMENTS

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REFERENCES


Soluble Interleukin-1 Receptor Accessory Protein Ameliorates Collagen-Induced Arthritis by a Different Mode of Action From That of Interleukin-1 Receptor Antagonist


Objective. To discern the mode of interleukin-1 (IL-1) inhibition of soluble IL-1 receptor accessory protein (sIL-1RAcP) by comparison with IL-1 receptor antagonist (IL-1Ra) in arthritis.

Methods. Adenoviral vectors encoding either sIL-1RAcP or IL-1Ra were administered systemically before onset of collagen-induced arthritis in DBA/1 mice. Anti-bovine type II collagen IgG and IL-6 were quantified in serum. Proliferative response of splenic T cells was determined in the presence of sIL-1RAcP or IL-1Ra. The effect on IL-1 inhibition of recombinant sIL-1RAcP and IL-1Ra was further examined in vitro, using NF-κB luciferase reporter cell lines. Quantitative polymerase chain reaction was used to determine the relative messenger RNA expression of the IL-1 receptors.

Results. Adenoviral overexpression of both sIL-1RAcP and IL-1Ra resulted in amelioration of the collagen-induced arthritis. Both IL-1 antagonists reduced the circulating levels of antigen-specific IgG2a antibodies, but only IL-1Ra was able to inhibit lymphocyte proliferation. By using purified lymphocyte populations derived from NF-κB reporter mice, we showed that sIL-1RAcP inhibits IL-1–induced NF-κB activity in B cells but not T cells, whereas IL-1Ra inhibited IL-1 on both cell types. A study in a panel of NF-κB luciferase reporter cells showed that the sIL-1RAcP inhibits IL-1 signaling on cells expressing either low levels of membrane IL-1RAcP or high levels of IL-1RII.

Conclusion. We show that the sIL-1RAcP ameliorated experimental arthritis without affecting T cell immunity, in contrast to IL-1Ra. Our results provide data in support of receptor competition by sIL-1RAcP as an explanation for the different mode of IL-1 antagonism in comparison with IL-1Ra.

The proinflammatory cytokine interleukin-1 (IL-1) is an important mediator controlling local and systemic effects on a wide variety of target cells, thereby regulating immunity and inflammation (1). IL-1 binds to IL-1 receptor type I (IL-1RI) (80 kd) (2,3), which results in the recruitment of the IL-1 receptor accessory protein (IL-1RAcP) (4–8). IL-1RAcP does not recognize the ligand but stabilizes IL-1 binding to the IL-1RI (9–14). Furthermore, IL-1RAcP is a crucial coreceptor in this complex by enabling recruitment and binding of intracellular adaptor proteins such as MyD88 and kinases such as IL-1R–associated kinases, ultimately leading to NF-κB activation (9–14). Another member of the IL-1 receptor family is IL-1RII (68 kd) (15), which upon binding of IL-1 also associates with IL-1RAcP. However, this does not lead to signal transduction because this receptor lacks the intracellular Toll–IL-1 receptor domain that is crucial for MyD88 binding (16–18). Therefore, the type II receptor is a decoy receptor as has been described for its transmembrane and soluble forms (19).

Another inhibitor of IL-1 signaling is the IL-1
receptor antagonist (IL-1Ra), which competes with IL-1 for occupation of only 1% of the IL-1RI by IL-1 instigates full-blown cell activation, and as a consequence, relatively high amounts of IL-1Ra (100–1,000-fold molar excess) are required to fully block IL-1. Fortunately, IL-1Ra binds poorly to IL-1RII (19), and both IL-1Ra and soluble IL-1RII (sIL-1RII) cooperate in IL-1 inhibition. We have demonstrated that administration of IL-1Ra protein or the IL-1Ra gene ameliorates disease in several models of experimental arthritis, with a profound protective effect against cartilage and bone destruction (20,21).

Recently, an alternative splice transcript of the membrane IL-1RACP, encoding a smaller and soluble protein comprising the 3 extracellular Ig domains and a unique C-terminal domain, has been described (22,23). This sIL-1RACP is mainly produced by the liver and circulates systemically. We showed that systemic overexpression of sIL-1RACP by adenoviral gene transfer in mice markedly ameliorates collagen-induced arthritis (CIA) (24,25). A possible explanation is that sIL-1RACP can interact with sIL-1RII, thus forming a high-affinity IL-1 scavenger (26). Although the mechanism and efficacy of IL-1 inhibition might be different, it is expected that under optimal conditions both IL-1Ra and sIL-1RACP may exert similar biologic effects.

This study was conducted to compare the inhibitory effects of sIL-1RACP and IL-1Ra in CIA, and to discern their modes of action. Systemic overexpression of either sIL-1RACP or IL-1Ra using adenoviral vectors before onset of CIA resulted in amelioration of CIA, with both demonstrating their potential as IL-1 inhibitors in vivo. Treatment with both inhibitors resulted in a clear reduction of circulating levels of antigen-specific IgG2a and IL-6. However, sIL-1RACP treatment did not affect T cell function, whereas IL-1Ra inhibited both antigen- and mitogen-induced lymphocyte proliferation. Next, we studied the effect of both inhibitors on enriched T and B cells obtained from mice expressing an NF-κB reporter gene. Recombinant sIL-1RACP protein was unable to block IL-1 signaling on T cells, while IL-1Ra inhibited IL-1–induced NF-κB activation in both T and B cells. A study of an array of reporter cell types that differ in their expression of the IL-1 receptors (IL-1RI, IL-1RII, and the coreceptor IL-1RACP) provided a possible explanation for the cell type–specific inhibitory effect of sIL-1RACP, i.e., receptor competition with the membrane IL-1RACP.

**MATERIALS AND METHODS**

**Animals.** Male 10–12-week-old DBA-1/bom mice were obtained from Bomholtgård (Ry, Denmark). C57BL/6 mice were obtained from Charles River (Sulzfeld, Germany); all mice were housed in low-pressure isolator cages during the experiments. The animals were fed a standard diet with water and food ad libitum. All in vivo studies complied with national legislation and were approved by local authorities for the Care and Use of Animals with related codes of practice.

**Construction of adenoviral vector.** The sIL-1RACP or IL-1Ra gene was obtained from murine liver complementary DNA (cDNA) and cloned into the first-generation E1/E3-deleted serotype-5 adenoviral vectors and produced according to the method described by Chartier et al (27). All constructed viruses contained the RGD amino acid motif, which was incorporated into the H1 loop of the adenoviral fiber knob (28,29). Purified recombinant adenoviral vector DNA was linearized through digestion with Pac I and transfected into 293 viral packaging cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). The Ad sIL-1RACP, Ad IL-1Ra, and Ad luciferase were purified using 2 × CsCl gradient purification and stored in small aliquots at −80°C. Viral titer of the purified viral vectors was determined on 911 indicator cells by immunohistochemical detection of viral capsid protein 20 hours after transfection (30).

**Production and purification of sIL-1RACP recombinant protein.** C-terminal FLAG-tagged sIL-1RACP recombinant protein was obtained from a stable transduced NIH3T3 cell line and purified as described previously (24). Briefly, sIL-1RACP was purified at 4°C from conditioned supernatants through affinity chromatography using anti-FLAG M2 affinity gel beads (Sigma, St. Louis, MO), followed by competitive elution with a solution containing 100 µg/ml 3 × FLAG peptide (Sigma) in 50 mM Tris HCl, 150 mM NaCl (pH 7.4). The protein was concentrated using molecular weight cutoff filters of 10 kDa (Amicon; Millipore, Bedford, MA) and stored in silicon-coated reaction tubes at 4°C for later usage. Purified protein was quantified using Coomassie Protein Assay Reagent according to the manufacturer’s instructions (Pierce, Rockford, IL), and the average production of sIL-1RACP by stably transduced NIH3T3 fibroblasts was ~500 ng/24 hours/1 × 10⁶ cells.

**Inhibition studies using IL-1Ra and sIL-1RACP.** The IL-1–responsive murine fibroblast NIH3T3 cell line, the EL-4 NOB-1 thymocytic cell line (American Type Culture Collection, Rockville, MD), and the H4 chondrocyte cell line (31) were all stably transduced with an NF-κB luciferase reporter construct. Reporter cells were seeded in Krystal 2000 96-well plates (Thermo Labsystems, Brussels, Belgium) at a concentration of 2–4 × 10⁴ cells/well, and cultured for 1 day at 37°C in 5% CO₂/95% air. Cells were incubated for 24 hours with either sIL-1RACP or IL-1Ra (Amgen Boulder, Boulder, CO) followed by addition of different concentrations of murine recombinant IL-1α (1 ng/ml or 10 ng/ml). Six hours after IL-1α addition, the intracellular luciferase activity was quantified using a Bright-Glo luciferase assay system (Promega, Madison, WI) followed by luminometric detection according to the manufacturer’s protocol (Polarstar Galaxy, Offenburg, Germany).

**Induction of CIA.** Bovine type II collagen (BII) was dissolved in 0.05M acetic acid to a concentration of 2 mg/ml
and was emulsified in equal volumes of Freund's complete adjuvant (Mycobacterium tuberculosis strain H37Ra; Difco, Detroit, MI). The mice were immunized at the base of the tail with an intradermal injection of 100 µl of emulsion (100 µg BII). On day 21, mice without clinical manifestations of arthritis received an intraperitoneal booster injection of 100 µg of BII dissolved in phosphate buffered saline (PBS). To evaluate systemic effects of sIL-1RAcP and IL-1Ra on development of CIA, we applied adenoviral delivery of the transgenes to immunized mice. At the onset of arthritis, we injected 3 × 10^10 plaque-forming units of adenoviral vector intravenously in immunized mice without clinical signs of CIA (22–24). Arthritis development in the hind paw and ankle joint was macroscopically monitored through day 38, at which point the experiment was terminated and the mice were killed. Arthritis was scored by 2 independent observers in a blinded manner on a scale ranging from 0 to 2 for each limb, based on redness, swelling, and, at later stages, ankylosis, as follows: 0 = no changes; +0.25 = 1–2 toes red or swollen; +0.5 = 3–5 toes red or swollen; +0.5 = swollen ankle; +0.5 = swollen footpad; +0.5 = severe swelling and ankylosis. A cumulative score was derived for all 4 paws, yielding a maximal possible score of 8 per animal. Serum was obtained and stored for further analysis.

**Determination of specific IgG titers against type II collagen.** IgG2a anti-BII titers were determined by enzyme-linked immunosorbent assay. Briefly, 96-well plates were coated with 10 µg of BII, followed by blocking of nonspecific binding sites. Serial dilutions of the mouse sera were added, followed by incubation with isotype-specific goat anti-mouse peroxidase (Southern Biotechnology Associates, Birmingham, AL) and substrate (5-aminosalicylic acid; Sigma). Absorbance was determined at 492 nm.

**Lymphocyte stimulation assay.** Splenocytes were obtained from mice systemically treated with sIL-1RAcP, IL-1Ra, or the control adenoviral vector AdCMV Luciferase. Mice previously immunized against BII were injected with the adenoviral vectors. Splenocytes from mice which had already developed CIA were used for lymphocyte proliferation assays in the presence or absence of recombinant mouse IL-1Ra (10 µg/ml). Three days after receiving the viral load, mice were killed and their spleens were dissected. Splenocytes were disrupted and erythrocytes were removed by osmotic shock.

**Isolation of NF-κB reporter T and B lymphocytes.** Remaining cell fraction was washed twice and incubated in RPMI 1640 at 37°C in 5% CO₂, 95% air for 1 hour in order to remove the SAC fraction. Nonadherent lymphocytic cells were used for the cell stimulation assay. Lymphocyte stimulation was determined against the T lymphocyte–stimulating concanavalin A and the immunization antigen BII (heated for 10 minutes at 80°C). Thymocytes (1 × 10^5) were added to the stimuli and incubated for 3 days (37°C in 5% CO₂, 95% air). Six hours before harvesting of the cells, 0.25 µCi of 3H-thymidine was added. The amount of incorporated 3H-thymidine was quantified using a Micro Beta-plate reader (Perkin-Elmer, Brussels, Belgium).

**Isolation of NF-κB reporter T and B lymphocytes.** Spleen lymphocytes were obtained from 3 × NF-κB reporter mice (32). Spleens were disrupted and erythrocytes were removed by osmotic shock. Remaining cell fraction was

**Statistical analysis.** Nonlinear regression analysis was performed using GraphPad Prism 4.0 software (GraphPad Software, Inc., San Diego, CA).
The dose-response curves were analyzed and fitted according to a sigmoidal dose-response model (with variable slope). Significance of differences was determined using Student’s t-test or Mann-Whitney U test (GraphPad Software). P values less than 0.05 were considered significant.

RESULTS

Amelioration of CIA by adenovirus-mediated overexpression of sIL-1RAcP and IL-1Ra. Both adenoviral vectors were validated and gene transfer resulted in
high production of sIL-1RαCp and IL-1Ra as described previously (24,35). Systemic overexpression of sIL-1RαCp resulted in circulating amounts of sIL-1RαCp up to 900 ng/ml 3 days after injection of the adenoviral vector (24). Prophylactic treatment through systemic overexpression of both sIL-1RαCp and IL-1Ra resulted in a marked reduction of disease severity in both front and hind paws, which was significant from day 30 through day 38 (Figure 1A). Although sIL-1RαCp treatment reduced the severity of arthritis, progression was still evident. This was in contrast to systemic IL-1Ra gene transfer, which arrested development of CIA.

Reduction of circulating levels of anti-BII IgG and IL-6 by sIL-1RαCp treatment. Systemic overexpression of sIL-1RαCp in mice after immunization resulted in a significant reduction of circulating levels of IgG2a antibodies directed against BII after 7 days (Figure 1B). Therapeutic intervention using IL-1Ra also resulted in a significant reduction of circulating anti-BII IgG2a (Figure 1B). Reduced levels of the B cell–activating cytokine IL-6 preceded the observed reduction of circulating anti-BII IgG levels, which was evident 3 days after either sIL-1RαCp or IL-1Ra adenoviral injection. Mean ± SEM levels of IL-6, determined in serum from at least 3 treated animals per group, were as follows: control 527 ± 47 pg/ml, sIL-1RαCp 188 ± 31 pg/ml (P < 0.05 versus control), IL-1Ra 217 ± 23 pg/ml (P < 0.05 versus control).

Lack of effect of sIL-1RαCp, in contrast with IL-1Ra, on lymphocyte activity. Splenocytes were isolated from BII-immunized mice 3 days after systemic injection with Ad sIL-1RαCp, Ad IL-1Ra, or the control vector adenovirus luciferase. Lymphocytes obtained from IL-1Ra–treated animals showed a significantly reduced overall proliferative response toward the immunization antigen BII and the mitogen concanavalin A. Although both IL-1Ra and sIL-1RαCp ameliorated CIA, sIL-1RαCp treatment did not reduce lymphocyte proliferative activity, in contrast to IL-1Ra treatment (Figure 1C). To confirm that sIL-1RαCp was unable to inhibit lymphocyte proliferation, we performed an in vitro experiment using spleen-derived T lymphocytes from untreated DBA/1 mice with CIA. These lymphocytes were cultured in the presence or absence of either recombinant IL-1Ra or sIL-1RαCp protein and stimulated with the pan-T cell activator concanavalin A. Recombinant IL-1Ra (10 μg/ml) significantly inhibited the concanavalin A–induced lymphocyte proliferation in vitro, with 47% and 53% concentrations of concanavalin A at 1.25 μg/ml and 0.6 μg/ml, respectively. This revealed the importance of IL-1 in lymphocyte function, and confirmed our in vivo findings of IL-1Ra as a functional antagonist of T cell proliferative activity. Interestingly, and in contrast to IL-1Ra, sIL-1RαCp at a high dose (10 μg/ml) was not able to inhibit lymphocyte activity.

Inhibition of IL-1 signaling on B cells, but not T cells, by sIL-1RαCp. The different effect of sIL-1RαCp treatment on immunity, in which sIL-1RαCp mainly inhibited humoral immunity without affecting T cell

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**Figure 2.** Reduction of NF-κB activity by sIL-1RαCp in purified B cells. Purified spleen T and B cells from bovine type II collagen-immunized DBA/1 mice were analyzed for their relative expression of mRNA encoding for IL-1RI, IL-1RII, and IL-1RαCp. A, Relative expression was determined by quantitative polymerase chain reaction (PCR). Values in parentheses are the relative expression compared with the IL-1RI. Cycle threshold (Ct) values were corrected for the housekeeping gene GAPDH and expressed as $2^{-\Delta\Delta Ct}$ ($\times 10^{-4}$). Quantitative analysis was performed after 35 PCR cycles. Data are the mean expression of 2 samples; the SD was <0.5 cycles for each primer set. B, Marked reduction of IL-1–induced NF-κB activation in T cells incubated with IL-1Ra, but not in sIL-1RαCp–treated T cells. In contrast to T cells, splenic B cells were sensitive toward IL-1 inhibition by both IL-1Ra and sIL-1RαCp, which resulted in a marked reduction of IL-1–induced NF-κB activity in these cells. Data are the mean and SD relative light units (RLU) with at least 4 measurements per group. See Figure 1 for other definitions.
function, might reflect a discrepancy of sIL-1RαCp inhibition of IL-1 signaling on T and B cells. To demonstrate that in vivo effects of sIL-1RαCp were the result of a direct effect of sIL-1RαCp–induced modulation of IL-1 signaling on B cells, we tested the IL-1 responsiveness of T and B cells in the presence or absence of sIL-1RαCp, using IL-1Ra as a control. Purified T and B cell populations derived from transgenic mice expressing a 3 × NF-κB reporter gene were shown to be sensitive to IL-1Ra treatment, which resulted in reduced NF-κB activity upon IL-1 stimulation (Figure 2). Interestingly, incubation with a similar dose of sIL-1RαCp in the presence of IL-1 did not affect NF-κB activation in purified T cell populations, whereas the enriched B cell fraction was shown to be sensitive to sIL-1RαCp treatment, although these differences did not reach statistical significance (Figure 2).

Availability of the membrane IL-1RαCp accounts for the efficacy of sIL-1RαCp in inhibition of IL-1 signaling. T and B cell populations expressed all 3 IL-1 receptor subtypes. Interestingly, B cells strongly expressed IL-1R11, revealing 21 times more IL-1R11 messenger RNA (mRNA) compared with IL-1R1, while T cells expressed 5 times more IL-1R11 compared with IL-1R1 (Figure 2A). The levels of expression of the different types of IL-1 receptors may contribute to the antagonistic activity of sIL-1RαCp. Therefore, we wanted to further substantiate the mechanism of action in vitro using NF-κB reporter cell lines. For these experiments, the reporter cell lines were characterized by their expression of IL-1R1, IL-1R11, and IL-1RαCp. Detection of the IL-1 receptor components on cell lines using immunohistochemistry or fluorescence-activated cell sorting analysis was not possible due to low surface expression of all 3 IL-1 receptor components, which hampered their detection. Therefore, we used the more sensitive quantitative PCR for analysis of mRNA expression. On H4 chondrocytes, which, compared with B cells, also highly express IL-1R11, sIL-1RαCp efficiently inhibited IL-1 signaling (Figure 3A). A concentration of 1,000 ng/ml sIL-1RαCp inhibited the IL-1 (10 ng/ml) response by 47%; the maximal observed inhibition of 88% was observed at a concentration of 10,000 ng/ml sIL-1RαCp (Figure 3C).

The EL4.NOBI thymoma cell line expressed very low amounts of the IL-1R11 (Figure 4A); even at the highest concentration of 10,000 ng/ml sIL-1RαCp was unable to inhibit IL-1–induced NF-κB activity (Figure 4C). In contrast to EL4.NOBI thymocytes and H4 chondrocytes, the NIH3T3 cell line showed no expression of mRNA for IL-1R11 and low expression of the membrane IL-1RαCp (Figure 5A). However, the
NIH3T3 cells were clearly susceptible to dose-dependent IL-1 inhibition by sIL-1RaCp, revealing a
48% inhibition of the IL-1 activation at a concentration of 1,000 ng/ml in the presence of 10 ng/ml IL-1α. A 1,000-fold concentration of IL-1Ra (1,000 ng/ml IL-1Ra in the presence of 1 ng/ml IL-1α) was able to completely inhibit IL-1–induced NF-κB activity with all 3 reporter cell lines.

**DISCUSSION**

Membrane IL-1RAcP is a crucial component of the IL-1 signaling complex, stabilizing the IL-1–IL-1RI complex and mediating IL-1 signaling (7,16,17,36). The recently discovered naturally occurring alternative splice transcript of the membrane IL-1RAcP comprises the 3 extracellular Ig-like domains (4,22,23). Due to the conservation of the ligand-binding domains of sIL-1RAcP, this molecule may still share the receptor complex stabilizing function, thereby acting as an inhibitor of IL-1 signaling through formation of an IL-1 trap. This action of sIL-1RAcP could therefore be distinct from IL-1Ra–mediated inhibition of IL-1 signaling, since binding of IL-1Ra to the IL-1RI does not lead to recruitment of IL-1RAcP (4,37,38). In this study, we compared the effect of both inhibitors on CIA.

Adenovirus-mediated intravenous transfer of sIL-1RAcP or IL-1Ra gene resulted in a clear-cut protective effect on bone and cartilage in murine CIA, as was shown previously (20,21,24,25). Accordingly, we expected similar effects of both inhibitors on the parameters of immunity and inflammation. Indeed, both inhibitors reduced circulating levels of antigen-specific IgG and IL-6, a well-known B cell activating and maturing cytokine (39). This observation supports the contention that IL-1 is an adjuvant for antibody production (40,41). In contrast, IL-1Ra gene transfer in mice inhibited both mitogen- and antigen-induced lymphocyte proliferation, whereas sIL-1RAcP gene transfer has no effect on this parameter. It is known that IL-1 plays an important role in the activation of T lymphocytes in CIA (42). Furthermore, the proliferative activity of T cells is enhanced in IL-1Ra−/− mice while decreased in IL-1α/β−/− mice, highlighting the importance of IL-1 in T cell activation and proliferation (43,44). However, this unexpected discrepancy in T cell modulation could be due to differences in the pharmacokinetics of the IL-1 inhibitors. Therefore, we tested both inhibitors in vitro using recombinant protein.

Splenocytes from mice with CIA were obtained and incubated with either sIL-1RAcP or IL-1Ra. Recombinant IL-1Ra reduced mitogen-specific T cell proliferation, in contrast to sIL-1RAcP, which did not affect the T cell proliferative response. Likewise, in experiments using enriched T and B cell populations from NF-κB reporter mice, sIL-1RAcP inhibited IL-1–induced NF-κB activity in B cells but had no effect on IL-1 signaling in T cells. Notably, IL-1Ra inhibited IL-1–induced NF-κB activity in both T and B lymphocytes. These results confirmed the selective mode of action of sIL-1RAcP compared with IL-1Ra as observed in vivo. A possible explanation is that sIL-1RAcP acts as an IL-1 antagonist by competing for the association of IL-1RI with membrane IL-1RAcP. Therefore, differen-
tial expression of the IL-1 receptor components between cell types could determine the antagonistic activity of sIL-1RAcP. This was further emphasized by the fact that T and B cell populations are known to differ in their receptor expression (15,45). B cells derived from BII-immunized mice revealed abundant expression of the IL-1RII, in contrast to purified T cells.

To elucidate the mechanism behind the cell-specific antagonistic activity of sIL-1RAcP, we compared 3 NF-κB luciferase reporter cell lines that differ in their IL-1 receptor composition (IL-1R expression in relation to antagonist activity is summarized in Table 1). We demonstrated that sIL-1RAcP was able to inhibit IL-1–induced NF-κB activation in cell lines that express either low levels of membrane IL-1RAcP or high levels of IL-1RII. It has already been shown that membrane-anchored sIL-1RAcP functions as a coreceptor and competitive IL-1 inhibitor (23). Those same authors were, however, unable to observe IL-1 inhibition using sIL-1RAcP on Hep-G2 cells, which they attributed to a low sIL-1RAcP concentration at the membrane level. Alternatively, the inability of sIL-1RAcP to inhibit IL-1 signaling on Hep-G2 cells could be a consequence of the high expression of membrane IL-1RAcP (46), in comparison with the low expression of NIH3T3 fibroblasts, for which we were able to induce sIL-1RAcP–mediated IL-1 antagonism.

Taken together, our results show that the underlying mechanism of cell specificity is probably dependent on the availability of membrane IL-1RAcP for IL-1 signaling. As previously reported, sIL-1RAcP can interact with soluble IL-1RII, thus forming an IL-1 scavenging molecule (26). Hence, the formation of this IL-1 trap, together with the observed inhibition of IL-1 at the cell membrane, are both mechanisms of IL-1 antagonism which could operate simultaneously in vivo. We observed IL-1 antagonism by sIL-1RAcP on cells highly expressing IL-1RII (B cells and H4 chondrocytes). Since we cannot exclude the formation of a membrane-bound IL-1 decoy receptor comprised of IL-1RII and sIL-1RAcP, this could still be a possible mechanism of IL-1 antagonism on cells expressing high levels of IL-1RII. However, the IL-1RII can also indirectly determine the availability of the IL-1RAcP. The amount of IL-1RII expression on the membrane determines the sensitivity of the cell toward IL-1, and membrane IL-1RAcP is able to interact with the IL-1RII (18,47). Therefore, abundant expression of the IL-1RII will not only lead to scavenging of IL-1, but will also reduce the available IL-1RAcP to form a signaling complex with the type I receptor, shifting the balance in favor of the sIL-1RAcP for receptor competition with the IL-1RAcP for formation of receptor complexes with IL-1RI.

This study provides evidence for an additional antagonistic mechanism by which sIL-1RAcP could directly inhibit IL-1 signaling at the cell membrane level. Due to the distinct action of sIL-1RAcP compared with IL-1Ra, both functionally and mechanistically, combining both antagonists offers additive therapeutic effects (25). With the sIL-1RAcP, we can now distinguish the roles of IL-1 in T cell– and B cell–driven processes, which may provide opportunities for treatment of diseases in which B cell derangement plays a prominent role.

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Effective Treatment of Collagen-Induced Arthritis by Adoptive Transfer of CD25+ Regulatory T Cells

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Objective. Regulatory T cells play an important role in the prevention of autoimmunity and have been shown to be effective in the treatment of experimental colitis, a T cell–mediated and organ-specific disease. We previously demonstrated that intrinsic CD25+ regulatory T cells modulate the severity of collagen-induced arthritis (CIA), which, in contrast to colitis, is a systemic antibody-mediated disease and an accepted model of rheumatoid arthritis. We undertook this study to determine whether regulatory T cells have the potential to be used therapeutically in arthritis.

Methods. We transferred CD4+CD25+ T cells into mice exhibiting arthritis symptoms, both immunocompetent mice and mice subjected to lethal irradiation and rescued with syngeneic bone marrow transplantation.

Results. A single transfer of regulatory T cells markedly slowed disease progression, which could not be attributed to losses of systemic type II collagen-specific T and B cell responses, since these remained unchanged after adoptive transfer. However, regulatory T cells could be found in the inflamed synovium soon after transfer, indicating that regulation may occur locally in the joint.

Conclusion. Our data indicate that CD25+ regulatory T cells can be used for the treatment of systemic, antibody-mediated autoimmune diseases, such as CIA.

Collagen-induced arthritis (CIA) in mice is a commonly used model for studying rheumatoid arthritis (RA) in humans. Although CIA is not identical to RA, the two diseases share many key features, such as synovitis, erosions of both bone and cartilage, and class II major histocompatibility complex–linked susceptibility (1). CIA is induced through immunizations with bovine type II collagen (CII) emulsified in Freund’s complete adjuvant (CFA), which leads to CII-specific antibody production. These antibodies are crucial and sufficient for disease induction. Therefore, CIA can be considered a systemic B cell–dependent and antibody-mediated autoimmune disease.

It is hypothesized that the bovine CII–specific antibodies recognize murine CII in articular cartilage, leading to the activation of the complement system and the consequent release of C5a (among other components), which acts as a chemoattractant for immune cells. In the joint, these cells are activated through Fc receptor (FcR) triggering, eventually resulting in chronic inflammation and destruction of the joints (for review, see refs. 2 and 3). This concept is supported by the hampered ability of B cell–deficient, C3-deficient, C5a receptor–deficient, and FcyR-deficient mice to develop disease (4–9). CD4+ T cells are also important during the initiation of arthritis by virtue of their ability to help B cells, a finding supported by CD4+ T cell depletion experiments using monoclonal antibodies (mAb) (10). T cells are less crucial during the effector phase, since depletion of CD4+ T cells in established arthritis does not influence disease severity (10). Instead, the main effector cells appear to be innate immune cells, which
are capable of maintaining joint inflammation and initiating erosions of cartilage and bone (3).

CD4+,CD25+ regulatory T cells are potent suppressors of T cell responses both in vitro and in vivo (11). In vivo, they are important in the prevention of organ-specific autoimmune diseases, as demonstrated by experiments in which splenocytes that had been depleted of CD25+ cells prior to adoptive transfer to immunocompromised mice caused the development of gastritis and thyroiditis as well as several other organ-specific autoimmune diseases (12). Currently, research focuses on their therapeutic value, and they have been successfully used to treat established colitis, a T cell–mediated and organ-specific autoimmune disease caused by the transfer of CD4+,CD45RB<sup>high</sup> T cells to SCID mice (13). Additionally, prophylactic treatment with CD4+,CD25+ regulatory T cells has been reported to diminish the severity of other T cell–mediated autoimmune diseases, such as experimental autoimmune encephalomyelitis (14) and diabetes (15).

We have previously demonstrated that depletion of CD25+ regulatory T cells before vaccinating mice with bovine CII results in an exacerbation of arthritis that is associated with an increase in CII-specific antibodies (16). These data indicate that regulatory T cells are also involved in the control of systemic B cell–dependent and antibody-mediated autoimmune disease. However, it is not known whether regulatory T cells can also be used therapeutically in such diseases. To investigate the potential of treating CIA with CD25+ regulatory T cells, we adoptively transferred CD4+,CD25+ regulatory T cells into mice during the early stage of arthritis. Our data indicate that CD4+,CD25+ regulatory T cells were able to diminish the clinical severity of arthritis despite a lack of reduction in systemic CII-specific T and B cell responses. Using immunohistochemistry, CD4+,CD25+ regulatory T cells were traced to the synovial tissue in affected joints, indicating that these cells may modulate inflammation locally.

**MATERIALS AND METHODS**

**Mice.** DBA/1 mice were bred at the animal facility of The Netherlands Organization for Applied Scientific Research–Prevention and Health (Leiden, The Netherlands) and then transferred to the animal facility at the Leiden University Medical Center (Leiden, The Netherlands). Mice that underwent bone marrow transplantation (BMT) were kept in filter-top cages under sterile conditions; otherwise, mice were kept under conventional conditions. Mice that underwent BMT were given an irradiated sterile diet (Hope Farms, Woerden, The Netherlands) and acidified water containing ciprofloxacin (85 mg/liter; Bayer, Leverkusen, Germany) and polymyxin B (70 mg/liter; Bristol-Myers Squibb, Woerden, The Netherlands). Treatment and maintenance were in accordance with the national guidelines for animal care. The Experimental Animal Commission of the Leiden University Medical Center approved the experiments described in this article.

**Induction and evaluation of CIA.** Bovine CII (Chondrex, Redmond, WA) was dissolved in a 0.1M acetic acid solution overnight at 4°C at a concentration of 2 mg/ml. The dissolved bovine CII (100 μg/mouse) was emulsified with an equal volume of CFA (Difco, Detroit, MI), and a total of 100 μl was injected subcutaneously into the base of the tail. Mice were examined 3 times each week beginning 2 weeks after immunization. The front and hind paws were evaluated for signs of arthritis. The severity of arthritis was graded for each paw using the following scoring system: 0 = normal joint, 1 = 1 or 2 swollen joints, 2 = >2 swollen joints, and 3 = extreme swelling of the entire paw and/or ankylosis. An arthritis score (range 0–12) was assigned to each mouse by summing the scores of each paw. In all experiments except those involving BMT, mice were killed when they had 2 paws with maximal swelling, as dictated by the Experimental Animal Commission.

**BMT.** BMT was performed when ~50% of the mice were clinically affected with arthritis. Bone marrow cells were collected by flushing femurs and tibiae of syngeneic donor DBA/1 mice with 1% bovine serum albumin (BSA)/phosphate buffered saline (PBS). For T cell depletion of the graft, cell suspensions were incubated first with fluorescein isothiocyanate (FITC)–conjugated anti-CD3 mAb and then with magnetic-activated cell sorting (MACS) anti-FITC microbeads (Miltenyi Biotec, Auburn, CA), and cells were removed by magnetic sorting. Prior to BMT, arthritic mice were subjected to lethal doses of total body irradiation (TBI; 9 Gy). On the day following TBI, purified CD4+ T cell subsets (5 × 10<sup>5</sup>) were injected intravenously (IV). The second day after TBI, an injection of T cell–depleted bone marrow cells (2.5 × 10<sup>5</sup>) was administered.

**Isolation, activation, and transfer of CD4+ T cell subsets.** Purified CD4+ cells were isolated from spleens obtained from naive DBA/1 mice, first by staining splenocytes with FTC-conjugated anti-mouse CD4 and then by using a MACS anti-FITC Multisort kit (Miltenyi Biotec), which allows for multiple positive selections of cells through the removal of microbeads from the cells. CD25+ cells were isolated from the CD4+ cells by first staining with biotin-conjugated anti-CD25 mAb (BD Pharmingen, San Diego, CA) followed by incubation with MACS antibiotin microbeads (Miltenyi Biotec)). CD4+,CD25+ T cells were then positively selected on a MACS LS-sized column, and the flow-through was collected as CD4+,CD25− T cells. The purity of all cell subsets using this method was >95% as determined by fluorescence-activated cell sorting analysis (data not shown). Isolated cells were activated by overnight incubation on 24-well plates coated with 2 μg/ml anti-CD3 (145-2C11) in the presence of 100 IU/ml interleukin-2 (IL-2; Sanver Tech, Heerhugowaard, The Netherlands), in RPMI 1640 medium supplemented with 1 unit/ml penicillin, 1 μg/ml streptomycin, 20 mM L-glutamine, 50 μM β-mercaptoethanol, and 8% fetal calf serum (FCS),
For IV injection, cells were suspended in 0.1% BSA/PBS, and a total volume of 200 µl was injected per mouse. Transfer was performed when ~50% of the mice had developed arthritis.

Isolation of messenger RNA (mRNA) and reverse transcription–polymerase chain reaction (RT-PCR) for Foxp3 expression in T cell subsets. Total RNA was isolated from T cell subsets (after overnight stimulation with anti-CD3 and IL-2) using RNA-Bee (Tel-Test, Friendswood, TX) according to the manufacturer’s recommendations. RT-PCR was performed with 2 µg of total RNA for complementary DNA (cDNA) synthesis. The following primers for Foxp3 expression were used: 5′-ACA-CCA-CCC-ACC-ACC-GCC-CTC-G-3′ (forward) and 5′-CAT-TTG-CCA-GCA-GTG-GGT-AG-3′ (reverse). For the generation of cDNA, a total of 40 cycles were run under the following conditions: 1 minute at 95°C, 1 minute at 57°C, and 1 minute at 72°C. The cDNA products were verified by sequencing.

Collagen-specific cell proliferation and suppression assay. Splenocytes were isolated from treated or naive mice. Cells were restimulated with 20 µg/ml bovine CII and cultured with Dulbecco's modified Eagle’s medium (Life Technologies, Paisley, UK) supplemented with 1 unit/ml penicillin, 1 µg/ml streptomycin, 20 mM l-glutamine, 50 µM β-mercaptoethanol, and 8% FCS in 96-well round-bottomed plates at a concentration of either 2.5 × 10^4 cells/well or 5 × 10^4 cells/well. Proliferation was measured 3–5 days later by addition of 0.5 µCi/well of tritiated thymidine (3H-TdR). Values are the mean (minus average medium values) and SEM of triplicate experiments.

After isolating and activating CD4+,CD25− and CD4+,CD25+ T cells, cells were cultured in 96-well round-bottomed plates in RPMI 1640 medium with equal numbers of freshly isolated splenocytes from bovine CII–immunized mice. Proliferation was stimulated by adding phytohemagglutinin (PHA) at a dilution of 1:400 and/or bovine CII at a concentration of 20 µg/ml. Incorporation of 3H-TdR was measured after 4–6 days of culture. Values are the mean (minus average medium values) and SEM of triplicate experiments.

Measurement of serum collagen-specific antibodies. Antibodies were measured by enzyme-linked immunosorbent assay (ELISA). Immuno-Maxisorp Plates (96-well; Nunc, Roskilde, Denmark) were coated with ELISA-grade bovine or murine CII (Chondrex) overnight at 4°C. After washing with PBS–0.05% Tween 20, plates were blocked with PBS/10% milk for 2 hours at 4°C. Serially diluted mouse serum was then incubated on the washed plates overnight at 4°C and washed again the following morning. Rabbit anti-mouse SAP (Calbiochem) was incubated for 2 hours at room temperature to detect the bound SAP. A secondary antibody was used (HRP-conjugated swine anti-rabbit; Dako, Heverlee, The Netherlands) to allow for detection. Substrate reactions were detected in the same manner as for the bovine or murine CII-specific antibody protocol.

Detection of cells in synovial tissues. T cell subsets were isolated and activated as described above. After activation, cells were stained with 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes Europe, Leiden, The Netherlands). Cells were suspended in 1% BSA/PBS containing 0.5 µM CFSE and then incubated for 10 minutes at 37°C in the dark. FCS was then added until a final concentration of 5% was reached. Cells were then washed twice with 1% BSA/PBS before IV transfer to arthritic mice. Between 1 and 2 days after transfer, mice were killed, and various tissues were sampled. Synovial tissue was harvested by first removing the surrounding muscle tissue and then removing the tissue lining the joint. Tissue samples were snap-frozen in Tissue-Tek (Sakura, Tokyo, Japan). In order to detect CFSE-labeled cells from the sorted tissues, we prepared acetone-fixed frozen sections. These sections were blocked with 10% mouse serum in PBS. Incubating the slides in a 1% H2O2,0.1% NaN3 solution for 20 minutes blocked endogenous peroxidase activity. CFSE+ cells were detected using an HRP-conjugated anti-FITC antibody (Dako) as previously described (17). A substrate reaction with 3,3′-diaminobenzidine (Dako) was then used to visualize the cells. Concomitant staining of CD4+ T cells was performed by first staining with FITC-conjugated anti-mouse CD4 and then with the HRP-conjugated anti-FITC secondary antibody.

Statistical analysis. Significant differences in the severity of arthritis symptoms in CD25+ T cell–treated and untreated mice on individual days and between levels of T cell proliferation were examined with Student’s t-test. Significant differences between antibody titers and between SAP levels were determined by the Mann-Whitney U test. P values less than 0.05 were considered significant.

RESULTS

Reduction of arthritis severity in lymphopenic hosts by adoptive transfer of CD25+ regulatory T cells. We have previously demonstrated that depletion of CD25+ regulatory T cells 2 weeks before immunization with bovine CII leads to increased severity of arthritis symptoms in CIA (16). Furthermore, we found that these magnified clinical manifestations were accompanied by augmented T and B cell responses against CII. These findings indicate that CD4+,CD25+ regulatory T cells are involved in the control of CII-directed responses in vivo.

To investigate whether CD4+,CD25+ regulatory T cells could be successfully used in a therapeutic setting, we studied the effects of infusions of CD4+,CD25+...
regulatory T cells in mice with early arthritis. In general, adoptive transfer of T cells for the treatment of disease appears most successful in immunocompromised hosts, presumably as a consequence of homeostatic proliferation and/or the absence of regulatory mechanisms (13,18,19). For example, studies addressing the potency of adoptively transferred tumor-specific T cells are, in general, performed using tumor-bearing T cell–deficient mice. Indeed,
studies in human cancer patients have shown that adoptive T cell therapy is most successful in lymphopenic patients (20). Similarly, in a T cell–mediated colitis model, SCID mice have been used to study the potency of regulatory T cells (13). Therefore, we chose to first examine the effects of regulatory T cells on chronic arthritis, as seen in CIA, in lymphopenic hosts.

Since CIA can only be induced in immunocompetent mice due to the necessity of T and B cells for disease initiation, we lymphodepleted bovine CIA–immunized DBA/1 mice by lethal irradiation at the time the first clinical symptoms of arthritis appeared. In this way, a lymphopenic environment is created in mice that have already displayed high titers of CII-specific antibodies (data not shown) and that will have chronic arthritis as a result. The next day, $5 \times 10^5$ prestimulated CD4+,CD25+ T cells were transferred, followed by an injection of syngeneic bone marrow cells to rescue the host. The prestimulated CD25+ regulatory T cells were capable of blocking PHA-induced T cell proliferation in vitro as well as bovine CII–specific proliferation (data not shown), and they expressed high levels of Foxp3 mRNA (21) (Figure 1A), demonstrating that the infused cells were effective suppressors. In contrast, similarly isolated and stimulated CD4+,CD25− T cells used as a control did not display these suppressive capabilities (Figure 1A and ref. 16). Although an initial decrease in clinical symptoms was noted soon after TBI in all irradiated groups (Figure 1B), most likely as a consequence of the irradiation, only mice treated with CD4+,CD25+ regulatory T cells showed a long-lasting reduction in disease severity (Figure 1B).

The finding that adoptively transferred CD4+,CD25+ regulatory T cells are able to dampen ongoing inflammatory reactions was further strengthened by the results of analyses of the levels of SAP, an acute-phase protein detectable in sera during arthritis-associated inflammation as well as after lethal irradiation (22,23). All groups that underwent BMT without CD25+ regulatory T cells had increased levels of SAP, which occur as a result of TBI (24). However, the SAP levels in mice that underwent BMT and were treated with CD4+,CD25+ regulatory T cells were significantly lower than those detected in controls or mice receiving other treatments ($P < 0.0001$) (Figure 1C). Taken together, these observations demonstrate that CD25+ regulatory T cells can be used to decrease arthritis-associated inflammation as well as acute-phase responses.

Lack of association between improved clinical outcome and CII-specific immunity. CII-specific T cells and antibodies play a crucial role in the development of CIA (for review, see refs. 3 and 25). To clarify the mechanism responsible for our clinical observations, we examined both the CII-specific T cell responses and antibody titers in the treated mice. Because no differences were found with respect to disease progression and immunologic parameters between groups of mice treated with CD4+ or CD4+,CD25− T cells, for these experiments we only used CD4+ T cells as a control for CD4+,CD25+ T cells. In order to examine bovine CII–specific T cell responses, mice were killed at least 30 days after BMT and cell transfer. Spleens were harvested and analyzed in a proliferation assay against bovine CII.

As expected, bovine CII–specific proliferation was strongly diminished after lethal irradiation in mice that underwent BMT only, indicating that irradiation combined with BMT resulted in a strongly reduced T cell reaction. This overall immunocompromised state was confirmed using a control antigen, keyhole limpet hemocyanin (data not shown), as well as PHA (data not shown). In contrast, splenocytes from mice that underwent BMT and were treated with CD4+ T cells still reacted significantly to bovine CII (Figure 2A). However, bovine CII–specific proliferation was strongly reduced in splenocytes from mice that underwent BMT and were treated with CD4+,CD25+ T cells. Togeth

Reduction of disease progression in nonlymphopenic hosts with arthritis by therapy with regulatory T cells. Although high-dose immunosuppression (i.e., lethal TBI) followed by syngeneic BMT and CD4+,
CD25+ regulatory T cell transfer was highly effective in reducing arthritis severity, a less toxic treatment regimen is preferable. Therefore, we investigated the efficacy of CD4+CD25+ regulatory T cell transfer without BMT in immunocompetent hosts. Initial experiments using $5 \times 10^5$ cells/mouse showed little effect on disease progression (data not shown), but when $1 \times 10^6$ CD4+CD25+ regulatory T cells were adoptively transferred IV at the time arthritis appeared, the mice demonstrated significantly reduced arthritis symptoms (Figure 3). These results indicate that the treatment of arthritis by infusion of CD4+CD25+ regulatory T cells positively affects clinical outcome when sufficient numbers of CD4+,CD25+ regulatory T cells are transferred.

Since CIA is mediated by bovine CIA–specific immune responses and is crucially dependent on the presence of CIA-specific autoantibodies, we wished to investigate whether adoptive transfer of CD4+CD25+ regulatory T cells leads to inhibition of CIA-directed immunity in nonlymphopenic recipients. Therefore, we measured the bovine CIA–specific T cell responses and determined CIA-specific antibody titers in the different groups. In all groups, CIA-specific proliferative responses were retained, and no apparent differences between groups were noted (Figure 4A). When we measured CIA-specific antibody titers in sera obtained 43 days after immunization, a slight reduction of CIA-specific antibodies was detected in mice given CD25+ regulatory T cells,
but the difference did not reach statistical significance (Figure 4B). Since even a slight reduction in total CII-specific Ig titers may be indicative of significant changes in specific IgG isotypes, we examined CII-specific IgG1 and IgG2a titers. No significant differences between groups were detected for both isotypes (Figures 4C and D). Therefore, we consider it unlikely that a reduction in CII-specific T cell– or antibody-mediated systemic immunity can account for the reductions in inflammation seen clinically.

**Localization of adoptively transferred CD4+, CD25+ regulatory T cells in inflamed joints.** Because the improvements in disease outcome observed after transfer of CD25+ regulatory T cells did not correlate with differences in systemic CII-directed T and B cell responses, we wished to investigate whether the infused cells could exert their function locally by determining whether they had the capacity to travel to the inflamed joints as well as to the lymphoid organs. After labeling the cells with CFSE, we were able to show that prestimulated CD4+,CD25+ regulatory T cells could easily expand both in naive mice and in arthritic mice (data not shown). To investigate whether they localized in the inflamed knee joints, we collected a variety of organs as well as synovial tissue 1–2 days after adoptive transfer into arthritic mice. By using an HRP-labeled FITC-
specific antibody that was cross-reactive with CFSE, we were able to visualize CFSE-labeled cells by immunohistochemistry (17).

As expected, CFSE-labeled CD25+ regulatory T cells localized to CD4+ cell–rich regions in the spleen (Figure 5A) and lymph nodes (results not shown), but were not found in the B cell areas of these organs. Transferred CD4+,CD25+ T cells were also detected in joint-draining lymph nodes (popliteal), blood, liver, lungs, kidneys, and synovial fluid of arthritic mice (results not shown). More importantly, CD25+ regulatory T cells were also detected in inflamed synovial tissue (Figures 5C and D). Together, these results demonstrate that adoptively transferred regulatory T cells quickly appear in synovial tissue after injection (1–2 days), and they raise the possibility that the transferred CD25+ regulatory T cells control CIA locally in the inflamed tissue of the joint, rather than by inhibiting systemic CII-specific immunity.

**DISCUSSION**

Successful treatment of autoimmune disease with CD25+ regulatory T cells has been reported in an animal model of colitis in which the infusion of $1 \times 10^6$ regulatory T cells successfully treated early-stage disease induced through the transfer of CD4+,CD45RB$^{high}$ cells to SCID mice (13). Unlike colitis, which is a T cell–mediated, organ-specific autoimmune disease, CIA presents different challenges for CD25+ regulatory T cells. CIA is a systemically induced autoimmune disease that is initiated mainly through CII-specific antibodies and is strongly influenced by innate immune cells (3). In the colitis model, regulatory T cell therapy was successful in lymphopenic hosts, but the applicability of regulatory T cell therapy in nonlymphopenic hosts has yet to be analyzed. In general, adoptive cell therapies against, for example, tumors are most successful after immunodepletion, largely due to the elimination of regulatory mechanisms and/or the homeostatic proliferation of transferred cells (18). These observations are consistent with our findings, since a transfer of $5 \times 10^5$ regulatory T cells was sufficient to inhibit disease progression in hosts that were myeloablated following irradiation, but were insufficient in nonlymphopenic hosts. However, our data show that regulatory T cell therapy can be used successfully in nonimmunocompromised animals when more regulatory T cells ($10^6$) are transferred into mice with early arthritis.

The observation that regulatory T cell therapy can be an effective treatment in systemic autoimmune disease is important in the context of the observations that proinflammatory cytokines such as IL-6 can hinder the suppression mediated by regulatory T cells (26) and that regulatory T cells from RA patients display a compromised function that is reversed by anti–tumor necrosis factor (anti-TNF) treatment (27). Mice with CIA, as well as RA patients, display highly elevated levels of proinflammatory cytokines, including IL-6, TNF$\alpha$, and IL-1 (3). These cytokines lead to the production of acute-phase proteins such as C-reactive protein in humans and SAP in mice (28). TBI, which was used to generate lymphopenic mice, has also been reported to result in proinflammatory cytokine release and, as a consequence, in acute-phase protein production (29). Despite high levels of proinflammatory cytokines such as IL-6, lymphopenic mice receiving CD25+ regulatory T cells showed large reductions in serum
levels of SAP compared with the levels in control groups, indicating that systemic autoimmune diseases associated with elevated levels of proinflammatory cytokines can be treated with CD4+CD25+ regulatory T cells.

In vitro suppression assays with CD4+CD25+ regulatory T cells have indicated that these cells primarily require cell–cell contact in order to regulate (30,31). However, in vivo experiments have indicated that the secretion of antiinflammatory cytokines such as IL-10 may be necessary for sufficient regulation (11). Therefore, CD25+ regulatory T cells may need to be in the direct vicinity of the cells responsible for the inflammatory responses. A direct cell–cell contact between transferred CD4+, CD25+ T cells and CD11c+ cells (dendritic cells in the mouse) and pathogenic T cells has been observed during the treatment of colitis with CD4+, CD25+ regulatory T cells (13). Although the mode of action of regulatory T cell–mediated suppression of ongoing colitis is not known, these contacts are particularly noteworthy, since colitis is a T cell–mediated disease (32). We also detected CD4+, CD25+ regulatory T cells in the inflamed synovium after adoptive transfer (Figure 5). However, we consider it unlikely that regulatory T cells act in the CIA model via the suppression of T cell responses, since the effector phase of arthritis depends on immune responses that are independent of T cells (10,33). Likewise, we did not observe a decrease in systemic CII-specific antibody titers. Therefore, we believe that transferred CD4+, CD25+ regulatory T cells are interacting locally with innate immune cells (e.g., mononuclear phagocytes and neutrophils), rather than with T cells or B cells.

Local control of innate immune cells by CD4+, CD25+ regulatory T cells could be achieved in various ways. Local IL-10 delivery has been shown to decrease TNFα and IL-1α production (34), and direct regulation through cell–cell contact between innate immune cells (which are abundantly present in inflamed joints) and CD4+, CD25+ regulatory T cells is possible and has already been shown in a bacterially induced colitis model (35).

We have now demonstrated that the adoptive transfer of CD4+, CD25+ regulatory T cells during the early phase of chronic arthritis can offer therapeutic benefits. Furthermore, we have found that these cells are capable of traveling to the joint, where they could conceivably have the potential to suppress inflammation locally. Although a number of practical difficulties still have to be overcome for clinical application, improvements in techniques for expansion of CD4+, CD25+ regulatory T cells that would allow for multiple transfers of regulatory T cells (36), along with the detailed exploration of the interactions between CD4+, CD25+ regulatory T cells and the immune cells of the joint, may allow for the development of optimized CD4+, CD25+ regulatory T cell therapies for chronic arthritis and systemic autoimmune disease in general. However, such interventions will represent a laborious treatment option, and it is therefore unlikely that adoptive cellular therapies will become part of the standard array of antirheumatic therapies. Nonetheless, our results do provide a rationale for targeting regulatory T cell activity in the treatment of patients with chronic systemic autoimmune diseases.

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Association of the PTPN22 locus with rheumatoid arthritis in a New Zealand Caucasian cohort

It is becoming evident that distinct inflammatory autoimmune pathologic conditions, including rheumatoid arthritis (RA), share common genetic causative factors. Expression of autoimmunity in both human and animal models generally requires specific class II major histocompatibility complex (MHC) alleles. Non-MHC loci are, however, also required. Evidence is accumulating that alleles at some of these loci predispose to autoimmunity per se. Becker et al (1) observed that ~65% of human linkages map nonrandomly into 18 distinct clusters. Meta-analyses of published linkage studies of autoimmune diseases in humans and rodents strongly implicated the human chromosome 18q12–q23 (IDDM6) region and the rodent orthologous region on distal chromosome 18 in the susceptibility to autoimmunity (2). Recently, convincing evidence has been presented implicating the CTLA4 gene in susceptibility to both type 1 diabetes and Graves’ disease in humans (3).

The PTPN22 gene encodes the lymphoid protein tyrosine phosphatase (Lyp), which is a suppressor of T lymphocyte activation (4). Lyp interacts in a synergistic manner with the protein kinase Csk to regulate antigen-triggered T lymphocyte activation by the sequential action of the Src and Syk/Zap-70 family of protein tyrosine kinases. A single-nucleotide polymorphism (SNP) in the PTPN22 gene (nucleotide 1858C→T, residue Arg620Trp) has been reported to be associated with autoimmune phenotypes, including RA (5–11). In vitro, the disease-associated allele of Lyp (Trp620) disrupts its association with Csk, potentially reducing the effectiveness of Lyp–Csk–mediated negative regulation of antigen-driven T lymphocyte activation (5,7). It is possible that T lymphocytes from individuals with the Lyp Trp620 allele have a reduced amount of the Lyp–Csk complex.

Given the evidence for association of the PTPN22 C1858T variant with RA in 3 US Caucasian cohorts (5,6), we tested the hypothesis that the PTPN22 locus influences predisposition to RA in New Zealand Caucasians. This was done by testing the variant for association with disease in 869 Caucasian patients with RA and 563 ethnically matched healthy controls from New Zealand.

The RA patients were drawn from the Auckland, Bay of Plenty, Wellington, Canterbury, Otago, and Southland regions of New Zealand and were recruited on the basis that they satisfied the 1987 American College of Rheumatology (ACR; formerly, the American Rheumatism Association) criteria for RA (12). The control group comprised 563 healthy subjects older than age 17 years who were recruited from the Otago and Auckland regions and who had no history of inflammatory disorders. Recruitment was done with the approval of the ethics committee, and all subjects gave their informed consent. All patients and controls were of white (Caucasian) ethnic origin. Based on an estimated minor allele frequency of 0.10, this cohort had 96.0% power to detect an effect of the size previously reported in RA (5) (odds ratio [OR] 1.65, α = 0.01). At an OR of 1.5, the study had 81.1% power, with power decreasing dramatically at lower effects (e.g., at an OR of 1.3, the power was 35.1%). Power calculations were performed as previously described (13).

The PTPN22 (rs2476601) gene was genotyped over the case–control cohort by a polymerase chain reaction (PCR) restriction fragment length polymorphism–based assay using the primer pairs 5’-CTTCTCTAACAACTTAAATG-3’ (antisense) and 5’-GAACAAATGTTCAACTTATG-3’ (sense). The nucleotide 1858C allele creates a restriction site for Rsa I, with the 232-bp PCR product being cleaved into fragments of 199 bp and 33 bp. The 232-bp PTPN22 fragment was amplified in a 15-μl reaction containing 30 ng of genomic DNA, 2 mM MgCl₂, 0.2 mM dNTP, 10 pmoles of each primer, and 0.5 units Taq DNA polymerase in 50 mM KCl and 10 mM Tris HCl (pH 8.3). The amplified product was restricted with 1.5 units of Rsa I, and DNA fragments were visualized under ultraviolet irradiation, after electrophoresis, on a 3.5% agarose gel stained with ethidium bromide.

The PTPN22 1858T (620Trp) allele was more frequent in patients with RA than in healthy individuals (Table 1): 15.1% of patient chromosomes had the 1858T allele, compared with 9.9% of control chromosomes (OR 1.61, 95% confidence interval [95% CI] 1.27–2.03, P = 5 × 10⁻⁵). Individuals homozygous for the 1858T allele (2.4% of patients compared with 0.5% of controls) had a 5.06-fold increased risk of developing RA compared with those homozygous for the 1858C allele (P = 0.009; 95% CI 1.50–17.07), and heterozygotes had a 1.50-fold increased risk (P = 0.002; 95% CI 1.16–1.94). The inclusion of HLA–DRB1 shared epitope status in logistic regression analyses did not greatly affect the risk estimates (Table 1).

We tested for genetic interaction between PTPN22 and HLA–DRB1 and found no evidence for a departure from a multiplicative model of interaction between the 2 loci (P = 0.24; a likelihood ratio test was done comparing a logistic regression model assuming multiplicative interaction with a model generated using a combination of 2 locus genotypes). Similarly, no evidence for an HLA–DRB1/PTPN22 genetic interaction has previously been observed, in either RA or type 1 diabetes (5,6,9). The PTPN22 association was evident in patients of both sexes (data not shown).

A gradation of OR estimates was observed between TT homozygotes (OR 5.06) and CT heterozygotes (OR 1.50). To investigate the mode of inheritance, the likelihood ratio test was used to compare logistic regression models. We were able to reject simple dominant and recessive inheritance models (P = 0.027 and P = 0.0021, respectively). A complex codominant model (in which each allele contributes to disease risk) is most consistent with the data. These findings are consistent with the dose-dependent effect of PTPN22 C1858T on RA risk reported elsewhere (5,6).

Rheumatoid factor (RF) is an anti-IgG antibody that is present in the majority of patients with RA, and its role in disease etiology remains unclear. Determination of any genetic differences between RF-positive and RF-negative patients may shed light on the role of RF in disease pathogenesis. Measurements of RF were available for 775 (89.2%) of the New Zealand patients. (RF levels were determined by either latex fixation or laser nephelometry, and RF positivity was defined as positive results on 1 or more occasions.) We stratified the patients according to RF status and found evidence for asso-
### Table 1. Allele and genotype frequencies in New Zealand patients with RA and controls

<table>
<thead>
<tr>
<th>Allele</th>
<th>Patients</th>
<th>Controls</th>
<th>OR†</th>
<th>95% CI</th>
<th>P</th>
<th>OR†</th>
<th>95% CI</th>
<th>P</th>
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<tr>
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<td></td>
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<td></td>
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<tr>
<td>CC</td>
<td>628 (72.3)</td>
<td>454 (80.6)</td>
<td>1.00</td>
<td>–</td>
<td>–</td>
<td>1.00</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>CT</td>
<td>220 (25.3)</td>
<td>106 (18.8)</td>
<td>1.50</td>
<td>1.16–1.94</td>
<td>0.002</td>
<td>1.45</td>
<td>1.10–1.92</td>
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<tr>
<td>TT</td>
<td>21 (2.4)</td>
<td>3 (0.5)</td>
<td>5.06</td>
<td>1.50–17.07</td>
<td>0.009</td>
<td>7.25</td>
<td>1.63–32.16</td>
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<td>CT + TT</td>
<td>241 (27.7)</td>
<td>109 (19.3)</td>
<td>1.60</td>
<td>1.24–2.07</td>
<td>0.0003</td>
<td>1.56</td>
<td>1.18–2.05</td>
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<td>CT + TT</td>
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<tr>
<td>CC</td>
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<td>454 (80.6)</td>
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<td>–</td>
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<tr>
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</table>

* Values are the number (%). RA = rheumatoid arthritis; 95% CI = 95% confidence interval; RF = rheumatoid factor.
† All odds ratios (ORs) were calculated using logistic regression with the Stata statistical package (http://www.stata.com; additional Stata routines were downloaded from http://www.gene.cimr.cam.ac.uk/clayton/software/stata).
‡ Patients and controls were subclassified according to whether they had inherited 0, 1, or 2 HLA–DRB1 shared epitope (SE) alleles (DR1, DR4, or DR10).
§ Overall P = 0.00012 by logistic regression, and P = 0.0003 when adjusted for HLA status.
# To enable direct comparison with previous studies (5,6), the CT and TT genotypes were combined.

* Overall P = 0.0006 by logistic regression, and P = 0.007 when adjusted for HLA status.

Citation of PTPN22 C1858T with disease in both the RF-positive group (81.3% of patients; for the CT/TT genotype, OR 1.58 [95% CI 1.20–2.07], P = 0.001) and the RF-negative group (18.7% of patients; OR 1.81 [95% CI 1.20–2.74], P = 0.005). Interestingly, these data contrast with those from 2 other studies. In the first of these studies, involving US multiplex families, Begovich et al (5) reported that the TT and CT genotypes were strongly associated with disease (OR 2.36, 95% CI 1.76–3.16, P < 0.0001) in RF-positive patients (83.2%), but that there was no evidence for association in patients with RF-negative disease (16.8%; OR 1.17, 95% CI 0.60–2.31, P = 0.64). In the second study, involving 1,413 RA patients (49.9% of whom had early-onset disease), Lee et al (6) also reported that association was evident in RF-positive patients (68.5%) with the CT/TT genotype (OR 1.69, 95% CI 1.38–2.07, P = 3.1 × 10⁻⁷) but not in RF-negative patients (31.5%; OR 1.17, 95% CI 0.89–1.54, P = 0.27).

In contrast with the studies by Begovich et al and Lee et al, in which patients represented the probands in multiplex families and patients with early-onset disease (~50%), respectively, the phenotypic recruitment criterion for the New Zealand patients was simply a clinical diagnosis of RA according to the ACR criteria. Therefore, there is clinical heterogeneity between cohorts. There also may be underlying genetic and environmental heterogeneity between the US and New Zealand Caucasian populations from which the RA cohorts were derived (certainly the cohort of index patients from multiplex families [5] would be expected to have a higher proportion of genetic susceptibility factors). This heterogeneity may explain why PTPN22 C1858T was associated with RA in RF-negative patients in our cohort but not in the US cohorts (5,6).

Post priori power calculations (for review, see ref. 13), in which α = 0.01 (assuming an OR for a putative association in the RF-negative group equivalent to that in the relevant unstratified cohort), demonstrated that both the study by Begovich et al and the present study had <40% power to detect a possible association in each RF-negative group, whereas the study by Lee et al was adequately powered (84%). It is possible that a combination of heterogeneity between study populations (in both the country of origin and the clinical phenotype) and insufficient power in some studies is obscuring the answer to the question of whether there is association of PTPN22 C1858T with RF-negative disease. Further adequately powered studies in RA cohorts drawn from outside of the US and New Zealand should help to resolve this important question.

Recently reported data from 3 US Caucasian cohorts provide strong evidence for association of PTPN22 C1858T with RA (for the T allele, OR 1.65, P = 0.0007). These include the “discovery” cohort (OR 1.97, P = 5.6 × 10⁻⁵) and the “replication” cohort (OR 1.57, P = 2.1 × 10⁻⁵) described by Begovich et al (5) and the cohort of patients derived from several sources, including the Study of New Onset Rheumatoid Arthritis (SONORA), as described by Lee et al (6). Our data, with a comparable OR of 1.61 for the T allele (P = 5 × 10⁻⁵), replicate the US data and demonstrate association of PTPN22
C1858T with RA in a cohort of patients who were selected based only on fulfillment of the ACR criteria (compared with the US studies, in which additional criteria included RF positivity, having at least 1 sibling with RA, and having early-onset RA). Collectively, these data provide strong evidence that PTPN22 is associated with RA. PTPN22 can be claimed to be the first non-HLA locus that is convincingly implicated in genetic susceptibility to RA.

Studies in population- and family-based cohorts with type 1 diabetes, Graves’ disease, systemic lupus erythematosus, or RA unequivocally demonstrate that PTPN22 C1858T is associated with autoimmunity (5–11) (Table 1). However, this does not necessarily establish C1858T as the causal variant (or PTPN22 as the causal gene). This claim can be made when it is shown that the C1858T SNP is the most associated variant in the region, as was done to demonstrate association of specific CTLA4 variants with Graves’ disease (3). Unlike the CTLA4 situation, in which the flanking CD28 gene was also a very strong autoimmunity candidate gene, no other strong candidate genes map to the immediate proximity of PTPN22. Eleven named genes (SLC16A1, LRIG2, PHTF1, AP4B1, DCLRE1B, HIPKI, TRIM33, BCAS2, AMPD1, NRAS, SYCP1) map to the 2 megabases of DNA flanking PTPN22. Of these, there is evidence for an immune role only for N-ras; mice deficient in N-ras have decreased numbers of cytotoxic T lymphocytes (14). Thus, at this stage, PTPN22 is the most obvious autoimmunity gene in the region, and the functional Arg620Trp variant (which disrupts association of Lyp with Csk) is a very strong candidate etiologic variant.

The PTPN22 gene product (Lyp) interacts with Csk to regulate antigen-triggered T lymphocyte activation by sequential action of the Src and Syk/Zap-70 protein family of protein tyrosine kinases. The genetic data presented here and elsewhere (5,6) implicating this pathway in RA etiology are consistent with data suggesting that a mutation in Zap-70 results in spontaneous autoimmune arthritis in mice (15). Collectively, the PTPN22 and Zap-70 data emphasize the relevance of T lymphocytes to RA pathogenesis. Elucidating the molecular pathways of control of autoreactive T lymphocytes is an important focus in understanding the etiology of autoimmune disease.

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Clinical Images: Radiographic healing of osseous sarcoidosis

The patient, a 59-year-old woman, was diagnosed as having osseous sarcoidosis, which was documented by radiography and biopsy. She was treated with prednisone (15 mg/day, tapered to 2 mg/day over 6 months) and methotrexate (MTX) (15 mg/week). Posteroanterior radiographs of the hand were obtained on March 21, 2001 (A) and were compared with radiographs obtained on February 13, 2003 (B) (after 23 months of MTX and 17 months of low-dose prednisone). A, Four sites of involvement, including a lytic lesion (1) on the middle phalanx of the left little finger (arrow 1), and permeative lesions of osseous sarcoidosis (1) on the middle phalanx of the left index finger (arrow 2), the middle phalanx of the right middle finger (arrow 3), and the proximal phalanx of the right little finger (arrow 4). B, Resolution of the permeative lesions and remodeling of the lytic lesion. C and D, Close-up views of the middle phalanx of the left index finger from the radiographs shown in A and B, respectively. There has been one previously reported case of radiographic healing in a patient treated with steroids and chloroquine (2), and another report describes control of the manifestations of osseous sarcoidosis with MTX and hydroxychloroquine treatments, without reference to the fate of the bone lesions (3). This appears to be the first serial radiographic demonstration of healing osseous sarcoidosis.


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Successful treatment of refractory Schnitzler syndrome with anakinra: comment on the article by Hawkins et al

To the Editor:

We read with interest the recent report that described a dramatic response of some autoinflammatory syndromes to anakinra (1). We describe a patient with severe and refractory Schnitzler syndrome that responded dramatically to interleukin-1 (IL-1) blockade with anakinra. The similarities between Schnitzler syndrome and some hereditary periodic syndromes suggest that these entities belong to a group of disorders in which IL-1 plays a dominant role.

The patient, a 42-year-old man, was remitted for evaluation in May 2002. He reported a 1-year history of high spiking fever accompanied by a widespread urticarial and pruritic rash, together with severe bone pain. The laboratory studies revealed an erythrocyte sedimentation rate of 23 mm/1 hour and a C-reactive protein level of 2.5 mg/dl (normal <0.5 mg/dl). The serum electrophoresis revealed an IgM kappa gammapathy (790 mg/dl; normal 60–370 mg/dl). The abdominal radiograph showed a condesant lesion on the right iliac bone, and the skin biopsy yielded findings compatible with urticaria.

The patient was diagnosed as having Schnitzler syndrome (2) and was treated with celecoxib, high-dose corticosteroids, azathioprine, pamidronate, rituximab, cyclosporin A, interferon alpha, infliximab plus methotrexate, intravenous immunoglobulins, and phototherapy. All of these treatments were discontinued because of inefficacy or side effects. In April 2004, anakinra (100 mg/day subcutaneously) plus methotrexate (5 mg/week) was started as a compassionate therapy. Within 1 week, the patient was asymptomatic. Ten months later, his condition remained stable on 100 mg/day of anakinra plus methotrexate (5 mg/week).

A number of rare autoinflammatory disorders with rheumatic manifestations have recently been found to be associated with mutations in NALP3 and Nod2 (3–5). Another member of this family, NALP1, has also been suggested to play an important role in mediating inflammatory responses and secretion of IL-1β (5). Therefore, in this patient, we searched for mutations in the coding region of NALP1, NALP3, and Nod2 and found no sequence alterations.

Schnitzler syndrome is a rare and probably underrecognized disorder. The presence of the chronic urticarial lesions and the monoclonal IgM component, which define the syndrome, are virtually constant. Intermittent fever, joint symptoms, and the increase in acute-phase reactants are also very frequent, albeit nonspecific, characteristics of this entity (2). The clinical course of the disease is usually chronic, with no spontaneous remissions, and treatment is difficult and often ineffective (2). Due to the severity of the clinical features in our patient, he was administered most of the different therapies that have been reported to be effective in isolated cases of Schnitzler syndrome (2). Based on the effectiveness of rituximab and tumor necrosis factor antagonists in other systemic inflammatory disorders, these agents were also used as compassionate therapies in this patient, without any significant benefit. However, the patient responded dramatically to treatment with anakinra, suggesting that IL-1 plays a predominant role in Schnitzler syndrome. Due to the rarity of this condition, the pathophysiology of the disease remains unknown. An increased frequency of IgG autoantibodies to IL-1α has been reported (6), and those authors suggested that an antibody-mediated prolongation of the half-life of IL-1 might account for some of the symptoms and signs of Schnitzler syndrome (6).

Human autoinflammatory diseases are a heterogeneous group of genetically determined conditions that are characterized by seemingly unprovoked inflammation in the absence of infection or autoimmunity (7). Although each of these entities presents genetic and phenotypic peculiarities, globally these conditions share an intermittent expression in the form of acute attacks of fever variably associated with serosal, synovial, and/or cutaneous inflammation, which is usually self-limiting (7). Although Schnitzler syndrome does not begin in childhood, it shares many of the clinical features of these autoinflammatory syndromes. Based on this, we analyzed NALP1, NALP3, and Nod2, which are members of a gene family associated with autoinflammatory disorders with rheumatic manifestations (5). Although these NALP family members are likely candidates as susceptibility genes in Schnitzler syndrome, we could not find mutations in any of them. However, this family contains at least 19 members and it may be that the association is established with one of the genes that we have not studied. Alternatively, because the penetrance of these mutations is not complete, it could be that our patient has no genetic association with this family of inflammation-regulatory genes. In addition to the clinical features that are shared by Schnitzler syndrome and the hereditary periodic syndromes, it seems that all these entities also share a predominant role of IL-1 in their pathogenesis. In fact, recent reports have described a dramatic response of some of these disorders to anakinra (1,8,9).

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6. Saurat JH, Schifferli J, Steiger G, Dayer JM, Didierjean L. Anti-interleukin-1α autoantibodies in humans: characterization,
is healthy control subjects. From 7 patients with active RA were studied, as was PB from 9 by Ficoll-Hypaque density-gradient centrifugation. PB and SF blood (PB) and synovial fluid (SF) mononuclear cells isolated CD64 (fluorescein isothiocyanate labeled), on both peripheral coerythrin labeled), TLR-4 (allophycoryanin labeled), and flow cytometric analysis using antibodies against TLR-2 (phycoerythrin labeled), TLR-2 and TLR-4 in RA and would like to report our findings, which differ quantitatively from those obtained by Iwahashi et al. We found that in RA patients, monocyte/macrophage expression of TLR-2 and TLR-4 in RA patients compared with healthy controls, as well as increased surface expression of TLR-2 in CD16+ versus CD16− monocytes of both patients and controls. In contrast, monocytes from RA patients expressed higher levels of TLR-4, independent of CD16 status.

We have similarly been interested in the expression of TLR-2 and TLR-4 in RA and would like to report our findings, which differ qualitatively from those obtained by Iwahashi and colleagues. We found that in RA patients, monocyte/macrophage expression of both TLR-2 and TLR-4 was increased in comparison with control subjects. In addition, we found that the lymphocyte (nonmonocyte) population showed increased expression of TLR-2, but not TLR-4. We performed flow cytometric analysis using antibodies against TLR-2 (phycoerythrin labeled), TLR-4 (allophycocyanin labeled), and CD64 (fluorescein isothiocyanate labeled), on both peripheral blood (PB) and synovial fluid (SF) mononuclear cells isolated by Ficoll-Hyphaque density-gradient centrifugation. PB and SF from 7 patients with active RA were studied, as was PB from 9 healthy control subjects.

CD64, the high-affinity receptor for IgG (Fcγ receptor I), is expressed on both macrophages and monocytes and was used as our monocyte/macrophage marker. We found that nearly all CD64+ mononuclear cells (92–99%) were TLR-2+, and this proportion was independent of disease status. However, TLR-2 expression was higher in monocytes from RA patients than from controls (mean fluorescence intensity [MFI] 362.6 ± 80.3 versus 178.7 ± 19.8 [mean ± SEM]; P = 0.025 by Wilcoxon’s test) (Figure 1). The MFI was similar in the SF and blood compartments of RA patients (MFI on synovial monocytes 360.1 ± 107.4). CD64+ cells from normal subjects had a low frequency of TLR-4 expression (0.24%) compared with RA blood cells (mean ± SEM 2.98 ± 1.34%) or SF cells (2.52 ± 0.84%). The MFI of TLR-4 expression was also higher in patients than in controls (P = 0.025 and P = 0.005, respectively). FL = fluorescence; PE = phycocerythrin; SF = synovial fluid.

There is no previous report of a comparative study of TLR-2 and TLR-4 expression on mononuclear cells from SF and PB in RA. Our results show that both TLR-2 and TLR-4 are expressed at higher levels in the CD64+ population, but expression does not differ between PB and SF in RA patients. The difference between our results and those obtained by Iwahashi et al could be due to the antibodies used (CD16 versus CD64), which may identify different subpopulations of monocytes in vivo. Our findings suggest that systemic inflam-
mation in RA is capable of enhancing TLR expression on monocytes and macrophages in general, which, in the presence of their cognate ligands, may serve to further enhance the proinflammatory capacity of these cells.

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Clinical Images: Anetoderma in systemic lupus erythematosus with antiphospholipid antibodies

The patient, a 29-year-old woman, presented with a 5-year history of numerous, progressive, flaccid, elevated, well-circumscribed, skin-colored, non-itching skin lesions (diameter 10–30 mm), mainly on her trunk and upper arms (A). She had had symmetric, nonerosive arthritis of the wrists, recurrent depression, and headaches for the past 3 years and had deep vein thrombosis in her right leg 2 months prior to the development of the skin lesions. These symptoms, and the detection of antinuclear, double-stranded DNA, and antiphospholipid antibodies (aPL), led to the diagnosis of systemic lupus erythematosus (SLE) with antiphospholipid syndrome. Histopathologic examination of the lesions (B and C) showed an atrophic, flattened epidermis with abundant, collagenous, and decreased elastic fibers in the upper dermis. Anetoderma was diagnosed. Anetoderma is an uncommon skin disease that occurs in association with infections (human immunodeficiency virus, syphilis, tuberculosis, and Lyme disease) and autoimmune disorders (SLE, systemic-sclerosis, and others) (1–3), but perhaps most frequently with aPL (3–6). One hypothesis regarding the focal elastolysis is an increased release of elastase from inflammatory cells (1). Other investigators suggest that microthromboses may lead to local ischemia followed by degeneration of elastic fibers (5). The clinician should keep in mind that anetoderma can occur as the first sign of autoimmune diseases in general and the presence of aPL in particular. Hence, the evaluation should include tests for lupus anticoagulant, all isotypes of anticardiolipin, and anti–β2-glycoprotein I antibodies (6).