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INVITED REVIEW

ABSTRACT: Neuropathy is often a major manifestation of systemic amyloidosis. It is most frequently seen in patients with hereditary transthyretin (TTR) amyloidosis, but is also present in 20% of patients with systemic immunoglobulin light chain (primary) amyloidosis. Familial amyloid polyneuropathy (FAP) is the most common form of inherited amyloidotic polyneuropathy, with clinical and electrophysiologic findings similar to neuropathies with differing etiologies (e.g., diabetes mellitus). Hereditary amyloidosis is an adult-onset autosomal-dominant disease with varying degrees of penetrance. It is caused by specific gene mutations, but demonstration that a patient has one such mutation does not confirm the diagnosis of amyloidosis. Diagnosis requires tissue biopsy with demonstration of amyloid deposits either by special histochemical stains or electron microscopy. Transthyretin amyloidosis is treated by liver transplantation, which eliminates the mutated transthyretin from the blood, but for some patients continued amyloid deposition can occur from wild-type (normal) transthyretin. Presently, a study is ongoing to determine whether amyloid deposition can be inhibited by small organic molecules that are hypothesized to affect the fibril-forming ability of transthyretin. Proposed gene therapy with antisense oligonucleotides (ASOs) to suppress hepatic transthyretin synthesis is effective in a transgenic mouse model but has not yet been tested in humans.


THE MOLECULAR BIOLOGY AND CLINICAL FEATURES OF AMYLOID NEUROPATHY

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Neuropathy is a major feature of several types of systemic amyloidosis. It is most consistently seen in patients with hereditary transthyretin amyloidosis and is often present in patients with hereditary ApoAI amyloidosis caused by the glycine 26 arginine (Gly26Arg) mutation. Neuropathy is also a cardinal feature of hereditary gelsolin (Gel) amyloidosis. Peripheral neuropathy is not seen in reactive (AA, secondary) amyloidosis nor in most of the inherited amyloidoses characterized by renal, hepatic, or cardiac deposition. The most common cause of amyloid neuropathy is immunoglobulin light chain (AL, primary) amyloidosis, in which approximately 20% of patients have peripheral neuropathy and an even larger number have carpal tunnel syndrome. Because AL amyloidosis is a sporadic disease and little is known about the pathogenesis of immunoglobulin light chain amyloid neuropathy, we will focus on the hereditary types of amyloidosis for this discussion of the molecular biology and clinical features of amyloid neuropathy. However, AL amyloidosis needs to be considered in the differential diagnosis of patients with amyloidosis and neuropathy but with a family history insufficient to make a definitive diagnosis of a hereditary form of the disease.

TRANSTHYRETIN AMYLOIDOSIS

Transthyretin amyloidosis is the most common form of autosomal-dominant hereditary systemic amyloidosis. It is caused by mutations in transthyretin, a plasma transport protein for thyroid hormone and retinol-binding protein (RBP)/vitamin A.56 Transthyretin...
thryretin is a single polypeptide chain of 127 amino acid residues. The transthyretin monomer (approximately 14,000 Da) folds to have an approximately 60% β-structure (Fig. 1). Four monomers associate noncovalently to form the tetrameric plasma transport protein (approximately 56,000 Da), which has binding sites for thyroxine in a central channel (T₄) and surface receptors for RBP/vitamin A (Fig. 2, arrows). The transthyretin gene is localized to chromosome 18. It spans approximately 7 kb and has four exons. Exon 1 codes for a signal peptide of 20 amino acid residues and only the first three residues of the mature protein. Exon 2 codes for amino acid residues 4–47; exon 3, amino acid residues 48–92; and exon 4, 93–127. Essentially all plasma transthyretin is synthesized in the liver, but the protein is also expressed in the choroid plexus of the brain and the retinal pigment epithelium of the eye. Expression is present during embryonic development and throughout life, but transthyretin evidently is not essential for life because mice that have had the transthyretin gene inactivated show no abnormalities in development or fecundity.

Greater than 100 mutations in the primary structure of transthyretin have been discovered and the majority have been found in association with amyloidosis. Table 1 lists 98 amyloid-associated TTR mutations that have been reported, with some clinical and demographic features of each. In addition, there are a few newly identified TTR mutations that have not yet been entered into the scientific literature. No mutation has been reported for exon 1. Exon 2 has 37 mutations reported to be associated with amyloidosis; exon 3, 45; and exon 4, 16.
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* | Clinical features: AN, autonomic neuropathy; CTS, carpal tunnel syndrome; eye, vitreous deposits; LM, leptomeningeal; PN, peripheral neuropathy.
† | Double-nucleotide substitution.
Amyloidosis is an autosomal-dominant disease; only one mutant TTR allele is required to develop pathology. Most affected individuals are heterozygous for a pathogenic mutation and express both normal and variant TTR. The majority of the mutations are the result of a single nucleotide substitution in the transthyretin gene, although one is the result of the deletion of an entire 3-base codon (ΔVal122), and two of the amino acid substitutions are the result of double-nucleotide substitutions in a codon. The development of transthyretin amyloidosis is most likely the result of change in primary structure of the protein, but the disease must be modulated by a number of genetic and possible environmental factors. The variant TTR is present in the blood from the time of birth, but does not start to form amyloid until adult life. The reasons for amyloid formation at a particular point in adult life must be under control of factors not inherent to the mutant TTR gene itself and are likely to be related to the biochemistry of aging. Penetrance, the percentage of individuals who are gene carriers for a mutant transthyretin who develop clinical disease, varies considerably for the different mutations. In northern Portugal, the Val30Met mutation is associated with late-onset clinical disease (mean age, 55–60 years) and is reported to have less than 50% penetrance. The same mutation in northern Portugal is associated with earlier onset of clinical disease (30–35 years of age) and is now recognized to have a much higher penetrance, although early reports of elderly Portuguese patients without a family history of disease suggested a lower penetrance. The Ile84Ser mutation in an Indiana/Swiss kindred shows essentially 100% penetrance by age 50.

Of the approximately 100 amyloidosis-associated TTR mutations, many have been found in single individuals or families. Only a few mutations are present in extended kindreds and multiple loci worldwide. These include Val30Met, which is prominent in northern Portugal, northern Sweden, and Japan. This mutation, however, has been found in many other countries. The Thr60Ala mutation is prominent in the United States, but probably originated in Northern Ireland and has now been found in many countries with families of Irish descent. Leu58His is also frequently found in the United States in families originating in Germany, where it is also found. Probably the most prevalent transthyretin mutation in the United States is Val122Ile. Approximately 3–4% of African Americans have this mutation, which causes late-onset restrictive cardiomyopathy that is rarely diagnosed in a timely manner. This mutation most likely originated on the west coast of Africa, where its prevalence is greater.

Some of the transthyretin mutations cause specific clinical syndromes, but there is considerable variation in disease presentation. Most produce sensorimotor polyneuropathy as a prominent feature and, for this reason, it has long had the name of familial amyloidotic polyneuropathy (FAP). The neuropathy usually starts with small-fiber dysfunction in the lower extremities, very similar to the neuropathy of diabetes mellitus, with lack of thermal appreciation being an early feature. Dysesthesias, however, may be prominent with or without varying degrees of pain. Motor function tends to be well maintained until the sensory neuropathy has advanced considerably. The level of sensory loss in the lower extremities slowly progresses from feet to ankles to legs to knees, at which time similar sensory symptoms usually present in the upper extremities. Autonomic neuropathy tends to occur relatively early in the course and results in sexual impotence in men, gastrointestinal motility problems, and bladder retention. Some men may have sexual impotence as their initial clinical symptom.

Nerve conduction studies can be a valuable tool for detecting peripheral nerve involvement. Localized abnormalities like median neuropathy at the wrist (carpal tunnel syndrome) can be differentiated from polyneuropathy based on the pattern of electrophysiological findings. Serial studies can help follow the course of peripheral nerve abnormality. A group of amyloid patients referred to the Indiana University Amyloid Research Center in whom the transthyretin abnormality was known have been evaluated with conduction studies. Of the Val30Met and Thr60Ala patients, 60% showed abnormalities characteristic of polyneuropathy but a few had isolated median neuropathy at the wrist. Among these two patient groups, 40% were electrophysiologically normal when initially seen. In contrast, of the Ile84Ser patients with abnormal peripheral nerve conduction results, 40% had isolated median neuropathy at the wrist and 60% had polyneuropathy.

Considerable variability exists with transthyretin amyloidosis. Carpal tunnel syndrome is often an early feature and occasionally may be the only clinical manifestation. Vocal hoarseness can occur due to recurrent laryngeal nerve palsy, and the “scalloped pupil” deformity, which is essentially pathognomonic for FAP, is due to amyloid deposits in ciliary nerves of the eye (Fig. 3). Vitreous opacities are much more common, seen with approximately 20% of transthyretin mutations, and may be the first manifestation of FAP. Vitreous opacities may be a
part of the oculoleptomeningeal syndrome of amyloid deposition,\textsuperscript{10,23,70} or associated with amyloid cardiomyopathy without leptomeningeal involvement (Table 1).\textsuperscript{19,57} Oculoleptomeningeal amyloidosis may be restricted to intracranial and spinal fibril deposition and presents with symptoms of stroke, seizures, hydrocephalus, spinal cord infarction or, later, cerebral hemorrhage.

Historically, death was often the result of infected leg ulcers, osteomyelitis, or general inanition due to gastrointestinal dysfunction. It is now appreciated that restrictive cardiomyopathy is a major cause of morbidity and mortality in patients with transthyretin amyloidosis. The restrictive cardiomyopathy presents as generalized weakness and fatigue without the signs of fluid retention typically seen in other forms of cardiomyopathy. When significant restrictive cardiomyopathy develops in patients with concomitant autonomic dysfunction, orthostatic hypotension becomes a major clinical manifestation and maintenance of proper fluid balance is often a challenge. Cardiac arrhythmias may occur with atrial ventricular block, and sinus exit block is common. Atrial fibrillation usually occurs in the late stages of restrictive cardiomyopathy and adds to the congestive failure due to poor ventricular filling.

Pathogenesis. Several factors are important in the pathogenesis of transthyretin amyloidosis. First among these is the structure of the fibril precursor protein, because amino acid substitutions in the primary protein structure dictate the inheritance of the disease. Despite solution of the tertiary structure of a number of the mutant transthyretins by X-ray crystallography, it is not obvious how each change in primary protein structure leads to initiation of amyloid fibril formation.\textsuperscript{25} Indeed, it is known that normal transthyretin can cause tissue deposition of amyloid fibrils.\textsuperscript{78} This is most commonly seen as senile cardiac amyloidosis and suggests that transthyretin, which has a predominantly β-pleated sheet structure (Fig. 2), has an inherent tendency to form the β-structured fibrils that we call amyloid. In vitro studies also support the fibril-forming tendency of transthyretin. Several of the different mutant forms of transthyretin as well as normal transthyretin will form fibril structures in vitro when incubated at acidic pH.\textsuperscript{32,38} Although these physical chemical studies are valuable in studying the pathogenesis of transthyretin amyloidosis, in vitro fibril formation is a model of fibril formation and not a model of amyloidosis.

A second factor to consider in pathogenesis is metabolic processing of transthyretin. TTR is a very prominent plasma protein (20–40 mg/dl) and it has a plasma residence time of only 1–2 days.\textsuperscript{5,72} It must represent a significant burden to the catabolic mechanisms of plasma protein turnover. Much is known about the synthesis of transthyretin by the liver, but the sites of catabolic processing have not been well defined. There have been reports of metabolic processing by the liver, and the kidney is also usually a very significant site for catabolism of plasma proteins. Transthyretin amyloidosis, however, is most prominent in nerves and in the heart, organs that are not known for their catabolism of plasma proteins.

The question arises as to whether organs that have high degrees of protein catabolism such as the liver, which is rarely involved with transthyretin amyloidosis, and the kidney, which may be involved but not as extensively as one would expect, are relatively protected from the downstream metabolic events necessary for amyloid fibril formation and deposition. Biochemical analysis of tissue transthyretin amyloid fibrils suggests that proteolytic processing may well be an important factor in pathogenesis. Although full-length transthyretin protein may be present in amyloid fibril deposits, a large percentage of the protein has been proteolyzed to give fragments of the carboxyl-terminal portion starting at amino acid positions 49 and 52.\textsuperscript{5,17,41} Is this relevant to amyloid pathogenesis or an epiphenomenon? It should be noted that TTR amyloid fibrils isolated from tissues of patients heterozygous for a transthyretin gene mutation contain normal as well as the variant TTR protein. Because plasma TTR exists as a group of TTR tetramers with one, two, three, or four variant monomer subunits, it is logical that normal TTR might find its way to the final amyloid fibril structure. Indeed, tissue deposits of TTR amyloid usually contain only 65%–75% variant TTR; the rest is normal TTR.

Considerable data on in vitro fibril formation suggest that the TTR monomer is the intermediate moiety in the pathway to aggregation and fibril formation, and the stability of the tetramer is the most important factor in amyloid fibril initiation.\textsuperscript{42} This is difficult to reconcile with the fact that both normal and variant TTR monomers are found in the final end-product (the in vivo amyloid fibril). One would have to hypothesize parallel metabolic pathways for the two proteins (variant and normal monomers) and they would need to converge on the final step of fibril assembly. In addition, although TTR tetramer stability may well be important in amyloid pathogenesis, there are conflicting observations when experimental data are compared to observed biologic data.
For example, for the Leu55Pro TTR variant, which causes an early-onset, aggressive form of amyloidosis, the tetramer is thermodynamically unstable. However, the Val122Ile tetramer, which is also thermodynamically unstable, is associated with one of the latest-onset and slowest progressive forms of amyloidosis. Although the Val122Ile tetramer is less stable than the normal TTR tetramer, the age of onset of clinical disease and the rate of disease progression are very similar to those of senile cardiac amyloidosis, in which no TTR mutation is present.

The importance of other amyloid fibril components has not been addressed for transthyretin amyloidosis. It is known that glycosaminoglycans are integral parts of AA amyloid and Aβ Alzheimer amyloid plaques, but similar data have not been documented for TTR amyloidosis.

Another aspect of transthyretin pathogenesis that must be addressed is the selective involvement of certain organ systems. The peripheral nervous system is the most common site of amyloid deposition in the transthyretin amyloidoses (Fig. 4).1 The gastrointestinal tract is also usually involved, but restrictive cardiomyopathy is probably the leading cause of death. The kidney frequently has amyloid deposits, but clinically significant renal amyloidosis is not as common in transthyretin amyloidosis as in AA or AL amyloidosis. Although amyloid deposits occur in blood vessel walls throughout the body, the spleen and hepatic parenchyma do not have amyloid deposits.

In peripheral nerve, amyloid deposits start around perforating arterioles (Fig. 5A). This deposition occurs in a sporadic fashion and, in advanced stages, large globular deposits of amyloid are seen displacing nerve fibers (Fig. 5B). At this stage, severe demyelination and loss of nerve fibers are seen (Fig. 5C). Figure 5A–C shows postmortem sciatic nerve specimens; similar findings are seen in biopsies of sural or other sensory nerves done for purposes of diagnosis (Fig. 5D). In the few studies that have been done on dorsal root ganglia, fibril deposition and neuronal loss appear to be an important part of the peripheral neuropathy. Similarly, little attention has been paid to the autonomic nervous system, although detailed studies revealed amyloid deposition within autonomic ganglia, nerve trunks, and adjacent vascular structures. In leptomeningeal amyloidosis, deposits were seen in vascular and surrounding connective tissue structures.

![FIGURE 3. Scalloped pupil deformity in a patient with Val30Met FAP. This sign of amyloid neuropathy disappears with dilation of the pupil.](image)

![FIGURE 4. Congo red–stained section of posterior tibial nerve from a patient with TTR Val30Met amyloidosis: (A) viewed under bright light; and (B) viewed with polarized light to show characteristic green birefringence of amyloid.](image)
leads to a clinical presentation with cerebral infarcts or, in later stages, cerebral hemorrhage. Infarcts may involve basal ganglia and the spinal cord. Although not proven, the amyloid fibril precursor of leptomeningeal amyloidosis is probably synthesized by the choroid plexus.\textsuperscript{15,63}

Patients dying from leptomeningeal amyloidosis have been found to have little or no amyloid deposition in other organ systems. Transthyretin amyloid in the vitreous of the eye is probably the result of synthesis by the retinal pigment epithelium. The predilection for fibril formation in the vitreous of the eye is not clear. The mechanisms involved in amyloid deposition in the vitreous may be similar to peripheral nerve or cardiac pathology, but an intriguing variation is that amyloid fibrils in the vitreous are greatly enriched (approximately 90\%–95\%) in variant TTR compared to the proportion (60\%–65\%) in nerve and cardiac tissue of heterozygotes.\textsuperscript{41} Vitreous opacities are found with approximately 20\% of the known amyloid-producing TTR mutations and may be seen in conjunction with leptomeningeal amyloidosis.\textsuperscript{6} This may, however, be found with other mutations that primarily cause peripheral neuropathy or cardiomyopathy.

**APOLIPOPROTEIN AI (ApoAI) AMYLOIDOSIS**

Twelve different mutations in the ApoAI gene have been associated with systemic amyloidosis.\textsuperscript{2} The amyloidosis of only one of these mutations, however, has been shown to cause peripheral neuropathy. This ApoAI mutation, Gly26Arg, was first described by Van Allen et al., in a family residing in Iowa.\textsuperscript{73} Unlike transthyretin amyloidosis, the main clinical feature of ApoAI amyloidosis is renal amyloid deposition.

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**FIGURE 5.** (A) Longitudinal section of sciatic nerve from a subject with Val30Met TTR amyloidosis. Amyloid deposits are present around and in walls of intraneural vessels (arrows). (B) Amyloid deposits in sciatic nerve give appearance of displacing nerve fibers (arrows). (C) Cross-section of sciatic nerve bundle showing marked amyloid deposits and loss of nerve fibers. (D) Section of sural nerve of a patient with Thr49Ala TTR amyloidosis (hematoxylin–eosin stain).
and death is usually caused by azotemia. Other organ involvement, including liver, spleen, and occasionally heart, has been seen in this syndrome. The renal amyloid deposition is unlike most cases of AL and AA amyloidosis, which usually cause significant proteinuria from glomerular fibril deposition. In ApoAI amyloidosis, amyloid deposits are predominantly in blood vessels and medullary structures. Proteinuria is usually very limited and nephrotic syndrome does not develop.

ApoAI amyloidosis is an adult-onset autosomal-dominant disease. In the Iowa kindred the first clinical manifestation appeared most commonly between age 30 and 40 years, but some individuals were affected by their late 20s, and others after 50. Most of the patients presented with neuropathic symptoms, but some were initially identified with renal failure. In most subjects, the neuropathy and renal failure were slowly progressive, with death occurring approximately 10 years after initial presentation. The disease duration varied from a few months to as long as 25 years.

Clinically, the neuropathy of ApoAI is very similar to transthyretin amyloidosis. It usually starts with sensory changes in the lower extremities. This is progressive and eventually involves the upper extremities. Weakness in leg muscles and foot drop often occur early in the disease. Impotence is a common feature in men. Ataxia, absent muscle stretch reflexes, and muscle atrophy are common as the disease progresses, and the patient may eventually become tetraparetic. Despite the extensive neuropathy, death is usually related to renal insufficiency.

A number of families have been described with ApoAI Gly26Arg amyloidosis and not all have neuropathy as part of their clinical picture. The reason for this is not known, nor is it known why the systemic amyloidoses caused by other mutations in ApoAI are not associated with peripheral neuropathy. In the Iowa kindred, neuropathy seems to have been delayed in the most recent generation; several individuals have begun to have neuropathy, but only after significant renal impairment was documented.

Amyloid deposition in neural structures with this form of amyloidosis was well described by Van Allen et al. and is very similar to the pathology of transthyretin amyloidosis. Large deposits of amyloid are found in peripheral nerves with displacement of nerve fibers. Extensive deposition is present in dorsal root ganglia. The leptomeninges around the spinal column and the brain show amyloid deposition, but, as with transthyretin, the central nervous system (CNS) parenchyma is not involved. Intracerebral hemorrhage has not been described with ApoAI amyloidosis. Leptomeningeal deposition is probably the cause of elevation in spinal fluid protein, which may exceed 200 mg/dl. Vitreous opacities are not part of this syndrome.

**Pathogenesis.** Mutations in the ApoAI gene, present as a single copy on chromosome 11 (11q23), are the cause of this form of amyloidosis. It is an autosomal-dominant disease and all affected subjects identified to date have been heterozygous for 1 of the 12 known mutations. Degree of penetrance has not been established but is probably greater than 50% of gene carriers. Both normal and variant ApoAI are found in blood. ApoAI is mainly contained in the high-density lipoprotein portion of plasma, as are SAA and ApoAl, two other amyloid precursor proteins. Pathogenesis appears related to incomplete degradation of the abnormal ApoAI, which normally is a single polypeptide chain of 243 amino acid residues. The amyloid deposits contain only the amino-terminal 83–93 amino acid residues of the protein. Despite the fact that patients are heterozygous for the variant ApoAI, there is no evidence that normal ApoAI contributes to fibril formation. With the neuropathic Gly26Arg protein, only the variant peptide is found in amyloid fibrils. Because ApoAI is mainly an α-helical apolipoprotein, major reconfiguration of the amino-terminal portion of this molecule must occur in order for it to be incorporated into the β-pleated sheet structure that is typical of amyloid fibrils. As with transthyretin, there is good evidence that the variant ApoAI has an increase in metabolic rate. Studies with radiolabeled ApoAI have shown that the Gly26Arg protein has approximately half the plasma residence time of wild-type ApoAI. This indicates an increase in the catabolic rate of the variant protein, and it is hypothesized that this increased intracellular degradation leads to generation of the pathologic amino-terminal peptide.

**GELSOLIN AMYLOIDOSIS (AGel)**

The neuropathy of gelsolin amyloidosis starts as a progressive cranial neuropathy in middle age (approximately 40 years of age) and may be followed much later by peripheral neuropathy involving the limbs. The disease is first manifest as lattice corneal dystrophy, often by age 20–30. This unusual association is typical of AGel amyloidosis and makes diagnosis of the condition relatively easy.

Amyloid formation is the result of a mutation (Asp187Asn) in plasma gelsolin, an actin-modulat-
ing protein. Most patients are heterozygous for this mutation and have modest involvement of internal organs, mostly vascular. Individuals homozygous for the mutation may have severe systemic disease with renal failure. A second mutation in gelsolin (Asp187Tyr) has been described and gives a similar phenotype. There are few patients with AGel in the United States and Japan; most affected families are in southwestern Finland.

DIAGNOSIS OF AMYLOID NEUROPATHY

The diagnosis of any type of amyloidosis is made by tissue biopsy. The biopsy may be from a clinically affected organ such as heart, nerve, kidney, or liver, or, for systemic amyloidosis, a nonspecific site such as rectal mucosa, abdominal fat, or gingival or minor salivary gland. Occasionally, systemic amyloidosis will be detected as an incidental finding on bladder, prostate, or gastric biopsies. The greatest impediment to making a timely diagnosis is lack of consideration of amyloidosis in the initial evaluation of the patient. This is true for cardiac and renal amyloidosis where more common pathologic conditions cause similar organ dysfunction. This is also true in the neuropathic forms of amyloidosis.

Once the diagnosis of amyloidosis is considered, a rectal biopsy or abdominal fat aspirate may demonstrate amyloid deposition, but many patients require nerve biopsy to confirm the specific neuropathology (Fig. 5D). The sural nerve is most often used for diagnostic biopsy. It is purely sensory and, by the time biopsy is considered, the degree of sensory loss to the foot is usually such that further loss of sensation from the biopsy is well tolerated. In light of the spotty nature of amyloid deposition, however, a sural nerve biopsy that does not demonstrate amyloid deposits should not be taken as conclusive evidence that amyloid is not the cause of the neuropathy (Fig. 6). At this point, consideration of other organ systems should be undertaken: Are there associated signs of cardiomyopathy, renal disease, autonomic dysfunction, hypotension, erectile dysfunction, or alternating constipation and diarrhea?

After the diagnosis of amyloidosis is made, for the patient with neuropathy, the most important question is whether this is an hereditary form of amyloidosis or a sporadic immunoglobulin light chain (AL) primary amyloidosis. AL amyloidosis frequently causes carpal tunnel syndrome, and 20% of patients with AL will present with peripheral neuropathy. On nerve biopsy, there are no distinct features to differentiate hereditary amyloidosis from AL amyloidosis (Fig. 7A). Pathogenic mechanisms for initiation of amyloid deposition in endoneurial vascular structures are probably similar (Fig. 7B).

Immunohistochemistry with specific antibodies may be helpful, but unfortunately this technique is not completely reliable. The demonstration of a monoclonal plasma cell dyscrasia and presence of monoclonal immunoglobulin protein in serum or urine in approximately 80% of AL patients helps to distinguish these patients from those with hereditary syndromes.

For hereditary neuropathic amyloidosis, family history can be very important, but is often not sufficient to confirm a diagnosis. Lack of penetrance and advanced age of onset of some of the transthyretin amyloidoses, as well as variable expression of the disease, hinder timely diagnosis. In rare cases de novo mutations in transthyretin cause amyloidosis. Of the hereditary amyloid neuropathies, gelsolin amyloidosis is the easiest to detect because all affected individuals have lattice corneal dystrophy from an early age. Careful ophthalmologic examination will reveal this pathognomonic sign. Neuropathy associated with the ApoAI Gly26Arg mutation is found in patients whose primary disease is renal and, unlike the typical AL amyloidosis patient, renal disease is associated with little proteinuria and presents as progressive azotemia. Transthyretin amyloidosis is the most frequent cause of hereditary amyloid neuropathy, and the entity that most often must be distinguished from immunoglobulin (AL) amyloidosis.

Most of the mutations in transthyretin that have been associated with amyloidosis cause some degree
of peripheral neuropathy. Neuropathic symptoms usually occur early in the disease process but may remain mild for an extended period of time. By the time the patient seeks care from a neurologist, there are often signs of other system manifestations, such as enteropathy or cardiomyopathy. The mainstay of diagnosing this disease is DNA testing, which will usually demonstrate one of the known mutations (Table 1). Occasionally, a new mutation will be found by direct DNA sequencing and correlation of disease and genetics will be needed. DNA testing is now available commercially as well as in a few amyloid research laboratories. If a patient has a family history of transthyretin amyloidosis and the family mutation is known, a specific test can be ordered to determine whether the patient is a carrier of this mutation. If a family mutation is not known, full TTR DNA sequencing is recommended. Ordering a panel of the most frequent TTR mutations will not necessarily exclude the diagnosis and may be a great disservice to the patient with a new mutation.

DNA sequencing for gelsolin and ApoAI mutations is not commercially available. Help from an amyloidosis research laboratory is recommended. These DNA tests are easily accomplished, but these types of amyloidosis are too uncommon to generate any great commercial interest.

**Treatment.** Therapy for hereditary amyloidotic neuropathy involves both specific and nonspecific measures. Nonspecific measures include treatment of the painful neuropathic symptoms. Treatment with gabapentin, amitriptyline, and more recently, pregabalin, may be effective but efficacy is unpredictable and, even when a drug is initially effective, the response may change as the neuropathy progresses. Alternating constipation and diarrhea are common manifestations of transthyretin amyloidosis. Addition of fiber to the diet to increase bulk of stool may be efficacious. Occasionally, alteration of gastrointestinal flora by antibiotics is effective, but often agents such as loperamide are required to slow bowel function. In severe cases, tincture of opium may be necessary. Urinary bladder retention occasionally requires training in self-catheterization. Newer agents for erectile dysfunction may help some men with autonomic dysfunction.

The only specific therapy for transthyretin amyloidosis is orthotopic liver transplantation. The basis for this form of therapy is to rid the plasma of mutated transthyretin, which is synthesized by the liver. Since the first patient received this therapy in 1990, greater than 1200 liver transplantations have been accomplished. Statistical analysis indicates that patients with the Val30Met mutation are most likely to benefit and have 80% survival at 5 years. Patients with other transthyretin mutations, however, do not fare as well and show a 5-year survival of only 55%–60%. Many patients, especially those with TTR mutations other than Val30Met, have progression of disease after liver transplantation. Biochemical analysis of amyloid from tissues of patients who died after having a liver transplant indicates that this is the result of continued amyloid deposition from wild-type TTR. It has been suggested that liver transplantation early in the course of disease should be considered, but it is still unknown whether this

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**FIGURE 7.** (A) Sciatic nerve of a patient with AL amyloidosis showing large deposits of intraneural amyloid. (B) Cross-section of sciatic nerve of a patient with AL amyloidosis. As with TTR amyloidosis, amyloid deposition in intraneural vessels occurs and suggests a similar pathogenesis for the two protein deposition diseases (hematoxylin–eosin stain).
would increase the overall time of survival. Nutritional status is an important factor in determining longevity after liver transplantation. As far as peripheral neuropathy is concerned, there is only one report documenting regeneration of nerve fibers on nerve biopsy after liver transplantation. At the present time, there is a study to determine whether diflunisal, a modified salicylate nonsteroidal anti-inflammatory drug, will alter the progression of TTR in amyloidosis. The study is based on the demonstration that small organic molecules such as diflunisal bind to the transthyretin tetramer and thermodynamically stabilize the structure. This form of therapy has not been tested in an animal model, but because diflunisal is marketed as a nonsteroidal anti-inflammatory drug, it has passed many of the safety requirements required for new drug approval. Vitrectomy may be considered a specific therapy for transthyretin amyloid in the eye; although usually effective, it is really a mechanical means of restoring visual acuity. Amyloid deposition may recur after vitrectomy and require repeated surgery. It is important to remember that these patients have increased risk of retinal detachment and many develop glaucoma after vitrectomy. They need to be monitored closely for changes in intraocular pressure. At the present time there is no evidence that orthotopic liver transplantation alters the course of oculoleptomeningeal amyloidosis.

Orthotopic liver transplantation has also been accomplished in a number of patients with ApoAI Gly26Arg amyloidosis and, although apolipoprotein levels in the blood only decrease by approximately 50%, disease progression seems to be favorably altered. As most of these patients underwent combined liver and kidney transplants, and it is usually the slowly progressive renal disease that determines the patient’s prognosis, it is difficult to be certain whether the generalized amyloidosis is favorably affected. Recently, some patients with only modest renal impairment received liver transplantation alone. Close longitudinal evaluation of these patients may reveal more clearly the efficacy of liver transplantation for this disease.

For gelsolin amyloidosis the only effective therapy to date is corneal transplantation. This is very effective and can be repeated if the amyloid deposits infiltrate the new cornea. Plastic surgery can be effective in treating the cutis laxis and blepharochalasis, which are the result of bilateral facial palsy, but there is no specific therapy for this disease. Gelsolin is an essential protein for actin function and treatments aimed at eliminating its production are not likely to be tolerated.

It is obvious that specific treatments are needed, especially for transthyretin amyloidosis. Recently, studies in mice transgenic for the human Ile84Ser TTR mutant gene have demonstrated suppression of hepatic TTR synthesis by antisense oligonucleotides (ASO). Whether this will be the basis for treatment of TTR amyloidosis, either FAP or senile cardiac amyloidosis (SCA), must await safety and efficacy testing. Similar gene therapy approaches have been proposed using ribozymes that suppress TTR expression in hepatic cell cultures. Also, recent studies on targeted conversion of the TTR gene have demonstrated the feasibility of this approach. These studies have not progressed to the point that a therapeutic agent for TTR amyloidosis can be predicted in the near future. Most important is the development of an animal model to test any new form of therapy in a timely fashion, because the human disease is caused by so many different mutations with various phenotypes and usually has a slowly progressive but ingravescent course.

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REFERENCES


INVITED REVIEW

ABSTRACT: Over the years various steroid trials have been conducted in Duchenne muscular dystrophy (DMD). In children who are still able to walk as well as in those who are wheelchair-bound, corticosteroids have been found to stabilize muscle strength for a period of time. Controlled clinical observations have shown that some boys remain ambulatory for years longer than reported in natural history data. The two main steroids used are prednisone/prednisolone and deflazacort. They are probably equally effective in stabilizing muscle strength but may have different side-effect profiles; for instance, deflazacort causes less weight gain. The exact mechanism by which steroids slow the dystrophic process is under investigation. DMD children treated long term also seem to develop other complications of the condition less frequently. For instance, they develop respiratory insufficiency later and have fewer cardiac symptoms. The therapeutic value of corticosteroids is limited, but these drugs represent the best treatment option currently available.

THE ROLE OF CORTICOSTEROIDS IN MUSCULAR DYSTROPHY: A CRITICAL APPRAISAL

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Mutations in the dystrophin gene that preclude the synthesis of a functional dystrophin protein lead to Duchenne muscular dystrophy (DMD). In developing a treatment for DMD, compensating for the genetic defect is now recognized to be a much greater challenge than when the molecular defect was first identified. In fact, potential therapies for the future include reading through nonsense mutations, cell or gene replacement, and antisense oligonucleotide technology inducing “exon skipping.” At the present time, validated clinical trials have been conducted, but only on the positive outcome of therapy with corticosteroids.

STEROIDS AND DMD

The use of steroids in DMD was first tested in an open-label study by Drachman et al.,17 who showed a positive outcome. In contrast, an independent trial by Siegel et al.47 was reported as negative but was heavily criticized. Since then, many studies have been undertaken and patients in many countries are now offered steroid treatment, although there is still a lack of international consensus on the most effective steroid and optimal dosage regimens. There is evidence from randomized, controlled trials that steroids do improve strength and functional outcome in DMD. In an ideal trial, the primary outcome measure should be prolongation in the ability to walk, whereas the outcome measures more frequently used by various investigators include tests of muscle function and strength or the forced vital capacity.

Five such controlled trials are suitable for inclusion as class I trials (Table 1). These studies include those in which participants were given either prednisone/prednisolone or deflazacort treatment and compared to those receiving placebo. Only one study used prolongation of walking ability as an outcome measure. Two studies demonstrated improved muscle function and strength over a 6-month period. One trial showed stabilization of muscle strength and function for up to 2 years. In this study deflazacort (DFZ) was used; DFZ is an oxazolidine derivative of prednisone that has an equivalent dose of 6 mg for each 5 mg of prednisone. DFZ reportedly has less effect on loss of vertebral bone mass, and this effect seems particularly important in growing children; DFZ has therefore been used in DMD because of its potential “bone-sparing” ef-
Subsequent studies have demonstrated equal effectiveness with prednisone, possibly with less risk of weight gain. In a non-randomized study, long-term treatment with DFZ led to preserved lung function compared with untreated controls. A long-term benefit in cardiac function with DFZ was found after treatment for 5 years. Echocardiography showed a mean fractional shortening of 33% compared with 21% in the untreated group. Asymptomatic cataracts were found in 50% of the treated boys.

Thus, there is controlled evidence that in the short-term, for instance, at 6 months to 2 years, steroids significantly improve muscle strength and function in DMD, and there is increasing evidence of long-term benefits.

**EVIDENCE OF STERROID EFFICACY FROM CLINICAL TRIALS**

In 1974, Siegel et al. (class II) published the only negative controlled trial of prednisolone/prednisone in DMD, but the outcome measures were not quantitative. In 1981, the Clinical Investigation of Duchenne Dystrophy (CIDD) group was formed. This consortium of U.S.-based specialists performed randomized and non-randomized corticosteroid trials.

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<td>21</td>
<td>Open</td>
<td>92</td>
<td>5–15</td>
<td>Prednisone 0.75 mg/kg/day</td>
<td>2 years</td>
<td>Stabilization for 2 years</td>
<td>Cataracts, glycosuria in 10 patients, significant weight gain</td>
<td>I</td>
</tr>
<tr>
<td>35</td>
<td>Double-blind</td>
<td>28</td>
<td>5–11</td>
<td>Deflazacort 1 mg/kg/day</td>
<td>9 months</td>
<td>Improvement for 6 months</td>
<td>Slow decline at 18 months</td>
<td>III</td>
</tr>
<tr>
<td>44</td>
<td>Open</td>
<td>32</td>
<td>6–14</td>
<td>Prednisolone 0.75 mg/kg/day for 10 days/month</td>
<td>6–18 months</td>
<td>Improvement at 3 months</td>
<td>No additional benefit from azathioprine</td>
<td>I</td>
</tr>
<tr>
<td>24</td>
<td>Randomized</td>
<td>107</td>
<td>5–15</td>
<td>Prednisolone 0.75 mg/kg/day; azathioprine 2.5 mg/kg/day/placebo</td>
<td>18 months; 12 months</td>
<td>Improvement</td>
<td>Weight gain</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>Double-blind</td>
<td>28</td>
<td>5–10</td>
<td>Deflazacort 2 mg/kg AD</td>
<td>24 months</td>
<td>Improvement at 12 months; prolonged walking</td>
<td>Fewer side effects but bone fractures</td>
<td>I</td>
</tr>
<tr>
<td>5</td>
<td>Double-blind cross-over</td>
<td>38</td>
<td>4–19</td>
<td>Placebo 6 months; prednisone 6 months</td>
<td>12 months</td>
<td>Improvement</td>
<td>Weight gain hirsutism</td>
<td>I</td>
</tr>
<tr>
<td>10</td>
<td>Randomized</td>
<td>18</td>
<td>5–14</td>
<td>Prednisolone 0.75 mg/kg/day; deflazacort 0.9 mg/kg/day</td>
<td>12 months</td>
<td>Equal effect</td>
<td>Weight gain in prednisone group</td>
<td>II</td>
</tr>
<tr>
<td>8</td>
<td>Open</td>
<td>30</td>
<td>7–15</td>
<td>Deflazacort 0.9 mg/kg/day</td>
<td>3.8 years</td>
<td>Ambulation prolonged; FVC preserved</td>
<td>Cataracts in 30%</td>
<td>II</td>
</tr>
<tr>
<td>28</td>
<td>Open</td>
<td>4</td>
<td>3–4.5</td>
<td>Prednisolone 0.75 mg/kg at 10 days on and 10 days off</td>
<td>2.5 years</td>
<td>Stabilization of motor function</td>
<td>Irritability</td>
<td>III</td>
</tr>
<tr>
<td>14</td>
<td>Open, historical controls</td>
<td>20</td>
<td>5–10</td>
<td>Prednisolone 5 mg/kg/twice weekly</td>
<td>22 months</td>
<td>Improvement</td>
<td>Irritability</td>
<td>III</td>
</tr>
</tbody>
</table>

AD, alternate days.
**FIGURE 1.** Milestones of disease progression in 131 DMD patients. Time (in seconds) to perform four different motor functions, GSGC (filled squares: gait, walk 19 meters; filled circles: climb stairs; filled triangles: Gowers’ maneuver, stand from sitting; open circles: chair, stand from chair) shows a progressive increase with age. There is a sudden loss of all functions: ability to perform Gowers’ maneuver is lost at 9.5 years and gait is lost at 10.5 years. Modified from Angelini et al.1

A series of class I randomized trials in DMD patients aged 5 years and older. Of the 14 tested drugs, steroids were the only ones to provide any benefit. A sometimes overlooked, but important part of this work was to establish the natural history of DMD in quantitative terms. Disease milestones were established by the Italian collaborative group1 in 131 DMD patients (Fig. 1). Most of the CIDD work, notably measurement of the downward slope of strength loss over time, was done before the discovery of the dystrophin gene.

Numerous clinical trials have established both the effect of steroids in DMD and the well-known risk of side effects associated with their daily use. The CIDD showed a daily dose of 0.75 mg/kg to be the most effective in their randomized, controlled trials, in which dose–response analysis showed that 0.3 mg/kg per day was not as effective,24 although 1.5 mg/kg daily provided no additional benefit.34 Deflazacort 0.9 mg/kg is the equivalent daily dose to 0.75 mg/kg of prednisone, and appears to be equally effective (Fig. 2).1,2,9,12 Side-effect profiles of the two steroids differ slightly. Deflazacort appears to cause less weight gain, but is more likely to be associated with the development of asymptomatic cataracts. It is difficult to assess the long-term differences in these regimens with respect to their effect on bone mineral density. Some investigators have reported a high incidence of vertebral fractures with deflazacort, whereas others did not have this experience.

Only a few randomized, controlled trials of steroids in DMD have been presented in sufficient detail to enable efficacy to be compared for several outcomes. These trials did, however, present evidence that use of daily prednisone22 (0.75 mg/kg), or the equivalent dose of deflazacort (0.9 mg/kg), stabilizes strength in DMD.10,11 Several studies then confirmed in patients treated with one or the other of these regimes that this increase in strength was followed by improvement in function; open follow-up studies have demonstrated that age at loss of ambulation was postponed to the mid-teens (Fig. 3)

**FIGURE 2.** (A) Medical Research Council (MRC) score in deflazacort- and prednisone-treated groups shows stabilization of strength after initial, slight improvement. Natural history controls show a continuous decline in strength. (B) Functional score in both the deflazacort- and prednisone-treated groups shows a slight improvement at the beginning of therapy, whereas the natural history group tends to increase in grade and worsen progressively. (C) Percentage of body weight increase in the deflazacort- and prednisone-treated groups shows a high incidence of vertebral fractures with deflazacort, whereas others did not have this experience.

Modified from Bonifati et al.11

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and there was better preservation of respiratory and cardiac function.46

**VARIETY OF DOSAGES AND REGIMENS**

Most studies have shown useful benefits from steroids: the recommended daily dose is prednisone 0.75 mg/kg or deflazacort 0.9 mg/kg. Fenichel et al.22 reported less effect on alternate-day prednisone therapy. Other studies have been performed using steroids on alternate days,2 or with an intermittent schedule for 10 days per month,47 for 10 days on/10 days off,28 or on weekends only,14 and some have demonstrated benefit in functional parameters and fewer side effects. The weekend-only regimens allowed linear growth to be maintained in boys,14 but did not prevent contractures, which resulted in loss of ambulation at around age 10 years in 25% of treated DMD cases. Other regimens (daily low-dosing) aim to reduce the cumulative steroid dose.5

**How Good Are the Corticosteroid Clinical Trials?** A methodological evaluation of the studies is presented in Table 1. Most controlled trials were short term and addressed the issue of muscle strength rather than functional outcome. The ideal outcome measures should reflect issues relevant to the disease; be simple to administer and standardize across different evaluators; and be validated. Measures of function can be divided into five main groups described in what follows.

**Milestones of Disease Progression.** In some ways, disease milestones are the most meaningful endpoints to measure changes in function. This may involve monitoring age at loss of ambulation or wheelchair-bound (WCB) age, because changing this endpoint implies better quality of life, but this requires a long-term study. Only one class I randomized study with DFZ2 used prolongation of time to WCB age as an outcome measure, studying 28 patients over a minimum of 24 months. Other double-blind class I studies were of short duration (6 months) and not designed to demonstrate prolongation of walking ability.24,34

Another functional index that is easy to monitor is the loss of ability to arise from the floor (Gower’s maneuver).

**Myometry and Dynamometry.** Quantitative strength measurements by different techniques have been performed. Dynamometry was first used in the Drachman trial.17 Myometric recordings of individual muscles (e.g., quadriceps) have also been performed, using a Penny & Giles transducer, and recorded in kilograms of force.23 Accurate placement of the spreader is required below the patella on the anterior surface of the knee. The knee should be at 90°, and peak force is recorded. Such measurements correlate with loss of force in the natural history of DMD (Fig. 4). Quantitative muscle testing by standard techniques has been validated and direct assessment of muscle strength is a valid outcome measurement not only in DMD but in all chronic neuromuscular disorders.

**Graded and Timed Testing.** Many clinicians use timed and graded functional tests as a measure of disease progression. Commonly used validated measures include time and grading of gait (G); for instance, time to walk 10 meters, and to climb a set of stairs (S), rise from a chair (C), and rise from the floor (Gowers’ maneuver; G) (Table 2). The sum of grades proposed for performing such functional parameters varies from 1 to 7 and are grouped together to form the GSGC score.3,11 These are simple, repro-

![FIGURE 3. Kaplan–Meier curve of loss of ambulation shows a delay of at least 1 year between steroid-treated (filled triangles) and untreated or placebo-treated DMD (filled squares) patients.](image_url)

![FIGURE 4. Correlation between measurements of muscle strength by MRC grade in iliopsoas (open bars) and in quadriceps muscles (shaded bars) and myometry in quadriceps (filled bars). The correlation is plotted against each grade of gait and shows a similar degree of strength decline as measured by myometry and by manual muscle testing between gait grades 0 and 6. When a patient becomes wheelchair-bound (grade 7) muscle strength appears preserved.](image_url)
ducible, functional tests that vary with age (Fig. 5). Several trials have shown that the tests are standardized and the timed activities are truly the same. Gowers’ time was analyzed and improved significantly in two trials with prednisone. Walking and stair-climbing time showed statistically significant improvement in these two trials. Various composite scores of function have been defined and consider not only the time taken or actual ability to perform an activity but also the quality of its performance; these include both the GSGC score and Brooke’s score, which analyzes in detail lower- and upper-limb measures and might be particularly valuable in non-ambulant children.

**Pulmonary Function.** Measurement of forced vital capacity can be performed in cooperative children aged 5 years and older. This technique appears to offer a reliable test in ambulant children. Analysis of pooled data from two studies with daily prednisone (0.75 mg/kg) demonstrated a mean improvement of 0.17 L at 6 months in the prednisone-treated group compared with the placebo group. Mendell et al. compared 1.5 mg/kg prednisone daily with placebo and found a mean improvement of only 0.14 L.

**Neuroimaging.** Computerized tomographic scanning might be useful to document muscle atrophy and fat degeneration quantitatively. Magnetic resonance imaging offers a good index of muscle mass and of myoedema in STIR sequences. Magnetic resonance spectroscopy can also be used to assess metabolism in individual DMD cases.

**MECHANISM OF GLUCOCORTICOSTEROID EFFECT**

Steroids act on the regulation of signal transduction and have a direct nuclear effect. The beneficial effect of DFZ on muscle has been associated with activation of the calcineurin/nuclear factor of activated T-cells (NF-AT) pathway. Studies at the transcriptome level by microarray analysis and at a proteomic level are in progress in DMD. Such studies might identify gene and protein targets for steroid response. A direct effect on muscle degeneration has been observed in *C. elegans*, in which prednisone reduced muscle cell degeneration by 40%.

Other hypotheses are that steroids reduce muscle necrosis and inflammation, although possible alternative actions of steroids are in modulating the cell response to inflammation. Alternatively, steroids may enhance the proliferation of myogenic precursor stem cells or myoblasts, and thereby increase muscle regeneration and growth due to their anabolic effect. Skeletal muscle is made of multinucleated postmitotic fibers that arise from mononuclear precursors, such as satellite cells located between the plasma membrane and extracellular matrix. Muscular dystrophy is associ-

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**Table 2. Functional GSGC score.**

<table>
<thead>
<tr>
<th>Gait (G)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1-normal</td>
<td>1</td>
</tr>
<tr>
<td>2-mild waddling, lordosis and/or toe walking</td>
<td>2</td>
</tr>
<tr>
<td>3-moderate waddling, lordosis and/or toe walking</td>
<td>3</td>
</tr>
<tr>
<td>4-severe waddling, lordosis and/or toe walking</td>
<td>4</td>
</tr>
<tr>
<td>5-walks only with assistance (i.e., braces, cane, crutches)</td>
<td>5</td>
</tr>
<tr>
<td>6-stands, but unable to walk</td>
<td>6</td>
</tr>
<tr>
<td>7-confined to wheelchair</td>
<td>7</td>
</tr>
</tbody>
</table>

Time to walk 10 meters: __ seconds

Climbing stairs (S)

1-Climbs without assistance
2-Supports one hand on thigh
3-Supports both hands on thighs
4-Climbs in upright position but with aid of railing
5-Climbs while clinging to the railing with both hands
6-Manages to climb only a few steps
7-Unable to climb steps

Time to climb steps: __ seconds

Gowers’ maneuver (G)

1-normal
2-Butt-first maneuver, one hand on floor
3-Butt-first maneuver, two hands on floor
4-Unilateral hand support on thigh
5-Bilateral hand support on thighs
6-Arises only with aid of an object (table, chair, etc.)
7-Unable to arise

Time to standing from sitting: __ seconds

Arising from a chair (C)

1-Normal
2-With wide base and/or difficulty, but without support
3-With support on one thigh
4-With support on both thighs
5-With support on arms of chair or on a table
6-Not possible

Time to standing from sitting: __ seconds

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**FIGURE 5.** Natural history of 131 DMD children shows a continuous increase in grade up to 9.5–10 years, when most children reach grade 7 (loss of function) in climbing stairs and rising from a chair.
ated with a secondary inflammatory cell reaction (Fig. 6) and replacement of muscle fibers by fibrous tissue. Spuler and Engel demonstrated that complement deposition occurs unexpectedly in dystrophic fibers. Theoretical explanations of the beneficial effect of steroids include a reduction in the rate of muscle breakdown. A study on protein metabolism concluded that the beneficial effect of steroids in DMD are associated with increased muscle mass mediated by inhibition of proteolysis. Steroids may act as direct transcriptional modifiers to augment dystrophin expression in “reverted fibers,” or increase synergistic molecules, such as muscle glycoproteins, which complement the action of dystrophin. Methylprednisone selectively affects dystrophin in culture and increases utrophin and satellite cells. It is unlikely that steroids have a purely immunosuppressive function because azathioprine does not have a positive effect in DMD and mononuclear cell populations are differentially expressed in controls and patients treated with prednisone or azathioprine. Immunohistochemical studies on biopsies from boys with DMD treated with placebo or prednisone showed that the total number of T cells was lower in the prednisone-treated group, although B cells, natural killer cells, macrophages, and necrotic fibers did not differ in number. However, the mechanisms of action for steroids and azathioprine are profoundly different and may not be compared easily in this way.

SIDE-EFFECT MONITORING

Side Effects. Table 3 shows the most common side effects and a response protocol for steroid reduction.

Weight and Height. For any child with DMD it is essential to document weight and clearly define the point at which weight gain becomes a substantial adverse event. This is best related to age and height by calculating body mass index (BMI). An absolutely essential adjunct to any protocol for monitoring steroid use in DMD is dietary advice, preferably from an

FIGURE 6. Cross-sections of muscle biopsy from a 6-year old DMD patient stained with hematoxylin–eosin (A) and Gomori trichrome (D), and immunostained using antibodies against macrophages (B), CD4 lymphocytes (C), major histocompatibility complex (MHC) class I (E), and CD8 lymphocytes (F). Subtypes of inflammatory cells and molecules have been characterized and localized in a group of degenerating/regenerating muscle fibers. The asterisks indicate the same fiber in different sections. Original magnification ×200.
experienced dietician, supplemented with written parental consent. This should include the suggestion to cut down on high-calorie foods and maintain a healthy diet. Weight gain is the most frequently reported side effect for DMD children on steroids. In a trial comparing prednisone and DFZ, 10% of the participants dropped out of the trial protocol because of weight gain; dropouts occurred with both drugs, although DFZ is generally associated with less weight gain than prednisone.1,10 However, many DMD children gain excessive weight even in the absence of steroid treatment, a tendency probably accentuated by relative lack of activity and parental indulgence. Excessive weight can cause reduced mobility, so it is paramount to control this factor.

Parents should be warned that appetite can increase dramatically at the onset of steroid treatment and an attempt to control appetite should be made, preferably at an early stage.

Table 3. Adverse event monitoring and response protocol.

<table>
<thead>
<tr>
<th>Side effect</th>
<th>Prophylactic measures</th>
<th>Event to be recorded</th>
<th>Event necessitating dose reduction</th>
<th>Treatment: steroid withdrawal, specific treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight</td>
<td>Dietary advice</td>
<td>Change in weight from baseline</td>
<td>25% increase from baseline</td>
<td>Obesity, severe cushingoid appearance; reinforced dietetic input necessary</td>
</tr>
<tr>
<td>Height</td>
<td>Endocrinological follow-up</td>
<td>Change in height compared to predicted percentiles</td>
<td>Failure to gain height that is unacceptable to child/family</td>
<td>Growth treatment by endocrinologist</td>
</tr>
<tr>
<td>Behavioral changes</td>
<td>Advice on behavioral modification</td>
<td>Irritability</td>
<td>Behavioral changes disrupting family/school life</td>
<td>Psychological/psychiatric input may be necessary</td>
</tr>
<tr>
<td>Bone mineral density</td>
<td>Vitamin D, dietary calcium, sunshine, exercise; DXA baseline and annually</td>
<td>Recording of fracture history, fracture site, trauma</td>
<td>Vertebral fracture treated by intravenous bisphosphonates</td>
<td>Limb fracture treated with early mobilization</td>
</tr>
<tr>
<td>Glucose tolerance</td>
<td>Dietary advice</td>
<td>Fasting blood sugar &gt;100 mg/dl</td>
<td>After dietary modification, blood glucose 2 hours after meals &gt;200 mg/dl</td>
<td>Diabetes mellitus</td>
</tr>
<tr>
<td>Blood pressure</td>
<td>Advice about dietary sodium intake</td>
<td>Routine blood pressure</td>
<td>Increase in systolic pressure of 15 mm Hg or diastolic pressure of 10 mm Hg</td>
<td>Treatment for confirmed hypertension: increase of 15–30 mm Hg in systolic blood pressure or 10–30 mm Hg in diastolic pressure</td>
</tr>
<tr>
<td>Gastrointestinal symptoms</td>
<td>Advise to avoid nonsteroids anti-inflammatory drugs</td>
<td>GI distress</td>
<td>Persistent GI symptoms despite treatment</td>
<td>Treat peptic ulceration with Maalox or ranitidine</td>
</tr>
<tr>
<td>Cataract</td>
<td>Ophthalmology examination yearly</td>
<td>Initial cataract</td>
<td>Unilateral or bilateral cataract</td>
<td>Surgery if symptomatic</td>
</tr>
<tr>
<td>Skin changes</td>
<td>Avoid bruising</td>
<td>Skin changes, type and extent</td>
<td>Hirsutism</td>
<td>Skin ulcers require treatment</td>
</tr>
</tbody>
</table>

DXA, dual energy X-ray absorptiometry.

Long-term daily use of steroids has an effect on linear growth: we have often observed a loss of final adult height with DFZ, although this might confer an additional advantage on muscle strength.

Bone Mineral Density. Reduced bone density occurs in boys with DMD, even before steroid use, and is associated with an increased risk of limb fractures. This probably relates to relatively low levels of activity, although recent studies have also shown that children with DMD may have abnormally low levels of vitamin D and osteoporosis, even at diagnosis. Vertebral fractures are rarely seen in DMD patients who are not treated with steroids.12,31,33,51 Although several techniques exist to measure bone mineral density, there are problems with interpretation of the results and, specifically, the interpretation of what a single finding in different bones (e.g., spine, hip) might mean to an individual patient.

Vertebral bone density decreases once DMD children are using a wheelchair, and vertebral fractures
have been reported in boys treated with steroids.\(^{50}\)

There is much concern about the prevention and treatment of decreasing bone mineral density in steroid-treated DMD. Routine practice and treatment of problems related to bone density vary considerably. Several centers give calcium and vitamin D supplementation and others use oral bisphosphonates, but only intravenous bisphosphonates are approved. In experienced hands, bisphosphonates are a good treatment for vertebral fracture or bone pain caused by microfractures in patients with low bone density. Oral bisphosphonates are not fully licensed for use in children. In general, dietary calcium and vitamin D are more effective than supplements at improving bone density, so dietary advice is important. Promoting exercise and some exposure to sunlight is another good way to maintain or improve bone health.\(^7,^{41}\)

In the context of steroid therapy, dual-energy X-ray absorptiometry (DXA) scans at initiation of treatment and at annual intervals are indicated to monitor osteoporosis. Dietary advice is useful and exercise should be promoted. Vitamin D levels should be measured before treatment, limb fractures treated with early mobilization, and vertebral fractures managed in consultation with a bone expert (Table 3).

**Monitoring Other Side Effects.** A well-known side effect of steroids is impaired carbohydrate tolerance. Baseline measurements include blood and urine glucose testing, and monitoring for glycosuria. Dietary modification should be promoted to increase the proportion of complex carbohydrates, reduce the intake of simple sugars, and divide food intake evenly throughout the day. Electrolyte disturbance should be monitored by routine blood analysis, and oral potassium supplements should be provided. Blood pressure monitoring should be performed based on criteria that envisage dose reduction of steroids and a possible criterion for dose reduction or sodium restriction when there is an increase in systolic blood pressure for age. Confirmed hypertension is a criterion for steroid withdrawal.

Gastrointestinal (GI) side effects have been reported rarely in steroid-treated DMD. Some clinicians use adjunct medications such as magnesium–aluminum hydroxide or ranitidine to prevent gastrointestinal disorders or ameliorate symptoms that are not responsive to Maalox.

Ophthalmological examination is necessary to monitor for the development of cataracts and increased intraocular pressure. Cataracts in children with DMD treated with DFZ have mostly been asymptomatic and few have required surgical treatment.

**Vaccinations.** Despite many years of treatment with steroids in DMD, there are few reports of serious immunosuppression. Children should be immune to chicken pox or have been immunized prior to starting steroids, as chicken pox can be serious in children receiving steroids. It is safe to use live vaccines in children who are treated with less than 2 mg/kg of prednisone daily (ENMC: steroids and DMD: material 1 patient information http://www.parentprojectmd.org/site/DocServer?docID=132).

**Quality of Life and Behavioral Changes.** No long-term studies have systematically addressed the issue of quality of life in children with DMD, and specific measures are not available for this disorder. Long-term trials of steroid regimes should incorporate at least a measure of quality of life. The ideal instrument should already have been validated, applied to the DMD age range (down to 4–5 years), and be practical to use. Ideally, the adopted instrument should already have been validated in different languages and among sociocultural backgrounds. The behavior of treated children must be monitored because the use of steroids in DMD frequently causes behavioral changes. This can be done by direct interview or by administering a questionnaire to parents.

**Cardiological Implications.** It is important to consider the effect of steroids on cardiac function because cardiomypathy is almost constant in DMD. Long-term cohort studies of boys treated with daily deflazacort suggest that steroids may have a cardioprotective effect.\(^{50}\) In steroid trials, cardiac function should be assessed on an annual basis using electrocardiography and echocardiography. In young boys with DMD treated with perindopril before the development of any signs of left ventricular dysfunction, the drug was well tolerated and, after a follow-up of 5 years, a smaller proportion of treated boys had left ventricular dysfunction than those in the placebo group.\(^{18}\) Management of any deterioration in cardiac function should be with angiotensin-converting enzyme inhibition and beta-adrenergic blockade.

**LONG-TERM USE OF STEROIDS**

Given the long periods of time that young people with DMD may be treated with steroids, it is important to address the prevalence and management of long-term side effects, which may include weight gain, behavioral changes, vertebral fractures secondary to osteoporosis, and cataracts.\(^7,^{31,33,41,56}\) Bone
mineral density must be followed by DXA; measurement of vitamin D level is useful, and any history of fracture (frequency, site, and trauma) should be recorded. In general, the beneficial response to steroids in functional tests is particularly evident in younger children (Fig. 7). However, non-randomized cohort studies, using both prednisolone and deflazacort, have recorded long-term improvement in functional outcomes, including prolongation of walking from a mean age of 10 years to a mean age of 14.5 years, preservation of lung function, reduced need for scoliosis surgery, and possibly a reduced incidence of cardiomyopathy.

The long-term use of daily steroids, introduced when patients are still ambulant and before they have lost significant function, alters the natural history of the disease and prolongs age of ambulation. Untreated DMD patients, however, have shown delayed loss of ambulation if they had reverted fibers and faint dystrophin on biopsy. Glucocorticoid receptor polymorphism may be implicated in the long-term response to glucocorticoids.

There are many possible side effects of long-term daily steroid use, such as adrenal suppression, susceptibility to infection, hypertension, impaired glucose tolerance, gastrointestinal irritation, and skin fragility, but clinicians with wide experience in the use of steroids in DMD do not see these complications very frequently, perhaps because the daily dose of corticosteroid is usually reduced over time.

**FUTURE TREATMENT OPTIONS**

In the last decade, outstanding progress has been made in the development of new approaches to treating DMD (Table 4). The use of oligonucleotides to induce single base-pair alterations in the dystrophin gene and restore gene expression in skeletal muscle has proven to be a feasible approach to dystrophin gene replacement. Oligonucleotide-mediated gene editing for dystrophin has the potential to treat the disorder effectively, but is still undergoing development. Targeted removal of one or more exons to restore a disrupted reading frame or omit a nonsense mutation could lessen the consequence of 80% of dystrophin gene mutations. Despite anticipation and enthusiasm for upcoming clinical trials, expectations must be realistic. Exon skipping cannot cure DMD but may reduce its severity, inducing synthesis of some functional dystrophin in children.

Gene therapy has great potential to treat or prevent genetic conditions. Despite great promise, non-viral approaches for gene transfer, such as injected plasmid DNA and naked DNA, still lack efficient methodology. Gene therapies involving viral vectors underlie most preclinical studies and are undergoing human clinical trials, but they require prolonged immunosuppression.

Following the description of myostatin, null phenotype myostatin blocking strategies have been used to promote muscle growth. A multicenter therapeutic trial is presently underway using anti-myostatin antibodies in 108 adult patients with various types of muscular dystrophy (Becker, facioscapulohumeral, and limb-girdle), but the results are not yet available. Although these antibodies could potentially be useful in some cases of limb-girdle muscular dystrophy, they may have dangerous cardiac complications. Utrophin upregulation is another potential therapy because overexpression of utrophin, the autosomal paralog of dystrophin, as a transgene in the mdx mouse, has demonstrated that utrophin can prevent muscle pathology. Slow muscle fibers in normal and dystrophic mice have more utrophin-A transcript and increased utrophin-A protein than fast fiber types. This may in part explain the preferential loss of fast muscle fibers in DMD. Pharmacological upregulation of utrophin may well be a feasible approach in DMD, because this protein is already present in muscle of affected children; its modulation may change the phenotype but not bring about full recovery.

Another approach, potentially beneficial in 10%–15% of DMD children, is the use of a read-through drug (gentamycin or PTC 124) for stop mutations. The cell therapy approach with mesangioblasts has recently been shown to be efficacious in improving muscular dystrophy in a dog model and could be tried in select DMD children, along with immunosuppressive treatment.
These approaches, although feasible, are beyond the clinical setting in the near future and will have to be compared with steroids, which currently remain the gold standard and best treatment option. In view of the multiplicity of steroid regimens, advice given to patients is usually based on the personal experience of the physician or institution administering treatment. Nevertheless, a practice parameter on corticosteroid treatment of DMD has been published by the Quality Standards Subcommittee of the American Academy of Neurology and is useful for patient management based on analysis of evidence.

Table 4. Future treatment options.

<table>
<thead>
<tr>
<th>Type</th>
<th>Rationale</th>
<th>Potential</th>
<th>Drawbacks</th>
<th>Current status</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antisense oligonucleotides</td>
<td>Antisense oligonucleotides induce exon skipping</td>
<td>Potentially applicable to 80% DMD patients</td>
<td>Has to be designed individually</td>
<td>Phase I trials underway</td>
</tr>
<tr>
<td>Plasmid DNA</td>
<td>Direct DNA injection in muscle with full-length dystrophin</td>
<td>Some dystrophin expression</td>
<td>Low efficiency</td>
<td>Arterial delivery to be developed</td>
</tr>
<tr>
<td>Adeno-associated virus gene therapy</td>
<td>Viral vector delivery of mini-dystrophin</td>
<td>Potentially partially effective</td>
<td>Immunosuppression Production of viral vectors difficult</td>
<td></td>
</tr>
<tr>
<td>Myostatin inhibitors</td>
<td>Use of antibodies to inhibit myostatin (a negative regulator of muscle growth), increase muscle bulk</td>
<td>Apply to several dystrophies</td>
<td>Does not affect dystrophin</td>
<td>Multicenter trials in adult muscular dystrophies performed</td>
</tr>
<tr>
<td>Utrophin upregulation</td>
<td>Utrophin is a paralog of dystrophin</td>
<td>Small molecules identified in screening</td>
<td>Utrophin already present in DMD</td>
<td>Needs clinical development</td>
</tr>
<tr>
<td>PTC 124/gentamycin</td>
<td>Read-through stop mutations</td>
<td>Applicable to 10%–15% DMD boys with stop-codon mutations</td>
<td>Dosage to be determined</td>
<td>Phase I trials underway with oral administration</td>
</tr>
<tr>
<td>Stem cells, mesangioblasts</td>
<td>Cells repopulate muscle</td>
<td>Production of cells applies to several dystrophins</td>
<td>Engrafting problem to be solved Needs immunosuppression</td>
<td>Mesangioblasts and cyclosporine beneficial in dog muscular dystrophy</td>
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This study aims to assess the potential of the electrophysiological muscle scan or stimulus–response curve as a diagnostic instrument. If stimulus intensity is gradually increased from subthreshold to supramaximal values, all motor units in a muscle are successively activated. Thus, by plotting response size versus stimulus intensity, an impression (scan) of the entire muscle can be obtained. We recorded 54 detailed scans from 34 patients and 11 healthy subjects, and analyzed them visually and quantitatively. The scan summarized much diagnostic information in a single picture. Specific patterns in or properties of the scan (steps, maximum, variability, decrements, stimulus intensities used) provide clinically relevant information regarding motor unit number, size, and stability, and neuromuscular transmission and axonal excitability. The scan can be recorded noninvasively in about 5 minutes and is fairly easy to interpret. Because it is built up from contributions of all functioning motor units, the scan shows if and how many large motor units are present. There is no sample bias. For these reasons, further exploration and exploitation of this tool in the clinical setting are warranted.

THE ELECTROPHYSIOLOGICAL MUSCLE SCAN

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Motor units (MUs) differ with respect to the stimulus intensity required to activate their axon by transcutaneous electrical stimulation. These differing thresholds imply that if stimulus intensity is gradually increased from subthreshold to supramaximal values, all MUs in the muscle are successively activated. Thus, by plotting response size against stimulus intensity, a visual assessment of the entire muscle can be obtained. Such a plot is commonly known as the muscle’s stimulus–response curve or the input–output curve, although the statistical method of motor unit number estimation (MUNE) employs the alternative name “scan”.6 This latter name suggests that the curve allows a fairly global but quick assessment of the MUs constituting the muscle. Because this corresponds exactly to our proposed use of the stimulus–response curve, we will henceforth refer to it as the electrophysiological muscle scan.

The electrophysiological muscle scan is rarely used in electrodiagnosis. As far as we have been able to determine, it only has a place in nerve excitability testing (threshold tracking).17–19,38 In these applications, it is primarily employed to provide background information for more specialized tests. Applications that aim to extract clinical information directly from the scan are rare.9,13,23,34,36 A recent study by Henderson et al.13 has confirmed our hypothesis that a scan that is recorded in sufficient detail (with adequate stimulus number) has much to reveal of clinical interest. The purpose of the present study was to explore these possibilities. More specifically, we aimed to determine what kind of clinically relevant information can be extracted from the scan in a variety of neurogenic disorders. On the basis of the information from this exploratory study, we evaluate the potential role that the scan might play in electrodiagnosis, to stimulate future applications.

METHODS

Subjects and Patients. A total of 43 scans were recorded from 34 patients (20 men, 14 women) who
were referred to us for electrodiagnostic evaluation with regard to a range of pathologies. Because our hospital has major research interest in Guillain–Barré syndrome (GBS), such patients are overrepresented. Of a total of 17 GBS patients, 10 were studied in the acute phase, 3 at 6 months after disease onset, and 4 both in the acute phase and 6 months later. In addition, electrophysiological muscle scans were obtained from 11 healthy subjects without neuromuscular complaints (5 men, 6 women; mean age 33 years). The experimental protocol was approved by our institutional medical ethics committee. All subjects gave informed consent.

**Electrophysiology.** The scan was performed using the MUNE500 program implemented on an electromyography (EMG) machine (Viking Select; Nicolet Biomedical, Madison, Wisconsin). This program allowed us to store the amplitude and area of each of the elicited compound muscle action potentials (CMAPs) to a file that could later be exported to Excel (Microsoft, Redmond, Washington). CMAP recordings were usually obtained from the thenar muscles in response to transcutaneous electrical stimulation of the median nerve at the wrist (stimuli of 0.1-ms duration at 2 Hz). Occasionally, recordings were made at other locations such as the hypothenar muscles (ulnar nerve stimulation) or the extensor digitorum brevis muscle (peroneal nerve stimulation) if a polyneuropathy had affected a patient’s legs more than the arms. To record the potential at the skin surface, we used cup electrodes, 10 mm in diameter, which were attached to the skin with electrode paste and tape. The active electrode was placed over the belly of the muscle under investigation. Its position was adjusted to maximize the negative peak amplitude of the CMAP. The passive electrode was attached to the metacarpophalangeal joint of the thumb or the fifth digit for recordings from the thenar and hypothenar muscles, respectively. For foot recordings, the passive electrode was placed over the metatarsophalangeal joint of the fifth digit. The ground electrode was placed nearby, for hand recordings at the base of digits 3 and 4.

After determining the optimal position for the stimulating electrodes by locating the point with lowest threshold, these electrodes were taped securely to the wrist. Subjects were asked to recline and relax without speaking, with their hand comfortably by their side. Recordings started with the determination of the threshold of the lowest-threshold MU (S0) and the lowest intensity at which a maximal CMAP could be recorded (S100). Next, a brief conventional scan, consisting of 30 evenly spread stimuli, was performed over the intermediate range S0–S100, and the responses were recorded. Based on visual assessment of this scan (an assessment of acceptability identical to that made preceding the statistical MUNE3), lower and upper limits were adjusted until the scan covered the entire CMAP range.

Next, the corrected total range S100–S0 was divided into 10 equal-width stimulus ranges that were each scanned automatically with 30 stimuli using the MUNE scan software, by manually adjusting the lower and upper limits of the scan range. This procedure resulted in a full scan recorded in response to 300 stimuli that were equidistant in intensity. For healthy subjects, additional recordings were made in the intermediate CMAP range, because, at these intermediate values, the most rapid recruitment of new MUs occurs. In patients, recordings were occasionally repeated at a particular stimulus intensity level if their online visual assessment indicated a deviation from normal scans that merited a more detailed study.

**Data Analysis.** After the recording session, the data were exported to Excel. The negative peak amplitude of the CMAP was first plotted against stimulus number, to check for any irregularities that might have occurred during the session. For example, slight movement of the stimulus electrodes was found to result easily in a shift of the scan to higher (or, rarely, lower) stimulus intensities, interrupting the smooth course of the scan. Such data were discarded. The scan was derived subsequently, by plotting CMAP amplitude against stimulus intensity.

From the scan, we determined the stimulus intensity at which the recorded CMAP equaled 50% of the maximum CMAP (S50). S0 was redefined as the lowest stimulus intensity that generated a CMAP ≥50 μV. S100 was taken to be the stimulus intensity that generated the largest CMAP in the scan (occasionally, S100 required manual correction for outliers). The stimulus intensity range S100–S0 (absolute range width) and (S100–S0)/S50 (relative width) were then calculated. Changes in any of these measures likely reflect changes in axonal excitability at the site of the stimulus. The relative width compensates to some extent for overall shifts in the curve that are due to differences in nerve proximity, conductive properties of the intermediate tissue, or changes in stimulus duration.

The recorded CMAPs were quantified as follows. First, we derived the maximum CMAP. Next, the scan was analyzed semi-automatically for the presence of steps, which are clearly visible size differ-
ences between consecutive CMAPs that are present if a MU size is large or MU number is severely decreased. This analysis was performed using MatLab (The MathWorks, Natick, Massachusetts) according to the following procedure. All recorded CMAPs were sorted according to their size, after which consecutive differences were calculated. Steps were derived as consecutive differences exceeding a subject-dependent threshold. The threshold value was adjusted by the operator to agree roughly with visual estimates of step number and location. In outcome, this procedure is similar to a completely manual step determination. It carries the advantage, however, that steps found could be quantified automatically by their size and their summed size as percentage of the maximum CMAP. If very few MUs are left, the summed step size (Step%) approaches 100% of the maximum CMAP, implying that the number of steps provides a good indication of the number of remaining MUs. If the percentage Step% is intermediate to large, it suggests moderate to severe MU loss.

Simulations. The scan is basically the result of two simultaneous changes to the MU pool with increasing stimulus intensity. First, with increasing intensities, ever more MUs are activated, resulting in a gradual increase of the recorded CMAP. Second, MUs have a range of stimulus intensities over which their firing probability gradually increases from 0 to 1.\(^1,4,10\) If these ranges overlap between MUs, any combination of probabilistically active units can be activated upon successive stimuli with equal strength. This alternating behavior of MUs results in a band of CMAP variability around the gradually increasing mean. The pattern with which the CMAP increases is therefore determined by the number of MUs, their activation order, and their sizes, as well as by alternation. To provide a better understanding of the effect of each of these variables on the scan, we have extended a previously described computer model of alternation\(^2\) to generate scans.

Briefly, this model simulates the firing probability of all MUs in a muscle as a function of stimulus intensity by defining the threshold and recruitment range for each. (“Recruitment range” is the stimulus intensity range over which the activation probability of an MU increases from 0 to 1.) Although physiologically the MU recruitment curve is known to follow a sigmoid curve,\(^10,32\) in the model it has a linear course with a slope that is the same for all MUs. The model further simplifies reality by assuming that the distribution of the recruitment thresholds is normal. Model parameter values for MU recruitment range width were determined for normal controls in a separate study (unpublished data). A normal MU size distribution was obtained from the literature.\(^7\) As previous work has not provided evidence of a relation between MU size and activation order (threshold),\(^7,21\) we assigned MU sizes randomly to modeled thresholds.

Using this model, the CMAP size distribution was simulated for a range of stimulus intensities, from 0 to supramaximal. For each stimulus and each MU, it was determined by chance (a chance equal to the firing probability of the MU at that stimulus intensity) whether the MU contributed its MU potential size to the CMAP size. In consecutive simulation runs, the number of MUs in the muscle was gradually decreased from 300 to 5. At the same time, the MU size distribution was shifted to a distribution with a relatively large number of MUs with increased size, as in conditions such as amyotrophic lateral sclerosis that show reinnervation. In this process, the properties of the threshold distribution, which reflect nerve excitability, were left intact. Occasionally, the model was used to reproduce observed pathological scans, to provide further support for interpretation of abnormalities.

RESULTS

Figure 1A provides an example from our collection of normal scans, all of which were obtained from the median nerve and thenar muscles. These scans generally follow a smooth sigmoid course. One scan (Fig. 1B) showed a pattern that deviated from the others, with a fairly large stimulus intensity range and multiple steps. This scan was excluded in the determination of the following overall results. On average, the negative peak of the maximum CMAP from the remaining 10 recorded scans was 10.2 mV (range, 7.1–16.3 mV). The mean threshold S0 for stimulation was 7.9 mA (range, 5.0–11.0 mA), and the maximum CMAP was obtained at a mean S100 of 14.5 mA (range, 9.6–26.5 mA). This resulted in an average absolute range width of 6.6 mA (range, 3.7–16.5 mA). The stimulus intensity S50, which generates half of the maximum CMAP, was 10.7 mA on average (range, 7.0–19.2 mA). The relative range width (S100–S0)/S50, which compensates for overall shifts in the curve, was 0.6 (range, 0.4–0.9). Four of 10 scans showed one step, accounting for 2%–4% of the maximum CMAP (Fig. 1A, upper end of scan). One additional scan showed two steps, which added up to 6% of the maximum CMAP. The maximum step size observed in all normal scans was 327 \(\mu\text{V}\). The aforementioned outlying normal scan had an absolute range width of 18 mA, relative range width...
of 1.4, and showed (at least) five clearly discernible steps that together formed 11% of the maximum CMAP (Fig. 1B). However, subsequent multipoint stimulation MUNE inferential performed in this subject yielded a normal value of 347 MUs.

An overview of patient diagnoses and the results from quantitative analysis of their scans are provided in a supplementary table available on this journal’s website. The size of the maximum CMAP (the amplitude of the negative peak relative to baseline) ranged from 0 (unmeasurable) to normal values. To quantify stimulus intensity, in the table S50 is provided together with the absolute range (S100−S0). Values for S50 were between 7 and 75 mA, and the absolute range varied from 3 to 57 mA. We found S50 more reliable to determine than either the threshold (S0) or supramaximal (S100) intensities. The error in S0 and S100 may well be several milliamps, whereas S50 can usually be determined with an accuracy of 1 mA or less. Absolute range is provided rather than relative range, because it is easier to interpret. Steps were quantified by their number (Step #, between 0 and 17), their summed size (Step%, the percentage of the maximum CMAP made up by steps, with values ranging from 0% to 83%), and the size of the largest step (between 0.13 and 0.98 mV).

Some statistical analyses were performed, primarily to provide a first assessment of the potential value of the aforementioned quantitative variables: some will be more sensitive to changes in disease than others. Only the groups of normal subjects and acute GBS patients were sufficiently large for these preliminary analyses. Independent-sample t-tests showed that all of the quantitative variables were significantly different between these two groups (P < 0.01), except relative range width and maximum step size. In 13 of 14 patients with acute GBS, either S50 or absolute range width, or both, were outside the normal range. In 6 patients, multiple steps (3–10) were observed, which added up to 12%–83% of the maximum CMAP.

In addition to being a source of quantitative information as noted earlier, we consider the scan to be first and foremost a visual tool that can provide a quick overview of the muscle under investigation. Its possibilities can best be demonstrated by a few illustrative cases. For each of these, future studies will be needed to assess the general validity of the observations and to test the derived hypotheses.

**MU Number.** Figure 2 shows the results of the simulations for changing MU number. With increasing number, the steps that are most prominent when 5–10 MUs are present, gradually disappear in the overall variability of the scan. Irregularities decrease and the scan takes an increasingly sigmoid shape, with a band of variability that appears to decrease in width with increasing number of MUs present. (In fact, the absolute width in millivolts of the band of variability increases. This increase is approximately proportional to the square-root of the number of MUs, while the maximum CMAP increases linearly with MU number. As a consequence, the width of the band relative to the maximum CMAP—as shown in Fig. 2—decreases.) A comparison of recorded scans with these simulation results can provide a rough estimate of the number of MUs present, particularly if this number is strongly reduced (Fig. 3).

**Motor Unit Size.** The lower left plot of Figure 3 represents a scan of patient G2 in the acute phase of GBS. It shows clear steps and a strongly diminished number of functioning MUs: probably 5 or 6, whereas normal numbers for the thenar muscles range from 150 to 350. The steps are remarkably
large. With sizes between 80 and 489 μV, they are much larger than the normal mean thenar MU size as recorded with surface EMG, which tends to be around 40 μV. \(^4\) Although less pronounced, scans in a few other GBS patients also showed a trend toward fairly large step size. There was no evidence in any patient of a precondition that could have resulted in enlarged MUs. Because the scans were made in the acute phase of GBS before reinnervation could have occurred, our observation seems to suggest either preferred affection of the smaller MUs, with the largest MUs remaining, or a kind of systematic coupling (e.g., by ephaptic transmission) of multiple smaller MUs. On needle EMG, this observation would be overlooked easily if it could be made at all.

**Stimulus Intensity.** Our results in normal controls show that, as a rule of thumb, absolute stimulus range width is less than or equal to the threshold value (e.g., for a subject with a threshold of 4 mA, the supramaximal intensity S100 would be less than 8 mA whereas, for someone with a threshold of 12 mA, S100 could be expected to be less than 24 mA). This general rule, which agrees with published data,\(^1\) clearly does not hold for the GBS patient whose scan is shown in Figure 4A. Although the threshold is approximately normal at 15 mV, range width is increased to 44 mA. The latter finding suggests large differences in excitability of the remaining axons. At the same time, variability in the scan is strongly reduced. This was most notable from series of recordings made at a fixed stimulus intensity (Fig. 4B). These recordings, at 10%, 30%, 50%, and 70% of the maximum CMAP, show no or hardly any variation (alternation). In turn, this suggests that in this patient, recruitment thresholds are so widely spaced that the MU recruitment curves overlap only marginally. Such a situation was simulated by the computer model and the results are presented in Figure 4C and D. These data show that a model configuration with widely separated thresholds and normal recruitment range widths indeed generates a scan that shows much similarity to that of the GBS patient, whereas recordings at fixed intensity are devoid of alternation.

**Longitudinal Data.** For the aforementioned GBS patient (G1), follow-up data were available at 6 months after the acute phase (Fig. 5A; acute phase scan in black, follow-up scan in gray). This figure shows a

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**FIGURE 2.** Simulated scans (physiological computer model of MU recruitment thresholds, ranges, and sizes) for varying number of MUs.

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normalization of the range width (24 mA at an S0 of 20 mA), but overall stimulus intensity remained increased. Possibly, during the course of the disease, all MUs had become affected, resulting in increased thresholds for all, whereas the recovery process reduced the worst effects by improving the excitability of the least excitable axons. Frequent longitudinal studies may shed more light on this process and associated changes, which clearly differ between patients. For example, Figure 5B and C show the scans made during the acute and late phase of two further GBS patients (G4 and G8), who initially showed elevated S50 and stimulus intensity range, whereas after recovery (at 6 months) both had decreased to approximately normal values. Figure 5D shows the reverse process, by two recordings that were 1 week apart in a GBS patient (G17) who was in the progressive phase of the disease.

**MU Stability and CMAP Variability.** The scans in Figure 5D show a clear increase in CMAP variability over the course of 1 week. Theoretically, this variability may result from three sources: noise, alternation, and within-MU variability. If MUs are unstable (i.e., if the number of activated muscle fibers in the MU varies from one stimulus to the next), the shape and size of the corresponding MU potential will vary. At intermediate stimulus intensities, such an effect is practically indistinguishable from alternation. However, if noise is minimal and alternation can be excluded (at supramaximal intensities and in cases such as presented in the lower left of Fig. 3), then the scan allows assessment of MU stability. The lower right plot of Figure 3, for example, suggests that, in this post-polio patient, instability (up to 8%) may be present, whereas in the GBS scan shown on the left it is nearly absent (note the different vertical scales). In the case of the scans in Figure 5D, noise is similar in both recordings and alternation may be expected to be less rather than more if the maximum CMAP is reduced (suggesting fewer MUs). Therefore, it is likely that the within-MU variability is increased compared to the earlier recording.

**Conformity of the Scan with NCS and Needle EMG.** Findings in the scan should be consistent with results from NCS and needle EMG. Although we have not yet systematically analyzed this correspondence, we did check for anomalies by comparing scan results with conventional neurophysiological findings. Thus far, no discrepancies have been found. For example, in a patient with an ulnar neuropathy (S4), conventional NCS showed the mean CMAP to be normal, and inching revealed very focal conduction slowing at the elbow. Concentric needle EMG showed signs
of active denervation and reinnervation, with MU potentials up to 8 mV. Consistent with these findings, the scan showed a normal maximum CMAP at normal stimulus intensities (as expected from the very focal nature of the lesion). The abundance of steps in this scan (at least 17) clearly showed that multiple MUs were enlarged. Similarly, a conventional F-wave study in patient S15 revealed a low persistence of F-waves. In response to a series of stimuli, only one giant F potential was generated, suggesting it to be a single axon response. The scan in this patient revealed a single large step of exactly the same size (see Fig. 6; because both recordings involve surface EMG, these measures are directly comparable).

**DISCUSSION**

The electrophysiological muscle scan is a noninvasive technique that summarizes much diagnostic information in a single picture. In the first place, it is a visual tool that can be readily understood by those accustomed to the basic principles of electrodiagnosis. Our experience shows that a training session of less than 1 hour suffices to instruct referring neurologists as well as technicians regarding its basics: recognizing and interpreting deviations from normal scans, against the background of the clinical information from individual patients. In addition to being relatively easy to interpret, the scan is easy to record if the following are available: (1) a means to stimulate at gradually increasing stimulus intensities, preferably automatically; (2) software that can automatically determine and record the amplitude (or area) of CMAPs; and (3) software to plot the results. The first two requirements in particular involve standard features of all modern EMG equipment, although not all commercially available software allows for their use in scanning. If the scan is recorded immediately after the NCS of a particular nerve, all

![Image](https://example.com/image.png)

**FIGURE 4.** (A) Observed scan and (B) CMAP variability at four different but fixed stimulus intensities from patient G1 with GBS. (C) Matched simulated scan and (D) corresponding simulated CMAP variability. In recordings and simulations, the CMAP variability at fixed stimulus intensity is extremely small. The correspondence between A and C and between B and D suggests that the assumed model configuration with widely separate MU recruitment thresholds and normal recruitment range widths provides an adequate representation of this patient’s pathophysiology.
electrodes are already in place. With proper software, the scanning procedure can then be completed by an EMG technician in 5–10 minutes at most. The major disadvantage of the scan is the number of stimuli that it requires, including many at higher intensities. However, in our experience, the test is well-tolerated by both normal controls and patients. Thus far, not a single test has had to be interrupted (paused) or aborted because of subject intolerance. Although some subject selection bias may have been present in this respect, inconvenience has usually been reported as mild to moderate.

**FIGURE 5.** (A) Scan in the acute phase of Guillain–Barré syndrome (black) and follow-up scan at 6 months (gray) in patient G1. Data for patients G4 (B) and G8 (C). (D) Two recordings with a 1-week interval in the acute phase of GBS patient G17. Further information about these patients is available in the supplementary table on this journal’s website.

**FIGURE 6.** F-wave study (left) and scan (right) in a patient with spinal muscular atrophy. Persistence of F-waves was decreased, with occurrence of only a single (giant) potential. The size of this potential, approximately 800 μV, corresponds with that of the largest step in the scan, suggesting that there is one (and only one) particularly large MU in this muscle, which occasionally generates an F-wave.
Many patients, particularly those with GBS, showed an increase beyond the normal range in S50 and the stimulus intensity range, suggesting changes in excitability at the site of stimulation. This observation is in line with previous excitability studies, some of which demonstrated that these changes may occur even at a subclinical level. From our preliminary statistical analyses, we tentatively conclude that the relative range width and size of the maximum step do not seem to hold much information. The latter finding, in particular, is not surprising. Potentials with sizes similar to those found in our study (from 0.13 to 0.98 mV) were previously reported to occur in normal controls. Potentials with sizes similar to those found in our study (from 0.13 to 0.98 mV) were previously reported to occur in normal controls. The number of steps and the part of the maximum CMAP made up by these steps (Step%) appear to be clinically more informative, as suggested by previous work. In normal subjects, Step% probably exceeds 10%–15% only rarely (accurate age-dependent normal values to be determined), and in many patients Step% is larger. Being defined as large consecutive difference, step size is not corrected for alternation, noise, decrements, and within-MU variability. This explains why, even in extreme cases such as in Figure 3 (lower left), the proposed variable Step% only reaches 83%, where a value close to 100% might be expected. Yet, Step% seems to capture to a reasonable extent the abnormalities in the scan that are most obvious on visual assessment.

Patients in this study were too heterogeneous and too few to draw firm conclusions with regard to trends observed. Dedicated studies are required to confirm or refute any of the hypotheses mentioned in the illustrative sections. In part, the purpose of these cases was to present deviant scans in order to support future users in their interpretation of recordings. The cases also demonstrate the potential of the scan for future studies that may shed more light on subclinical changes to MUs and on pathophysiological processes. However, it should be noted that MU recruitment and alternation together explain much of the “look” of the scan. Because alternation is an inherently random process, this look may vary somewhat for subsequent scans in individual subjects, but the major constituting elements can be expected to remain approximately the same.

Particularly after reinnervation, the maximum CMAP cannot be used as an accurate measure of the number of functioning axons. Assessments of MU number from the needle EMG (i.e., from interference pattern analysis at maximum voluntary contraction) are qualitative at best. Quantitative estimation of the number of MUs (MUNE) requires running of a separate test and at least 10 minutes per investigated muscle. The scan offers various possibilities to assess the number of MUs:

1. **Visual**—determining whether there are steps or changes to CMAP variability, which may be a sign of reduced MU number. In the case of “single patterns” on needle EMG, often all MUs present can be simply counted from the scan and followed over time.

2. **Semi-quantitative**—by comparing the scan with “validated” scans (obtained from either simulations or extensive analysis of scans with a similar number of stimuli) to obtain a rough estimate of the number of MUs present (Fig. 3).

3. **Quantitative**—by performing an inverse analysis that estimates the number of MUs together with their thresholds, recruitment slopes, sizes, and within-MU variability using a stochastic approach, or by a slight extension of the protocol so that statistical MUNE values can be calculated.

Standard electrodiagnostic procedures do not include measures of the number of muscle fibers per MU (MU size). The semiquantitative MU size estimates based on needle EMG are, in fact, assessments of fiber type grouping (reduced average distance between fibers of a single MU due to reinnervation within the surviving MU’s original territory) and only indirectly of the number of muscle fibers in the MU. By contrast, surface EMG MU potential size correlates fairly well with macro-EMG, which is the gold standard for MU size estimation. The scan therefore provides a measure of MU size for the largest MUs (steps). Because in neurogenic disorders normal MUs are also present, it is the number and sizes of these largest MUs that are of particular interest for the diagnosis. In addition, variations in MU potential size due to pronounced MU potential instability can probably be detected, either at maximum CMAP or at levels of constant MU activity.

Because of its noninvasive nature, the scan may be more readily applied than needle EMG, for example, in children or in longitudinal studies. Furthermore, the usefulness and reproducibility of needle EMG are limited by the fact that the amplitude and morphology of the needle EMG signal are largely determined by only a few muscle fibers close to the needle tip. Because not all of these fibers are equally affected by a pathological process, coincidence may become a factor of importance during the recording. Hence, multiple sites within a muscle should be sampled. The scan is built up from contributions of all functioning MUs within the muscle. For this reason, it will show if and how many large

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MU potentials are present. There is no sample bias. This implies that sampling of MUs with needle EMG at multiple sites in the search for (extremely) large MU potentials may become superfluous if the investigation is preceded by a scan. In addition, the scan may be able to elucidate subclinical changes that are not accessible with NCS or needle EMG, as illustrated by the GBS case presented in Figure 3 and discussed in the subsection on motor unit size (see Results). Such information is probably not accessible with any other EMG technique. The scan can also provide background information that aids or supports interpretation of NCS or needle EMG findings.

Finally, standard tests do not include systematic documentation of axonal excitability at the site of stimulation, even though this has been shown to yield diagnostically relevant information that is largely independent from the results that can be obtained with standard NCS tests. This possibility is offered in limited form by the scan. In addition, repetitive stimulation is often performed only if there is a clinical suspicion of a neuromuscular transmission disorder, whereas CMAP decrements may be seen (and interpretation of other findings) in other pathologies as well. For example, despite the fact that our protocol was not designed to study decrements, we have seen decrements in several patients with a polyneuropathy, including GBS. This observation is consistent with previous studies that have demonstrated neuromuscular transmission impairment in these conditions.

We conclude that the electrophysiological muscle scan seems able to provide much information in an accessible way. Some of this information is routinely obtained with NCS or needle EMG. Other information can be collected with additional tests such as MUNE, whereas still other aspects of the scan are probably not accessible with any other technique (particularly the overview of all enlarged MUs and their sizes). In our experience, additional tests are presently not often performed, possibly because the extra effort involved is believed to outweigh the clinical relevance of the information obtained with such tests. It is in this balance between effort and information yield that we find the scan superior—and useful. For this reason, we believe there is a need for further exploration and exploitation of this tool in the clinical setting.

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ABSTRACT: Inclusion-body myopathy with Paget’s disease and frontotemporal dementia (IBMPFD) is a disease of muscle, bone, and brain that results from mutations in the gene encoding valosin-containing protein (VCP). The mechanism of disease resulting from VCP mutations is unknown. Previous studies of VCP localization in normal human muscle samples have found a capillary and perinuclear distribution, but not a nuclear localization. Here we demonstrate that VCP is present in both myonuclei and endothelial cell nuclei in normal human muscle tissue. The immunodetection of VCP varies with acetone or paraformaldehyde fixation. Within the nucleus, VCP associates with the nucleolar protein fibrillarin and Werner syndrome protein (Wrnp) in normal and IBMPFD muscle. In patients with inclusion-body myositis (IBM), normal nuclear localization is present and some rimmed vacuoles are lined with VCP. These findings suggest that impairment in the nuclear function of VCP might contribute to the muscle pathology occurring in IBMPFD.


NUCLEAR LOCALIZATION OF VALOSIN-CONTAINING PROTEIN IN NORMAL MUSCLE AND MUSCLE AFFECTED BY INCLUSION-BODY MYOSITIS

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Mutations in the gene encoding valosin-containing protein (VCP) are associated with the clinical syndrome of inclusion-body myopathy with Paget’s disease and frontotemporal dementia (IBMPFD).18 VCP has a range of reported functions, including the assembly of nuclear membranes,10,15 but the specific mechanism that results in tissue pathology in IBMPFD is unknown. The location of VCP has been studied in several mammalian cell culture lines, where it is present in the cytoplasm and nucleus.17

VCP in normal human muscle tissue has previously been reported as present in small capillaries around muscle fibers9,18 and near muscle myonuclei,9 but not within nuclei despite the presence of a nuclear localization signal in its N-terminus.17 Here we demonstrate a nuclear localization of VCP in normal human muscle tissue, inclusion-body myositis (IBM), and muscle tissue with the most common IBMPFD-associated VCP mutation. Within myonuclei, VCP associates with fibrillarin and Werner syndrome protein.

MATERIALS AND METHODS

Muscle Tissue. Muscle biopsy samples were obtained from five subjects with no evidence of a neuromuscular disease, by clinical evaluation, laboratory studies, and muscle histopathology, and classified as normal. Muscle biopsy samples were also obtained from four subjects with definite IBM (according to European Neuromuscular Centre criteria1) and from three subjects with IBMPFD with the most common R155H mutation who were

This article includes Supplementary Material available via the internet at http://www.mrw.interscience.wiley.com/suppmat/0148-639X/suppmat/
undergoing diagnostic muscle biopsies. Our Institutional Review Board approved these studies.

**Immunohistochemistry and Microscopy.** Muscle samples were cryostat-sectioned at 10 μm at −23°C and placed on glass slides. Slides were dipped into acetone at room temperature and then frozen at −20°C for subsequent studies. Primary antibodies used were a monoclonal anti-VCP (cat. no. MA3-004, clone 5, isotype IgG2a; ABR-Affinity BioReagents, Golden, Colorado) and a rabbit polyclonal anti-VCP (kind gift of Dr Chou-Chi Li, National Cancer Institute, Frederick, Maryland). Cryostat sections were fixed in either cold (5°C) 4% paraformaldehyde (PFA) for 5 min and then soaked consecutively in cold (5°C) 0.05 M Tris buffer, pH 7.6, and room temperature Tris buffer, or were fixed in cold acetone (−10°C ± 5°C) for 5 min and soaked in Tris buffer at room temperature. All tissue sections were then moved to Tris buffer supplemented with 4% porcine serum for immunohistochemistry.

Multiple sections were studied to determine optimal fixation methods, antibody concentrations, primary antibody incubation duration, and secondary antibody conditions. Sections (PFA fixed) were incubated for 1 h with monoclonal anti-VCP (ABR, 1:50,000 dilution) and subsequently incubated for 30 min with horseradish peroxidase–labeled polymer conjugated to goat antimouse immunoglobulins (ImmunoVision Technologies, Daly City, California). Polyclonal anti-VCP (1:5,000) was incubated with PFA-fixed sections for 2 h. Sections were then incubated consecutively for 30 min each with swine antirabbit immunoglobulins (1:80 dilution; DakoCytomation), or for 30 min with horseradish peroxidase–labeled polymer conjugated to goat antirabbit immunoglobulins (30-min or 1-h incubation; ImmunoVision Technologies). Staining (DAB, methyl green) was detected over a range of conditions.

Immunofluorescence studies were carried out with polyclonal anti-VCP (1:5,000 dilution, 1-h or 2-h incubation), and monoclonal antibodies to dystrophin (clone Dy8/6C5, 1:50 dilution, 1-h incubation; Novocastra/Vision Biosystems, Norwell, Massachusetts), CD31 (clone JC/70A, 1:25 dilution, 1-h incubation; DakoCytomation), and fibrillarin (clone mAbcam18380, 1:50 dilution, 1-h incubation; Abcam). Secondary antibodies were Alexa Fluor 488–labeled goat antirabbit immunoglobulins (1:400 dilution, 1-h incubation) and AlexaFluor 555–labeled goat antimouse immunoglobulins (1:400 dilution, 1-h incubation) and AlexFluo 555–labeled goat antitumor immunoglobulins (1:400 dilution, 1-h incubation) and Molecular Probes/Invitrogen, Carlsbad, California), respectively. Subsequently, nuclei were stained with DAPI [1 μg/ml in phosphate-buffered saline (PBS)] for 1 min. For dual staining the monoclonal and polyclonal antibody pairs could be incubated sequentially or in admixture (each at their correct dilution) followed by incubation with the secondary antibodies sequentially or in admixture (each at 1:400 dilution).

In a similar fashion, immunofluorescence studies were carried out with monoclonal anti-VCP (1:30,000 dilution, 2-h incubation) and polyclonal anti-fibrillarin (1:100 dilution, 1-h incubation; Abcam) or 2 h when in admixture with monoclonal anti-VCP. Secondary antibodies were AlexaFluor 488–labeled goat antimouse immunoglobulins (1:400 dilution, 1-h incubation) and AlexaFluor 555 goat antirabbit immunoglobulins (1:400 dilution, 1-h incubation) alone or in admixture. Also, studies were carried out with a mix of monoclonal anti-VCP (1:30,000 dilution) and rabbit polyclonal...
anti-Werner syndrome protein, Wrnp (1:100 to 1:5,000 dilution; Abcam) with 3–5-h incubation followed by a 60–80-min incubation with the labeled secondary antibodies admixture. Tris buffer and normal rabbit serum at the same immunoglobulin concentration as the polyclonal anti-Wrnp were used as negative controls.

A Zeiss Axioimager with an Apotome optical sectioning device and Axiovision software (Carl Zeiss, Oberkochen, Germany) were used for 0.4-μm optical sections and 3D reconstructions.

RESULTS

Myonuclear and Endothelial Cell Nuclear Localization of VCP in Normal Human Skeletal Muscle. By light microscopy, both the polyclonal anti-VCP and monoclonal ABR anti-VCP antibodies stained structures that had the typical appearance and location of myonuclei, as well as structures in the typical location of capillaries (Fig. 1A–C). Weaker diffuse cytoplasmic staining was present as well, sometimes in focal accumulations typical of artifact often seen in muscle immunohistochemistry. The nuclear localization with the polyclonal VCP antibody was less apparent when tissue is fixed in acetone than with paraformaldehyde. (D) Acetone fixation. Nuclei show methyl green counterstaining but no VCP immunoreactivity. (E) Paraformaldehyde fixation. Adjacent 10-μm sections of the region corresponding to D of normal muscle show prominent nuclear staining. Other than fixation, identical conditions were used for each section, with a polyclonal anti-VCP antibody titer of 1:2,000. (F–H) Nuclear localization of VCP in normal muscle. VCP-ABR antibody. (F,G) True-color fluorescent images of DAPI and VCP immunoreactivity. (H) Digitally superimposed images F and G show colocalization of VCP (white speckling of blue nuclei) in 96% of the nuclei.
Because of the light microscopic appearance of VCP immunoreactive structures that had the typical shape and location of myonuclei, we performed double-fluorescent immunohistochemistry, which confirmed that VCP staining localized with the nuclear stain DAPI (Fig. 1F–H). To further confirm the location of these nuclei, we performed immunofluorescent studies with thin 0.4-μm optical sections using optical sectioning microscopy and 3D reconstructions of VCP combined with dystrophin, to outline the muscle sarcolemmal membrane, and with CD31, to outline vessel endothelium, both with DAPI. These studies demonstrated VCP within myonuclei and endothelial cell nuclei (Fig. 2).

Nuclear and Perinucleolar Localization of VCP in Myonuclei and Endothelial Cell Nuclei. The specific location of VCP within human myonuclei is of interest, as localization to nucleoli has been noted in some mammalian cell lines.17 We examined the relationship of VCP to the nucleolar protein fibrillarin. VCP typically localized to regions around nucleolar fibrillarin, often leaving a “pocket” for fibrillarin (Fig. 3). This arrangement was particularly evident with 3D reconstructions of optical sections (Fig. 3I–L).

Overlapping Localization of Werner Syndrome Protein with VCP. The Werner syndrome protein (Wrnp) is a DNA helicase. Mutations in Wrnp result in a multisystem disease likened to premature aging. Previous reports have demonstrated physical interaction between VCP and Wrnp in cell culture.12 We accordingly looked at Wrnp in myonuclei and its location with respect to VCP. Light immunohistochemistry demonstrated punctate and infrequently diffuse nuclear localization of Wrnp in muscle (Fig. 4). Triple immunofluorescent studies showed small deposits of Wrnp typically colocalized with larger regions of VCP (Supplementary Fig. 1). Unlike with fibrillarin, there appeared to be complete overlap of Wrnp-containing regions with a portion of a VCP region.

Normal Nuclear Localization of VCP in Human Mutant R155H VCP IBMpfd Muscle. Immunohistochemistry of muscle from three patients with IBMpfd and R155H VCP mutation showed intact nuclear staining (Fig. 5).
Immunofluorescent studies of VCP with fibrillarin and DAPI in IBMPFD muscle showed VCP within nuclei and with variable sometimes overlapping relationship to fibrillarin (Supplementary Fig. 2). Unlike the normal muscle, no perinucleolar VCP pockets were seen; the significance of this result is uncertain. Studies of Wnpl’s relationship to VCP showed no difference compared to normal.

VCP Nuclear and Rimmed Vacuole Localization in IBM. VCP was present in IBM myonuclei, definitively identified by their internal location away from the sarcolemmal membrane (Fig. 6A–C). Vacuoles were sometimes lined with VCP (Fig. 6D). As in normal muscle, nucleolar fibrillarin overlapped with a portion of VCP (Supplementary Fig. 3).

DISCUSSION
VCP has not previously been recognized within nuclei in normal human muscle tissue. Instead, it has been reported within capillaries but not to a more specific endothelial compartment. In muscle
from one patient with IBMPFD, VCP has been reported in nuclei. Although that finding was considered abnormal, the findings in normal muscle were not reported. Here we demonstrate a nuclear localization within normal myonuclei and furthermore demonstrate a more precise localization within capillaries that includes the endothelial cell nuclei. In IBMPFD with the R155H mutation, nuclear localization is not disrupted and the specific sublocalization of VCP appears normal. In IBM the nuclear localization is present and some vacuoles are lined with VCP.

Several possibilities exist for the lack of recognition of nuclear localization in the two previous reports describing immunohistochemical studies for VCP in normal human muscle samples. First, the method of tissue fixation affects immunodetection of nuclear VCP. In our studies, fixation with acetone showed little nuclear localization with polyclonal VCP, whereas PFA fixation readily showed nuclear staining (Fig. 1D,E). The failure of some nuclear antigens to demonstrate immunoreactivity with acetone fixation has previously been recognized in other tissues. Acetone fixation could alternatively result in extraction from the nuclei of certain proteins. The type of fixation used in those earlier studies is not reported or referenced in either of the two previous publications. Second, capillary staining recognized previously was not further localized, as we have done, to nuclei within capillaries. Lastly, as myonuclei are typically located just under the sarcolemmal membrane, there may be uncertainty in distinguishing them from capillaries located just external to the sarcolemma. The use of immunofluorescence with dystrophin staining of the sarcolemma, as we have done, is particularly helpful in this regard.

Although early studies did not find nuclear localization of VCP despite a putative N-terminal nuclear localization signal, subsequent studies demonstrated nuclear localization in a variety of transfected cell lines and confirmed through mutation studies a nuclear localization signal. Two patterns of VCP nuclear fluorescence have been reported in cell lines: a punctuate pattern throughout the nucleus and several foci of intense fluorescent signal colocalizing with the nucleolar protein fibrillarin. In normal human skeletal muscle, we find that VCP tends to be restricted to regions around, rather than overlapping with, the fibrillarin-rich portion of the nucleolus. In three patients with mutant VCP, overlap of fibrillarin within a larger region of VCP was seen more frequently. VCP has also been found to interact physically with Wrnp, a DNA helicase in which mutations produce a premature aging syndrome. We found uniformly that Wrnp colocalized to a region overlapping with VCP.

A prominent aspect of muscle pathology in patients with VCP mutations is the presence of rimmed vacuoles. Rimmed vacuoles in two other diseases, IBM and oculopharyngeal muscular dystrophy, occur in association with nuclear pathology. The localization of VCP to myonuclei shown here suggests that some muscle pathology in IBMPFD may result from a disturbance of the nuclear function of VCP, which includes the maintenance and assembly of nuclear membrane.

In this regard, nuclear pathology consisting of ubiquitinated nuclear inclusions and TAR DNA binding protein 43 (TDP-43) nuclear inclusions have been described in IBMPFD neurons, and a hypothesis has been suggested that
VCP gene mutations result in impaired degradation of TDP-43.16

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Figure 6. VCP staining in IBM muscle. Immunoreactivity of IBM myonuclei with both the (A) ABR-VCP antibody and (B) the polyclonal VCP antibody. (C) Longitudinal sections showing myonuclei as defined by their position inside the sarcolemmal membrane. (D) Vacuoles staining with VCP at their periphery (arrows). Panels A and B from Patient P237; Panel C from Patient P301; Panel D from Patient P170. Grayscale image.
ABSTRACT: Mutations in the fukutin-related protein gene (FKRP) are associated with a spectrum of diseases from mild limb-girdle muscular dystrophy type 2I to severe congenital muscular dystrophy type 1C, muscle-eye–brain disease (MEB), and Walker–Warburg syndrome (WWS). The effect of mutations on the transportation of the mutant proteins may constitute the underlying mechanisms for the pathogenesis of these diseases. Here we examined the subcellular localization of mouse and human normal and mutant FKRP proteins in cells and in muscle in vivo. Both normal human and mouse FKRP localizes in part of the Golgi apparatus in muscle fibers. Mutations in the FKRP gene invariably altered the localization of the protein, leading to endoplasmic reticulum retention within cells and diminished Golgi localization in muscle fibers. Our results therefore suggest that an individual missense point mutation can confer at least two independent effects on the protein, causing (1) reduction or loss of the presumed glycosyltransferase activity directly and (2) mislocalization that could further alter the function of the protein. The complexity of the effect of individual missense point mutations may partly explain the wide variation of the FKRP-related myopathies.


FUKUTIN-RELATED PROTEIN LOCALIZES TO THE GOLGI APPARATUS AND MUTATIONS LEAD TO MISLOCALIZATION IN MUSCLE IN VIVO

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The integrity of muscle fiber structure relies on an extensive network linking the extracellular matrix, through the cell membrane, to the cytoskeleton of the muscle fibers.13 The main complex of this network is the dystrophin-associated protein complex that anchors muscle fiber onto the extracellular scaffolding through the binding of membrane α-dystroglycan to the laminin-α2 chain and other matrix proteins such as perlecan, biglycan, neurexin, and agrin.7,17,25 Effective binding of α-dystroglycan to the laminin-α2 chain relies on the appropriate glycosylation of both molecules.22 Recently, mutations in several genes have been associated with a group of muscular dystrophies that share a common abnormality in the glycosylation of α-dystroglycan. These genes encode either glycosyltransferases or putative glycosyltransferases. Mutations in the Fukutin gene cause Fukuyama congenital muscular dystrophy (FCMD),23 and mutations in POMGnT1, POMT1, POMT2, and LARGE genes are linked to muscle–eye–brain disease (MEB),24 Walker–Warburg syndrome (WWS),1,27 and MDC1D,18 respectively.

The human FKRP gene is located on chromosome 19q13 and encodes a 493 amino-acid protein, consisting of a hydrophobic N-terminal transmembrane domain, a stem region, and a putative catalytic C-terminal domain.3 This domain-structure is similar to many proteins residing within the Golgi apparatus.
and functioning as glycosyltransferases, suggesting a similar role for FKRP. Mutations in the FKRP gene have been linked to several forms of muscular dystrophy, ranging from MEB, WWS, and congenital muscular dystrophy type 1C (MDC1C), with early manifestation and fatal consequence, to limb-girdle muscular dystrophy 2I (LGMD2I) with mild clinical symptoms. Asymptomatic carriers with mutations in both alleles have also been reported. The mechanisms by which mutations in the FKRP gene cause a wide variation in clinical manifestation are not clearly understood. No patients have been found with two FKRP null alleles, suggesting that complete lack of FKRP results in embryonic lethality. Furthermore, patients with a compound heterozygous mutation for one L276I mutant and one null allele can present with the mild phenotype of LGMD2I. This suggests that one copy of the L276I mutant FKRP allele is sufficient to protect the individual from severe muscle damage. These facts together suggest that individual missense mutations affect the function of the FKRP protein through several different mechanisms, and factors independent of the gene mutations could also play important roles in the wide variation of the diseases.

Several studies have investigated the localization of the FKRP protein for clues relating to its function and mechanisms of pathogenesis. Esapa et al. reported that, in a cell culture system, FKRP mutants (S221R, A455D, P448L) associated with the more severe clinical phenotypes are retained within the endoplasmic reticulum (ER), whereas the wildtype protein and the common mutant L276I, associated with LGMD2I, are found predominantly within the Golgi apparatus. They also demonstrated that the ER-retained mutant proteins have a shorter half-life than wildtype FKRP and are preferentially degraded by the proteasome. It was therefore suggested that the degree of ER-retention might explain, at least partially, the differential severity of the disease state with the mutant FKRP5s. This hypothesis, if proven, could help to establish clinical diagnosis, predict prognosis, and, perhaps more important, provide a basis for developing a new approach to improve the transition of mutant proteins to the Golgi as a new therapy for some of the FKRP-related myopathies. However, a recent study from Dolatshad et al. reported contradictory findings that both wildtype and mutant human FKRP5s, including those associated with MDC1C, can be localized to the Golgi in C2C12 myoblasts in vitro. They suggested that mislocalization is unlikely to play an important role in the pathogenesis of the FKRP mutations. Further investigation is therefore required to establish the pattern of localization for both normal and mutant FKRP5s, especially in muscle in vivo, for better understanding of the mechanism, and more important, to devise new therapies to address the diseases.

**MATERIALS AND METHODS**

**Antibodies.** FKRP-5643 (FKRP-STEM) rabbit polyclonal antibody raised against the stem region (amino-acid residues 29–130) of the mouse FKRP was used as described previously. Rabbit polyclonal antibody FKRP143 was raised against a synthetic peptide of amino-acid residues 140–159 of the human and mouse FKRP. Both antibodies are affinity-purified. The anti-GM130 monoclonal antibody was purchased from BD Biosciences (San Jose, California). The antibody against GRP78 (also known as BiP) was purchased from Santa Cruz Biotechnology (Santa Cruz, California). Anti-COPII and anti-GM130 rabbit polyclonal antibodies were generous gifts from F. Gorelick (Yale University) and E. Szutl (University of Alabama), respectively.

**Mouse FKRP Expression Constructs.** Four mutant FKRP5s, L276I, P448L, V405L, and C318Y associated with LGMD2I, MDC1C, MDC1C presenting with mental retardation and cerebellar cysts, and WWS, respectively, were examined. Mouse expression constructs of normal FKRP, L276I, and P448L mutants were tagged with eYFP as reported previously. Mutant mouse constructs V405L and C318Y were created by polymerase chain reaction (PCR)–directed mutagenesis from the normal mouse FKRP-eYFP construct. The expression of the transgenes is under control of the cytomegalovirus (CMV) promoter.

**Human FKRP Expression Constructs.** The full-length coding sequence of human FKRP was amplified by reverse transcription PCR (RT-PCR) from total RNA isolated from a normal control muscle using TRIZol Reagent (Gibco/Invitrogen Life Science, Carlsbad, California). The RNA was reverse-transcribed using the Stratascript One-Tube RT-PCR System (Stratagene, La Jolla, California). The primers used were: P1-hfkrp/F125-148, 5′-CAACCTACACAGAGCTTCTC-3′; P2-hfkrp/R1844-1821, 5′-TCACACAGAGCTTCTC-3′; P3-hfkrp/SalI191-216, 5′-CTGCGCTCGACAGATAAACGTCTCAGAG-3′; and P4-hfkrp/BamHI1777-1755, 5′-GGCTGGATCCGC- TTOCCGTAGACAG-3′. The P1 and P2 primers were used for the initial RT-PCR and the P3 and P4 primers incorporated with the restriction enzyme sites (underlined) were used for cloning. The SalI-hfkrp-BamHI 1572-bp
cDNA fragment containing the full coding sequence was ligated into the pEGFP-N3 vector (ClonTech, Mountain View, California) with and without the eGFP tag at the C-terminus. The recombinant clones were verified by DNA sequencing. The CMV promoter was used for transgene expression.

The human mutant FKRP expression vectors, C826A (L276I) and C1343T (P448L), were generated by PCR-directed mutagenesis and overlap extension. Two PCR reactions were performed on the normal human FKRP expression vector using primers with single base mismatch and Pfu DNA polymerase (Stratagene). The two primary PCR products harboring the point mutation were combined and used as a template for further PCR extension from the overlapping sequence. The resultant fragment was digested with the restriction enzymes, ScaI and BamHI, and ligated into the pEGFP-N3 vector. The constructed vectors were sequenced and termed the L276I and P448L expression vectors. The two pairs of the primers used are shown below with the mutated nucleotide underlined:

L276I-MF1-hFkrp, 5'-GGCATCGCCATGTCGAGCGCTG-3'; L276I-MR1-hFkrp, 5'-CAGCTCAGCTCGAGCAGCTG-3'; P448L-MF1-hFkrp, 5'-CTTCCTGCAGCTGCTGGTGC-3'; and P448L-MR1-hFkrp, 5'-GCCAAGCTGCCAGAAG-3'.

**Cell Culture and Transfection.** C2C12 cells were grown in Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum, 4 mM L-glutamine, and 100 µg/ml penicillin-streptomycin. Chinese hamster ovary (CHO) cells were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum, 4 mM L-glutamine, and 100 µg/ml penicillin-streptomycin. Cells were transfected with 2 µg (for each well of a 6-well plate or a 4-well chambered coverslide) of each construct using Lipofectamine 2000 as per the manufacturer's instructions. We counted 1,000 cells for expression of eGFP, and the percentage of eGFP-positive cells with Golgi localization was calculated. All culture and transfection reagents were purchased through Invitrogen/Gibco Life Sciences.

**Intramuscular Injection and Tissue Preparation.** We injected 20 µg of each construct in 40 µl saline and 0.025% F127 into the tibialis anterior (TA) muscle of 6-week-old C57BL/10 and mdx mice (Jackson Laboratories, Bar Harbor, Maine). Three muscles were injected for each construct. Animals were euthanized 2 and 5 days postinjection and TA muscles dissected and immediately snap-frozen in isopentane over dry-ice. Sections of 4 µm were collected onto 3-aminopropyltriethoxysilane–coated glass slides (Sigma-Aldrich, St. Louis, Missouri) using a Leica CM1850 cryostat and stored at −80°C for immunohistochemical analysis.

**Immunohistochemistry of Cultured Cells and Muscle Sections.** Cells cultured onto chambered glass slides were transfected 24 h after plating. The transfected cells were harvested 1–2 days later, fixed in 2% paraformaldehyde diluted in 0.01 M phosphate-buffered saline (PBS) at 4°C for 15 min, followed by thorough rinsing with PBS. Muscle sections of both transfected and control mice were collected as described, air-dried, then rehydrated in PBS. Cells and sections were first blocked with 10% normal goat serum and then incubated with AlexaFluor 594-conjugated antimouse or antirabbit secondary antibodies (Gibco/Invitrogen/Molecular Probes). Immunofluorescence was visualized using an Olympus BX51/BX52 fluorescence microscope and images were captured using the same exposure time for each fluorescence using the Olympus DP70 digital camera system (OPELCO, Dulles, Virginia). Confocal images were captured on a Leica LSM 510 microscope in MultiTrack mode(601,483),(935,503) using an HeNe laser at 543 nm and the Ar laser at 488 nm with the pinhole adjusted to 2 Airy units for both excitation wavelengths. The percentage of transfected cells was calculated by counting the GFP-positive cells among the total of 1,000 cells (DAPI-stained nuclei). All microscopic images of muscles presented in the figures originated from the C57BL/10 mice.

**Western Blot Analysis.** Two days after transfection, cells were lysed in Triton lysis buffer containing 1% Triton X-100, 50 mM Tris pH 8.0, 150 mM NaCl, 1 mM EDTA, and a Protease Inhibitor Cocktail (Sigma-Aldrich). The protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Hercules, California). Protein was also obtained from normal human and mouse muscle tissues. Cell and tissue lysates (20 µg) were analyzed on 4%–15% Tris-HCl gradient gels (Bio-Rad Laboratories), transferred onto supported nitrocellulose membrane, and probed with the FKRP-STEM and FKRP143 antibodies followed by horseradish peroxidase–conjugated antirabbit IgG (Bio-Rad Laboratories). Bands were detected using an enhanced chemiluminescence detection kit (PerkinElmer,
RESULTS

Characterization of FKRP Expression Vectors and Antibodies in Cell Lines. Previous studies have demonstrated that both untagged and eYFP-tagged full-length mouse FKRP s shared the same localization patterns and were recognized by the antibody FKRP 5643 (also called FKRP-STEM) against mouse FKRP s in cultured C2C12 and CHO cells. Therefore, eYFP-tagged mouse FKRP s (normal FKRP, L276I, P448L, V405L, and C318Y mutants) were used to determine their subcellular localization. Immunocytochemistry with two independent antibodies against FKRP (FKRP-STEM and FKRP143) showed signals for the FKRP s completely colocalized with the eYFP, further confirming that the eYFP tags represent the FKRP proteins faithfully (Fig. 1A). The FKRP143 antibody was raised against a synthetic peptide, common to the C-terminal region of both the mouse and human FKRP s. However, the intensity of the signals at the Golgi apparatus with the two antibodies was clearly weaker than eYFP signals at the Golgi apparatus, suggesting a possible partial masking of the epitope when the FKRP protein is located at the Golgi apparatus.

To determine whether there were any species-specific differences in the expression of FKRP in cell culture and in vivo, we also generated the full-length human normal FKRP and two mutants, L276I and P448L, cDNA expression vectors tagged with eGFP at the C-terminus. Effective expression was achieved in both C2C12 myoblasts and CHO cells (CHO data not shown). Immunocytochemistry revealed that the human FKRP proteins were also recognized by the two antibodies and the signals for both the normal and mutant human FKRP s registered well with the eGFP signals (Fig. 1A). Again, the intensity of the signals within the Golgi apparatus with both antibodies was weaker than eGFP signals within the Golgi apparatus. No staining was observed with the two antibodies in the cells expressing control eGFP alone (data not shown).

The expression of the FKRP s of both the mouse and human origins were also confirmed by the Western blot analysis with both antibodies (Fig. 1B). The transfection efficiency and the levels of expression with all vectors were similar for normal FKRP, L276I, V405L, P448L, and C318Y mutants (43%, 45%, 46%, 45%, to 51%, respectively) and as shown in Figure 1B by Western blot. Therefore, the eGFP and eYFP signals were considered to be faithful indicators for

FIGURE 1. FKRP detection with the antibodies FKRP-STEM and FKRP143 by immunohistochemistry (A) and Western blots (B). (A) C2C12 cells were transfected with the vector expressing normal mouse FKRP tagged with eYFP (nFKRP-YFP; upper and middle panels). The pseudo-colored green fluorescence signal (eYFP) colocalizes with the signal (red) detected by the antibody FKRP-STEM (upper panel) and FKRP143 (middle panel). C2C12 cells are transfected with the vector expressing human P448L mutant FKRP tagged with eGFP (P448L; lower panel). The green fluorescence (eGFP) colocalizes with the signal (red) detected by the antibody FKRP143. Note that signals (red) with both antibodies for normal FKRP in the Golgi apparatus are weaker than eYFP signals, but the red signals for mutant P448L and the tagged protein (eYFP) are of equal intensity. Nuclei are stained with DAPI (blue). Magnification: ×100. Scale bar, 50 μm. (B) Western blot detection of mouse normal and mutant FKRP s in C2C12 cells with the FKRP-STEM antibody. m, mouse; n, normal; eGFP cont, cells transfected with the vector expressing eGFP only; C2C12 cont, C2C12 cells without transfection. β-actin was used as the sample protein loading control. (C) FKRP detection by Western blot with antibody FKRP143. nhFKRP, normal human FKRP without tag; nhMuscle, normal human muscle; nmMuscle, normal mouse muscle.
the localization of the FKRP s of human and mouse origin. To identify the endogenous FKRP proteins with precise molecular weight, normal human and mouse muscle tissues were analyzed together with the C2C12 cells transfected with nontagged human FKRP expression vector by Western blot. Only barely detectable to weak signals were observed in the muscle tissues (Fig. 1C). No convincing positive staining for endogenous FKRP was observed by immunohistochemistry with the same antibodies.

Expression of Normal and Mutant Mouse FKRP s in C2C12 Myoblasts and CHO Cells. The normal mouse FKRP-eYFP fusion protein localized almost exclusively at one pole of the nuclei in C2C12 cells. A similar pattern of localization was observed with the L276I mutant. Immunofluorescence signals with the antibody GM130 specific to the Golgi apparatus in most cells expressing normal FKRP (nFKRP) and L276I mutant, respectively (A). C318Y mutant protein is concentrated in the Golgi apparatus in most cells although diffused cytoplasmic signals can also be seen. V405L and P448L mutant proteins are diffusely distributed throughout the whole cytoplasm with concentration in the Golgi apparatus in some cells expressing P448L. Diffused cytoplasmic signals for P448L and V405L mutant FKRP proteins are colocalized closely with the signals for GRP78 (B, left three columns) and only partially with COPII protein (B, right three columns). Nuclei are stained with DAPI (blue). Scale bar, 50 μm.
fuse cytoplasmic staining were occasionally present (5%), particularly with the L276I mutant (10%) (Fig. 2A). Patches of stronger signals near the nuclei, however, were also observed in these cells and colocalized with the signal specific to the Golgi apparatus (Fig. 2A). In contrast, the majority (more than 90%) of the cells transfected with the P448L mutant and nearly all the cells with the V405L mutant showed diffuse cytoplasmic expression, with signal throughout the whole cytoplasm. A small proportion of the cells with P448L mutant (less than 10%), and very few expressing the V405L mutant, presented higher levels of expression within the Golgi areas marked by the GM130 antibody. However, the majority (65%) of the cells with the C318Y mutant FKRP (associated with WWS) showed clear Golgi-localized expression, although diffuse cytoplasmic signals were also present in most of the cells. The diffuse cytoplasmic signals for the P448L and V405L mutant proteins colocalized well with the signals for the ER resident protein GRP78/BIP and partially with ER-exit sites/ER-derived transport vesicles detected by the anti-COPII antibody. The patterns of colocalization were further confirmed by confocal analysis (Fig. 2B). These results are consistent with our previous observations.\(^\text{15}\)

Similar patterns in subcellular localization of the FKRPs were observed in CHO cells. The normal FKRP localized at or close to, but did not completely register with, the Golgi apparatus in nearly all the cells. This is mainly due to the fact that the Golgi apparatus (as recognized by the antibody GM130) distributed as separated patches near the nuclei in most of the CHO cells. The localization of the L276I and C318Y mutants overapped between the Golgi and ER. In contrast, the V405L and P448L mutant FKRPs showed diffused cytoplasmic signals without clear concentration at the Golgi apparatus (data not shown).

Expression of Normal and Mutant Mouse FKRPs in Muscle Fibers. The mouse normal and mutant FKRPs (L276I, C318Y, P448L, and V405L) were investigated for their localization in the muscle fibers in vivo. Our delivery system consisting of naked plasmid DNA and the pluronic copolymer F127 created an expression gradient, ranging from weak to strong signals of tagged eYFP proteins in the transfected muscle fibers of normal C57BL/10 mice. The cytoplasmic eYFP signals in the fibers with the control eYFP vector were lost almost completely during the immunostaining of the sections without fixation, indicating a cytosolic localization of the proteins. The intensity and the localization of all of the eYFP-tagged FKRPs remained the same as those viewed prior to the immunostaining, indicating that the FKRP proteins, as predicted from the domain structure, were membrane bound. Fluorescence signals with the FKRP antibodies colocalized with the eYFP (data not shown). The levels of expression varied from barely detectable to intense bright fluorescence covering the whole cytoplasm with all FKRP constructs (Fig. 3). A clear signal gradient was also observed in some fibers with a polarized area of bright eYFP fluorescence that obscured any possible localization pattern in these areas. Interestingly, a differential localization pattern between normal FKRP and mutants was observed in the fibers expressing low to intermediate levels of the transgenes. Fibers expressing normal FKRP and the L276I mutant contained well-defined concentrated speckles regularly distributed along the membrane and within the cytoplasm, most of which were partially colocalized with the Golgi marker, GM130 (Fig. 3). These speckles were also partially colocalized with the signals for the COPII protein (Fig. 3). A weak but clearly visible diffused cytoplasmic signal was present in most of these fibers, particularly with the L276I mutant. Clear speckles, partially colocalized to both GM130 and COPII antibody staining, were also observed, albeit with less frequency in the fibers expressing C318Y mutant protein. By contrast, the P448L and V405L mutant FKRPs were diffusely expressed in the cytoplasm of all fibers with some patches of stronger signals that showed no clear colocalization to both signals for the Golgi and the COPII-marked vesicles (Fig. 3). Similar results were observed in the muscles of mdx mice (data not shown). No speckles were observed in the muscle fibers expressing control eGFP protein and in the muscle without transfection.

Expression of Normal and Mutant Human FKRPs in C2C12 Myoblasts and CHO Cells. The localization pattern of the normal human FKRP was similar to that observed with the normal mouse FKRP in the C2C12 myoblasts as well as in the CHO cells. Normal human FKRP in nearly all transfected C2C12 cells was localized at the Golgi apparatus, indicated by its colocalization with the signal for the GM130 marker (Fig. 4). Weak cytoplasmic signal was seen in less than 5% cells with Golgi expression. The L276I mutant FKRP was expressed predominantly within the Golgi apparatus. In contrast, the P448L mutant proteins were predominantly cytoplasmic, colocalized with the ER marker GRP78/BIP, and partially with COPII protein, although a proportion of the cells (about 40%) displayed stronger signal in the Golgi area (Fig. 4). P448L expression within the Golgi was only observed in less
than 5% of cells. A similar pattern of expression was observed in the CHO cells (data not shown).

**Expression of Normal and Mutant Human FKRP in Muscle Fibers.** An expression gradient from weak to strong signals of the tagged eGFP proteins was observed in the transfected muscle fibers with all the human FKRP constructs. Similar to the expression of the mouse FKRP-eYFPs in muscles, the FKRP-eGFP signal remained within the fibers after immunostaining without fixation. The localization pattern of the normal and the mutant human FKRP proteins was also similar to that observed with the mouse FKRP equivalents. In the fibers with high and frequently polarized expression and in those with very weak expression, no specific pattern was identified with all the FKRP variants. However, eGFP signals as speckles were clearly identified in the majority of the fibers expressing the normal FKRP and distributed sparsely within cytoplasm and along the inner membrane (Fig. 4). These speckles were also partially colocalized with the Golgi apparatus and the COPII-labeled transport vesicles. Such eGFP speckles were also present, although with less frequency, in the fibers expressing the L276I mutant FKRP. In contrast, speckles of the eGFP signals were almost absent in any fibers expressing the P448L mutant protein, although some patches of eGFP signals were present (Fig. 4). No difference in pattern of signal distribution for the FKRP variants was observed between muscles examined 2 or 5 days after intramuscular injections and between C57BL/10 and mdx mice. No speckles were observed in the muscle fibers expressing control eGFP protein and in the muscles without transfection.

**DISCUSSION**

In this study we investigated the localization of normal and mutant FKRPs of both human and mouse
origins and compared the patterns of localization between cultured cells and muscle fibers in vivo. Although it is conceivable that FKRP localizes within the ER/Golgi network, our results clearly show that normal FKRP is localized to a specific site within or close to the Golgi apparatus in muscle in vivo as well as in cultured cells. Mutations alter the pattern of protein transportation, leading to ER retention in cultured cells and loss of Golgi accumulation in fibers in vivo. However, the degree of mislocalization is not entirely related to the severity of the disease.

FKRP is a type II transmembrane protein containing an N-terminal transmembrane domain and an active site of DxD motif within a putative catalytic C-terminal domain. These basic structures are conserved in several other Golgi-resident proteins such as α-mannosidase-II and β4GalTI. This, together with the observation that normal FKRP transgene expresses exclusively within the Golgi apparatus in myoblasts, indicates strongly that normal FKRP most likely resides at the Golgi apparatus. However, immunochemical studies have so far failed to show convincing evidence for the exact localization site of the endogenous FKRP within the ER/Golgi network both in cell lines and particularly in muscle fibers in vivo. Dolatshad et al. and Matsumoto et al. using the same antibody against FKRP, reported the distribution of the endogenous FKRPs

![Image of colocalization of human normal and mutant FKRP](image)

**FIGURE 4.** Colocalization of human normal and mutant FKRP as recognized by antibodies specific to the Golgi apparatus (GM130), the ER-related transport vesicles (COPII protein), and ER (GRP78/BIP) in C2C12 cells (left three columns) and in the tibialis anterior muscles of C57BL/10 mice (right three columns). The green fluorescence represents the FKRP expression and the red signals represent the staining with the antibody to GM130, COPII, and GRP78. Normal FKRP and L276I mutant FKRP localize at the Golgi apparatus in all or nearly all of the cells, respectively. The P448L protein is seen diffusely within the cytoplasm, although stronger signals were also observed in the Golgi area. Speckled signals can be identified in the fibers expressing nFKRP and mutant L276I proteins and are partially colocalized with the signals (red) identifying the GM130 and COPII proteins. No clear speckled patterns are observed in the fibers expressing the mutant P448L protein, although patches of stronger signal can be visualized. Nuclei are stained with DAPI (blue). Magnification: ×100. Scale bar, 50 μm.
demonstrate that normal FKRPs are concentrated at markers for the Golgi and the transport vesicles, we FKRP. By colocalizing the FKRP with subcellular expressing low to moderate levels of the normal level and distribution of endogenous FKRP in vivo. However, a clear pattern was visualized in the fibers 

promoter such as CMV and unlikely represent the most likely only associated with the use of a strong expression constructs containing a single point mutation, Esapa et al.15 demonstrated that various regions of FKRP play different roles in its functions. Other studies also suggest that reduced or loss of function for some Golgi-resident proteins may not be the direct result of changes in its biological function, but rather can be the result of defects in transportation of the proteins within the ER/Golgi network. The most common mutation in cystic fibrosis, CFTR (cystic fibrosis transmembrane conductance regulator) ΔF508, is retained in the ER due to a temperature-sensitive defect in protein folding.8,9 When this mutant is expressed in oocytes, it functions as a chloride channel, demonstrating that the protein retains some activity.27 FKRP mutations might have a similar effect on the transportation of the mutant proteins. Using expression constructs containing a single point mutation, Esapa et al.15 demonstrated that mutations in the FKRP gene associated with the severe phenotypes (MDC1C with and without brain involvement) result in the accumulation of mutant proteins in the ER, where they are removed by the proteasome in a cell culture system. In contrast, normal FKRP and L276I mutant proteins, associated with milder allelic disorder LGMD2I, are trafficked to the Golgi apparatus exclusively at the nuclear and perinuclear sites in fibers of both normal and dystrophic muscles with FKRP mutations. This pattern is apparently in contradiction with the sequence prediction and the Golgi localization observed in cultured cells. We also observed similar nuclear and perinuclear staining with our antibodies against FKRP in some areas of muscles from both normal control and patients with FKRP mutations, but have been unable to attribute the staining specific to the FKRP protein (unpublished observations). The reasons for the difficulty in identifying endogenous FKRP by immunohistochemistry with our antibodies are not clear. One possibility is the low levels of the endogenous FKRP protein, beyond the sensitivity of the current antibodies available. This would be consistent with the results that endogenous FKRP protein could only be weakly detected by Western blots in normal muscles. It is also possible that the binding sites on the endogenous FKRP in situ to the available antibodies might be at least partially masked, possibly by other proteins in a complex essential for glycosyltransferase activity. This is supported by the fact that both our antibodies recognize the ER-retained mutant FKRPs more effectively than the Golgi-localized normal FKRPs (Fig.1).

To elucidate the precise localization of FKRP in muscle in vivo, Dolatshad et al.11 deployed electroporation to transfer the V5-tagged FKRP constructs into the TA muscles of mice. They achieved a widespread high level of expression throughout the sarcoplasm of the fibers in both normal and regenerative muscles with all FKRP expression constructs. No specific pattern was observed and the authors suggested that this might either indicate a similar pattern of localization between normal and mutant FKRPs or be due to a gross overexpression of FKRP. Results from our transfection experiments with the pluronic polymer F127 as a delivery adjuvant produced muscle fibers with FKRP expression at different levels. Fibers with high levels of expression often show polarized distribution within a single fiber. Indeed, a differential pattern of subcellular localization could not be identified in these fibers between the normal and mutant FKRPs. Thus, overly expressed and polarized FKRPs in the muscle fibers are most likely only associated with the use of a strong promoter such as CMV and unlikely represent the level and distribution of endogenous FKRP in vivo. However, a clear pattern was visualized in the fibers expressing low to moderate levels of the normal FKRP. By colocalizing the FKRP with subcellular markers for the Golgi and the transport vesicles, we demonstrate that normal FKRPs are concentrated at the Golgi apparatus close to the transport vesicles identified by the COPII protein. This would be in agreement with the pattern observed in cultured cells and the prediction from the protein sequence, although the localization of endogenous FKRP remains to be confirmed.

Mutations in the FKRP gene have been linked to several forms of muscular dystrophy, ranging from MEB, WWS, and MDC1C with early manifestation and fatal consequence, to LGMD2I with mild clinical symptoms.2,19,26 As noted, asymptomatic carriers have also been reported.10 One of the prominent biomolecular features in patients with FKRP mutations is the secondary deficiency in the glycosylation of α-dystroglycan. The severity of the diseases with FKRP mutations appears to correlate with the levels of hypoglycosylation of the α-dystroglycan in the diseased muscles, with lower levels of glycosylation in severe MDC1C than those in milder LGMD2I.3–5,14 However, it is not understood how missense point mutations affect the functions of FKRP differentially. The FKRP protein structurally consists of several functionally distinctive domains such as the hydrophobic transmembrane domain (amino acids 4–28), a conserved N-terminal motif (R/KxxR/K), a stem-like domain, and an active site of DxD motif within the putative catalytic C-terminal domain.3 It is therefore conceivable that mutations involving different regions would affect functions of the protein differentially. This has been supported by the results of Esapa et al.14 that various regions of FKRP play different roles in its functions. Other studies also suggest that reduced or loss of function for some Golgi-resident proteins may not be the direct result of changes in its biological function, but rather can be the result of defects in transportation of the proteins within the ER/Golgi network. The most common mutation in cystic fibrosis, CFTR (cystic fibrosis transmembrane conductance regulator) ΔF508, is retained in the ER due to a temperature-sensitive defect in protein folding.8,9 When this mutant is expressed in oocytes, it functions as a chloride channel, demonstrating that the protein retains some activity.27 FKRP mutations might have a similar effect on the transportation of the mutant proteins. Using expression constructs containing a single point mutation, Esapa et al.15 demonstrated that mutations in the FKRP gene associated with the severe phenotypes (MDC1C with and without brain involvement) result in the accumulation of mutant proteins in the ER, where they are removed by the proteasome in a cell culture system. In contrast, normal FKRP and L276I mutant proteins, associated with milder allelic disorder LGMD2I, are trafficked to the Golgi apparatus.
and are less susceptible to proteasomal degradation. The results from the current study now demonstrate a clear pattern of localization both in cell culture and in muscle in vivo with mouse and human FKRP. Normal and L276I mutant FKRP from both species localized predominantly within the Golgi apparatus in cultured cells. In contrast, both mouse and human P448L mutant and mouse V405L mutant FKRP were mainly localized to the ER compartments. More important, normal and L276I mutant FKRP from both species accumulate in the Golgi apparatus, whereas most mutant FKRP failed to do so in the muscle fibers in vivo, indicating that mislocalization is a general consequence of FKRP mutations. Therefore, these results further support our previous suggestion that the differences in disease severity associated with FKRP mutations can be explained partly by differential trafficking of mutant proteins in the cell.

Our results also indicate that, whereas mutations associated with severe disease courses are more likely to cause mislocalization of the mutant proteins outside the Golgi apparatus, there is no direct correlation between the degree of loss of Golgi localization and severity of clinical manifestations. Both the P448L mutant and the V405L mutant with and without involvement of central nervous system showed a similar pattern in their subcellular localization. Perhaps more revealing is that the expression of the C318Y mutation (associated with severe disease) localized within the Golgi apparatus in the majority of the transfected cells. More important, similar patterns of localization were demonstrated in muscles in vivo. The P448L and V405L mutant proteins were almost exclusively localized within the ER in all fibers. The C318Y mutant protein, in contrast, accumulated in the Golgi apparatus in most fibers. These observations suggest that individual missense point mutations can have two independent effects on the FKRP, either by causing mislocalization or by direct reduction/loss of its proposed glycosyltransferase activity. It remains to be investigated to what extent the mislocalization contributes to the deficiency of the protein functions.

In summary, our results demonstrate that normal FKRP localize within the Golgi apparatus, most likely at the cis-compartment, as they are in close proximity to the COPII-coated transport vesicles in muscles in vivo. Individual missense point mutations can have two independent effects on FKRP, causing either reduction or loss of functions directly or mislocalization, which, in turn, could affect the functions of the protein. The complexity of the effect from an individual mutation could therefore partly explain the wide variation of the FKRP-related myopathies. Further study is required to determine whether correction of mislocalization can restore at least partial function of some mutated FKRP, leading to a new experimental therapy for the diseases.

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REFERENCES


ABSTRACT: Transient decrease in the excitability of a reflex circuit following its activation by appropriate stimuli is a well-recognized phenomenon, but it is unclear how this applies to thermoalgesic stimuli during quantitative sensory testing (QST). We examined the effects induced by a thermoalgesic (conditioning) stimulus on the response to a subsequent (test) stimulus of the same characteristics. All tests were done using a Peltier thermode with a surface area of 12.5 cm² using ramp rates of 2°C/s and variable interstimulus intervals (ISIs) ranging from 10 to 60 s. Perception was measured with an electronic visual analog scale. No changes were observed in latency of pain perception. However, latency of warm perception was significantly delayed and pain perception intensity was significantly reduced with respect to conditioning stimuli at ISIs below 60 s. Our results indicate a transient saturation of warm and heat pain perception systems after a thermoalgesic stimulus. We therefore recommend that time intervals of >1 min be used between two consecutive thermoalgesic stimuli when examining QST.


TRANSIENT DECREASE OF SENSORY PERCEPTION AFTER THERMOALGESIC STIMULI FOR QUANTITATIVE SENSORY TESTING

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Quantitative sensory testing (QST) of warm and heat pain sensations is often used in clinical practice for functional evaluation of small peripheral nerve fibers and their central nervous system projections. In the method of limits, the conventional assessment of warm and heat pain sensory perception is done by applying a Peltier thermode over relevant areas of the skin, and requesting the subject to press a switch to mark when the sensation is perceived. In clinical practice, assessment of sensory thresholds with the method of limits is done by repeating the same stimulus a few times for consistency of the results. Usually, sensory perception changes slightly from one stimulus to the next, even in cooperative subjects. These small variations are attributable at least in part to oscillations in attention and affect, but transient refractoriness of the system could also theoretically have some effect if the interval between stimuli is short enough.

Although it is relevant for a better physiological understanding of the effects of warm and heat pain stimuli, the temporal profile of refractoriness in the perception system has not been properly analyzed. We reasoned that, as in many other physiological systems, the application of a thermoalgesic stimulus should be followed by transient refractoriness of the sensation induced by a subsequent stimulus of similar characteristics, as is the case for other forms of sensory processing. The aim of our study was to define the time course of recovery of sensory perception after thermoalgesic stimuli, using variable interstimuli intervals (ISIs).
MATERIALS AND METHODS

The study was carried out in 22 healthy subjects, 11 women, aged 21–43 years. Subjects who agreed to participate in the experiment were invited to undergo the thermoalgesic stimulus so that they could be confident of tolerating the stimulus when doing the actual recording. The study was approved by the ethics committee of the Hospital Clinic of Barcelona and accorded with the Helsinki Declaration, and all subjects gave their written informed consent.

Thermoalgesic stimuli were delivered through a Peltier thermode with a surface area of 12.5 cm² (Thermotest; Somedic, Stockholm, Sweden) controlled by Stim-it software. Baseline temperature was always set at 31.5°C, and ramp rate was fixed at 2°C/s. We first determined the individual warm and heat pain thresholds, using the method of limits and standardized procedures. The thermode was then attached with a Velcro strip to the ventral aspect of the subject’s left mid-forearm. Conditioning and test stimuli were delivered through the same thermode, using ISIs of 10, 20, 40, and 60 s from the end of conditioning to onset of test temperature stimuli. Peak temperature was set according to each individual’s pain threshold. We used subthreshold intensity (80%) in one session and suprathreshold intensity (120%) in another. The order of the two sessions was randomized and they were always separated by at least 1 day of rest. Three trials were applied for each ISI, at slightly different sites of thermode contact in the forearm, and separated by at least 5 minutes.

Psychophysical assessment was made using an electronic visual analog scale (VAS) made from a 10-cm linear analog potentiometer (RSA0N11S9002; Alps, Munich, Germany). This device was installed in a metallic box where it could be activated by a lever. We marked seven labels on the side of the lever: no temperature sensation; light warm; medium warm; high warm; light pain; medium pain, and high pain. Subjects were requested to pay attention to the thermal sensation and to avoid speaking during the experiment. Care was taken to ensure that the subjects were awake with eyes open during the whole session. They were instructed to move the lever with their right hand as soon as they felt any change in temperature, and to keep marking the changes in the intensity of their sensations until the stimulus was over. The lever could be moved without resistance along its course, and the use of intermediate positions was encouraged. Signals from the lever were recorded together with the temperature signal generated by the Thermotest during the entire trial. The signals were digitized at a sampling frequency of 200 Hz and fed into a computer equipped with software for off-line analysis (Acknowledgment: Biopac Systems, Bionic Iberica, El Masnou, Spain).

Data Reduction, Measurements, and Statistical Analysis. In all trials, we measured time variables with respect to onset of temperature change (time 0), as the moment when subjects marked the following psychophysical events (Fig. 1): first perception of light warm (warm onset); first perception beyond high warm (pain onset); highest VAS score (MaxVAS-onset); and onset of the descent after reaching maximum (MaxVAS-end). We calculated the duration of maximum perception (MaxVAS-dur) by subtracting MaxVAS-end from MaxVAS-onset. The corresponding thermode temperature was noted at each psychophysical event. We also assessed the relative intensity of the sensation by measuring the individual’s VAS score at MaxVAS-onset (MaxVAS-level), as a percentage of the maximum possible VAS lever displacement. We calculated the mean value of the responses to the conditioning and test stimuli grouped according to ISI and subject. For each subject we ended with five descriptors of VAS for the responses to each stimulus: four time variables (warm onset, pain onset, MaxVAS-onset, and MaxVAS-end) and one magnitude variable (MaxVAS-level). Normality of distribution of the data was assessed using the Kolmogorov–Smirnov test.

FIGURE 1. Latency measurements of psychophysical events with respect to onset of temperature change (time 0): (a) warm onset; (b) pain onset; (c) MaxVAS-onset; (d) MaxVAS-dur; and (e) MaxVAS-level.
### RESULTS

Mean data are summarized in Table 1 for VAS descriptors measured in the responses to conditioning and test stimuli, for both sub- and suprathreshold stimulus conditions. Because there was no occurrence of pain sensation in the subthreshold stimulus, no values are given for variables related to pain in this condition.

Warm onset in response to conditioning stimuli was not different between sub- and suprathreshold stimulus conditions (t-test; \( P = 0.6 \)). As expected, MaxVAS-level was higher in the supra- than subthreshold stimulus condition (mean 98.52 ± 2.2% vs. 52.02 ± 1.8%; t-test; \( P = 0.001 \)). A few subjects reported a ceiling effect in their marking of MaxVAS during suprathreshold stimuli. Responses to test stimuli were different from those to conditioning stimuli.

Statistical analysis showed that ISI accounted for significant differences in a number of VAS descriptors in both stimulus conditions. In the subthreshold stimulus condition, significant effects of ISI were seen in warm onset (\( F[4, 105] = 13.8, \ P = 0.001 \)) and MaxVAS-level (\( F[4, 105] = 27.5, \ P = 0.01 \)). In the suprathreshold stimulus condition, significant effects of ISI were seen in warm onset (\( F[4, 105] = 20.8, \ P < 0.001 \)), MaxVAS-level (\( F[4, 105] = 22.0, \ P = 0.02 \)), MaxVAS-end (\( F[4, 105] = 10.1, \ P = 0.002 \), and MaxVAS-dur (\( F[4, 105] = 16.6, \ P < 0.001 \)). Figure 2 shows representative recordings of the paired suprathreshold stimulus condition in all ISIs.

Bonferroni's post hoc analysis indicated that warm onset was delayed and MaxVAS-level was lower at ISIs of 10, 20, and 40 s for both sub- and suprathreshold stimuli \( (P < 0.05 \) for all comparisons). In the suprathreshold stimulus condition, MaxVAS-end and MaxVAS-dur were delayed at ISIs 10, 20, and 40 s \( (P < 0.02 \) for all comparisons).

No significant effects of ISI were found on pain onset (\( F[4, 105] = 0.08, \ P = 0.9 \)) and MaxVAS-onset (\( F[4, 105] = 0.17, \ P = 0.2 \)) in the suprathreshold stimulus condition, nor on MaxVAS-onset (\( F[4, 105] = 0.35, \ P = 0.1 \)), MaxVAS-end (\( F[4, 105] = 0.09, \ P = 0.09 \), and MaxVAS-dur (ANOVA; \( F[4, 105] = 0.80, \ P = 0.1 \)) in the subthreshold condition.

### DISCUSSION

Our results indicate that a single stimulus with a Peltier thermode induces a consistent delay in the

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<table>
<thead>
<tr>
<th>Conditions</th>
<th>VAS descriptors</th>
<th>Conditioning stimulus</th>
<th>10 s</th>
<th>20 s</th>
<th>40 s</th>
<th>60 s</th>
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<tr>
<td>Subthreshold</td>
<td>Warm onset (°C)</td>
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<td>MaxVAS-onset (°C)</td>
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<td>MaxVAS-end (°C)</td>
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<td>MaxVAS-dur (s)</td>
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<td>Suprathreshold</td>
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<td>Pain onset (°C)</td>
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<td>10.3 (0.7)*</td>
<td>10.3 (0.5)*</td>
<td>10.2 (1.0)*</td>
<td>9.2 (0.5)</td>
</tr>
<tr>
<td></td>
<td>MaxVAS-dur (s)</td>
<td>2.1 (0.5)</td>
<td>2.9 (0.7)*</td>
<td>2.8 (1.1)*</td>
<td>2.7 (0.9)*</td>
<td>1.9 (0.2)</td>
</tr>
<tr>
<td></td>
<td>MaxVAS-level (%)</td>
<td>98.52 (2.2)</td>
<td>92.13 (5.0)*</td>
<td>90.28 (6.1)*</td>
<td>95.14 (3.2)*</td>
<td>97.91 (4.5)</td>
</tr>
</tbody>
</table>

Data expressed as mean (standard deviation). MaxVAS-onset, latency of highest VAS score; MaxVAS-end, latency of onset of the descend after reaching the maximum perception; MaxVAS-dur, duration of maximum perception onset; MaxVAS-level, percentage of VAS lever displacement.

* Statistically significant differences \( (P < 0.05) \) between conditioning and test stimuli.

---

Statistical analyses were done with a paired Student's \( t \)-test for group comparisons between data obtained in the same subjects for sub- and suprathreshold conditions. Repeated-measures one-factor analysis of variance (ANOVA) was used to determine the effects of ISI as an independent variable on VAS descriptors. Bonferroni's post hoc test was used to explore the nature of significant effects found by ANOVA. The level of significance was set at \( P = 0.05 \).
perception of warm sensation to a subsequent stimulus applied at intervals of $<60 \text{ s}$. Therefore, an immediate implication of these results is that it would be appropriate in clinical practice to allow for a resting interval of $\geq 1 \text{ minute}$ between two successive stimuli in QST measurements. A longer ISI would require a longer time for QST assessment in clinical practice. This may lead to decreased levels of attention, but would avoid residual effects of the preceding stimulus on subjective perception of thermal sensation. We have been unable to find in the reviewed literature, or even in instruction manuals from the most commonly used QST devices, any specific recommendation for the minimum ISI required to avoid the influence of effects carried out by the preceding stimulus. Some investigators utilized ISIs between 20 and 30 s to assess heat pain thresholds in thenar eminence and averaged the results. According to our results, such stimulus repetition would not have caused a significant effect on heat pain thresholds, but warm thresholds would have been increased significantly.

We did not specifically evaluate how possible subgroup differences in thermal thresholds related to age or gender could affect recovery of thermal perception. It is known that thermal thresholds remain relatively stable in adult humans up to the age of 60 years. Thermal thresholds in our subjects, aged 21–43 years, showed little dispersion and, therefore, we did not carry out a subgroup analysis of age differences. We also did not perform a subgroup analysis of gender differences. Whether the thresholds for men and women are different is debat-

able and, according to Riley et al., a minimum sample size of 41 per group would be needed to guarantee adequate statistical power.

The transient decrease in the efficacy of input transmission after a thermoalgesic stimulus may be just one aspect of the habituation phenomenon that takes place if a subject is repeatedly presented with a stimulus. This is probably related to the reduced levels of selective attention and arousal that occur after a series of thermal stimuli. It is also possible that the conditioning stimulus causes refractoriness in the receptors mediating warm sensation or subsequent changes in perception, such as those related to context updating and context closure. The delay in perception of warm sensation occurred in both sub- and suprathreshold stimulus conditions, indicating that it is independent of whether heat pain receptors are stimulated. This could be an intrinsic feature of the warm, but not pain perception system, because, at the intervals tested in our study, the delay was only significant for perception of warm sensation. In fact, studies of refractoriness of A$\delta$-nociceptor afferents, involving application of two consecutive pinprick laser stimuli, have shown full recovery of the cortical evoked potentials at intervals beyond 2 seconds.

Interestingly, whereas pain onset did not change significantly in our study, the pain intensity, revealed by MaxVAS-level, was reduced in the responses to test stimuli in comparison to those to the conditioning stimuli. This dissociation suggests that there is a different physiological mechanism accounting for heat pain perception threshold and intensity of sensation. Heat pain perception threshold depends on A$\delta$-mediated nociception, whereas heat pain intensity may be also influenced by C-fiber-mediated nociception. Therefore, the differences in warm perception threshold and heat pain sensation intensity between conditioning and test stimuli found in our study could be due to refractoriness in circuits mainly dependent on C-fiber receptors.

Our finding of a decrease in the perception of pain intensity in test stimuli is opposite to the pain amplification reported in studies of temporal summation. Such an effect would have required a stimulus frequency of $>0.3 \text{ Hz}$. However, the fact that MaxVAS-dur was longer in test than conditioning stimuli may indicate a longer period of aftersensation. Both the aftersensation phenomenon and temporal summation may reflect enhancement of neuronal discharges in wide-dynamic-range neurons and are found to be altered in neuropathic pain states.

We conclude that after a thermoalgesic stimulus there is a transient delay of warm perception and a
transient decrease of pain intensity perception. This is probably due to a refractoriness in the pathway of the C-fibers carrying inputs from both warm and heat pain receptors. This effect declines progressively and is practically overcome at about 60 s after the end of the stimulus. Our results suggest that ISIs of >1 min should be used between consecutive thermoalgesic stimuli during QST assessment of small-fiber function. Further studies are needed to assess transient changes in perception using different thermode sizes and body regions.

This work was supported in part by a grant from CAPES, Brazil, to P.S. The authors also acknowledge the support of the FIS (PI040970).

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ABSTRACT: The effect of a disrupted immune system on the neuromuscular system is poorly characterized. We compared the strength and fatigue of $\text{RAG2}^{-/-}$ mice, which lack T-cells and B-cells, with immune intact controls. $\text{RAG2}^{-/-}$ mice demonstrated fatigue with shorter inverted hang-times (HT) and voluntary wheel-running (VWR) distance and total run times; they increased body weight more slowly but had proportionally normal forelimb grip strength (FGS) and VWR speed. Medial rectus femoris histopathology showed no change in fiber type proportions, no variation in type 2b fiber diameter, and no change in the percentage of central nuclei. There was no change in serum creatine kinase (CK) levels. Thus, in $\text{RAG2}^{-/-}$ mice body weight and fatigue were directly affected by a hypoactive immune system. Whether these effects were centrally or peripherally mediated is unknown. This model may help to explain fatigue in human conditions in which the immune system is suppressed or absent.


RAG2 GENE KNOCKOUT IN MICE CAUSES FATIGUE

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The human immune and neuromuscular systems are in simultaneous decline in multiple settings such as human immunodeficiency virus (HIV) infection, chronic fatigue syndrome, advancing age, chronic nervous system disease, and cancer with cachexia. Whether these are parallel or sequential effects have not been determined in human patients. Existing rodent models of fatigue have focused on hyperactivity of the immune system rather than immune hypoactivity. In these models, an immune response to bacterial antigen, e.g., Brucella abortus or Corynebacterium parvum, is elicited. Fatigue is then measured as a decrease in total voluntary wheel-running (VWR) time. These models may best represent fatigue associated with inflammatory or infectious diseases. They do not tell us about the interactions when the immune response is inactive or in immunosuppressed states.

To assess the effects of immune hypoactivity or absence leading to changes in strength, fatigue, and muscle makeup, we studied control and immunosuppressed mice carrying the RAG2 gene knockout. $\text{RAG2}^{-/-}$ mice lack a protein necessary to recombine the DNA at the loci of the T-cell receptor and the immunoglobulin genes, resulting in absent T-cells and B-cells. The $\text{RAG2}^{-/-}$ gene knockout effect on the immune system is more complete compared to the severe combined immunodeficiency (SCID) mouse strain with $\text{Prkdc}^{-/-}$ gene knockout. Thus, even older $\text{RAG2}^{-/-}$ mice have no detectable T-cells or B-cells. T-cells and B-cells play a pivotal role in coordinating and affecting responses to infectious and inflammatory insults; T-cells carry out cytotoxic attack on virally infected cells and B-cells are the sole source of immunoglobulins. Together, these cells have profound downstream effects on the activities of macrophage/monocytes, granulocytes, and complement systems. Rag2 protein has restricted expression to immune precursors. It is not known to have any direct action in the central and peripheral nervous systems or in muscle tissue. Changes in neuromuscular function should be a direct consequence of T-cell and B-cell absence.

MATERIALS AND METHODS

Mice. All mice were maintained in micro-isolator cages in a pathogen-free facility in accordance with our institutional and national guidelines. The C57BL/10 $\text{RAG2}^{-/-}$ mouse strain was crossed with the C57BL10/J $\text{RAG2}^{-/-}/+\text{mdx}$ mouse (Taconic, Ger-

Abbreviations: CK, creatine kinase; FGS, forelimb grip strength; HT, hang-time; PCR, polymerase chain reaction; RAG2, recombinase activating gene 2; SCID, severe combined immune deficiency; VWR, voluntary wheel-running

Key words: fatigue; RAG2 knockout; SCID; strength; voluntary wheel-running

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manton, New York; Jackson Laboratories, Bar Harbor, Maine, respectively) to produce RAG2<sup>+/−</sup> heterozygotes, which in turn were backcrossed to C57BL/10 RAG2<sup>−/−</sup> mice to produce sex- and age-matched mice for testing. All mice included in this analysis were male, with normal dystrophin genotype. The RAG2 mutation is strictly recessive, so that RAG2<sup>+/−</sup> and RAG2<sup>+/+</sup> mice are both fully immunocompetent. They were considered equivalent and were referred to as RAG2<sup>+</sup> or wildtype in this study. Bactrim antibiotic prophylaxis was given for 30 days on alternating months. Polymerase chain reaction (PCR) genotyping was done using standard methods from tailsnip DNA. RAG2 phenotyping was performed at 3 and 40 weeks of age using direct staining of a dried blood spot from tailsnips using phycocrythrin-labeled antomouse CD5 and/or antomouse CD19 (BD Pharmingen, San Diego, California).

**Forelimb Grip Strength (FGS).** FGS testing was done as described previously. FGS fatigue is a decrement of strength over five consecutive pulls. FGS testing, hang-time testing, and body weight measurement was done by blinded examiners at 3, 4, 5, 6, 8, 10, and 12 weeks and then at 4-week intervals up to the age of 36 weeks. Absolute FGS measures were divided by body weight to determine relative FGS.

**Hang-Time Test (HT).** The mice were suspended upside down from a metal screen and timed for their ability to hold on for a maximum of 60 s. Two consecutive tests were done and the greater value was used for analysis.

**Voluntary Wheel-Running (VWR).** Mice were tested at 38 weeks of age after being segregated to individual cages with a voluntary exercise wheel recorded by an automatic speedometer. After a 5-day acclimation period, two 24-h running periods were recorded for speed, total distance, and total time on the wheel.

**Serum Creatine Kinase (CK) Levels.** Serum levels were analyzed at 39 weeks after a 1-week rest period.

**Histology.** Mice were euthanized by CO<sub>2</sub> inhalation at 39 weeks of age. Serum was separated and frozen. We stained 10–14-µm sections from flash-frozen right quadriceps and biceps muscles with hematoxylin and eosin, Gomori trichrome, acid phosphatase, alkaline phosphatase, nicotinamide adenine dinucleotide, succinate dehydrogenase, cytochrome oxidase, esterase, myoadenylate deaminase level, glycogen phosphorylase level, periodic acid–Schiff, Sudan, Verhof van Giesson, and ATPase staining (at acid and alkaline pHs). This standard panel of stains is used for the clinical testing of human muscle biopsies at our institution. Fiber type staining was done with anti-type 1 myosin heavy chain (NCL-MHCs, Novocastra Labs, Newcastle upon Tyne, UK), anti-type 2a (SC-71), anti-type 2b (BF-F3), and anti-type 2d/x (6H1) immunostaining. BF-F3, SC-17, and 6H1 sera were gifts from Leslie Leinwand (University of Colorado).

Images of stained sections were processed using Adobe Photoshop (San Jose, California) to distinguish type 2a, 2b, and 2d/x fibers. Fiber type percentages were determined by counting all fibers in a deep, medial field from rectus femoris (200–300 fibers per field) stained for type 2a and 2b fibers from eight RAG<sup>−/−</sup> and nine RAG<sup>+</sup> mice. Since type 1 fibers were absent from quadriceps in all mice tested, fibers not staining for type 2a or 2b were scored as pure type 2d/x for statistical analysis. A blinded examiner measured 100 type 2b myofibers for diameter from eight RAG<sup>−/−</sup> and nine control mice.

**Statistical Analysis.** Two-way ANOVA, Student’s <i>t</i>-test (two-tailed, equal variance), Wilcoxon signed rank test, and Mann–Whitney test and frequency distribution histograms were completed using GraphPad Prism (San Diego, California).

**RESULTS**

**RAG2<sup>−/−</sup> Mice Had a Smaller Body Weight.** Weight gain by the RAG2<sup>−/−</sup> mice slowed, most notably after 16 weeks of age (<i>P</i> < 0.0001; Fig. 1A). This deficit was directly reflected in lower absolute FGS in the same time period (<i>P</i> < 0.0001; Fig. 1B). When corrected for weight, RAG2<sup>−/−</sup> mice had no loss of muscle strength per body weight (Fig. 1C). This resulted in a smaller mouse with proportionally preserved, normal strength. There was no progressive loss of weight or strength, as is seen in chronically infected mice or those with active inflammatory disease. There was no change in grooming.

**RAG2<sup>−/−</sup> Mice Had Increased Fatigue.** HT testing demands forced self-sustained exertion over a short period. After 12 weeks of age, RAG2<sup>−/−</sup> mice were not able to hang inverted as long as wildtype mice despite their proportional strength with lesser body weight (Fig. 2A). VWR was used to assess fatigue with chronic self-paced exertion. At 38 weeks of age the RAG2<sup>−/−</sup> mice had nearly comparable running speeds to wildtype mice (1.50 vs. 1.45 km/h). However, the RAG2<sup>−/−</sup> mice did not or could not main-
RAG2−/− Mice Had No Pathologic Changes in Muscles. Although all areas of the quadriceps and biceps brachii muscles were reviewed qualitatively at lower magnification, we concentrated on the deep, medial portion of the rectus femoris. Both these muscles play a role in gait. Quadriceps muscles are most often biopsied in human patients with nonfocal muscle complaints. The rectus femoris is the largest and

FIGURE 1. Body weight, absolute and relative strength comparison of RAG2−/− versus wildtype mice. RAG2−/− weigh less (A) and have proportionally decreased absolute strength (B) compared to wildtype mice (two-way ANOVA, 3–36 weeks, both \( P < 0.0001 \)). Correcting for body weight, there is little or no difference in relative strength between RAG2−/− and wildtype mice (C, two-way ANOVA, for 3–36 weeks, \( P = 0.03 \); for 3–12 and 24–36 weeks there is no difference). \( n = 18–22 \) mice per group. Error bars = SD.

FIGURE 2. Fatigue comparisons of RAG2−/− and wildtype mice. (A) RAG2−/− mice (open symbols) hang inverted for a shorter period of time (two-way ANOVA, 3–36 weeks, \( P < 0.0001 \)), and (B) run for a shorter time and distance in a 24-h time period (paired, two-tailed t-test, both \( *P < 0.004 \)) compared to wildtype mice (solid symbols). Running speed is not effected by RAG2 genotype. HT error bars represent SEM. HT, \( n = 18–22 \) mice per group at all timepoints. VWR, \( n = 10 \) per group.
histologically best preserved of the vasti muscles given its central location in the quadriceps. As reported in the literature, there was a relative increased abundance of highly oxidative fibers in the deep portions of the rectus femoris muscle compared with the superficial portion in both groups of mice (RAG2<sup>-/-</sup> and wildtype).<sup>29</sup> There was also a difference in fiber type makeup of the medial compared with the lateral portions of the rectus femoris muscle that was not changed by RAG2 genotype. Quantitative analysis of the deep, medial rectus femoris muscle of mice revealed no muscle pathology. There was no significant increase in the number of muscle fibers with central nuclei, implying fatigue is not associated with increased degeneration and regeneration of muscle due to RAG2 gene knockout. There was no change in the type 1, 2a, 2b, or 2d/x fiber proportions in RAG2<sup>-/-</sup> mice compared with wildtype (Table 1). Direct type 2d/x myosin staining showed some overlapping expression of the 2d/x myosin among type 2a and 2b muscle fibers, as previously described.<sup>20,22</sup> Regardless of RAG2 genotype, there were no type 1 fibers by low and high pH ATPase staining or by immunostaining for myosin slow chain in the quadriceps. The deep, medial rectus muscle is rich in type 2a and 2b muscle fibers, as previously described. There was no change in the type 2b muscle fiber diameter with RAG2 genotype (Fig. 3).

**DISCUSSION**

Although mice with the RAG2<sup>-/-</sup> gene mutation have increased fatigability by two measures, they have normal running speed, proportionally normal muscle strength, and normal muscle histology. Unlike other mouse models of fatigue, it was the presence of markedly increased muscle fibers with central nuclei, a characteristic feature of the mdx mouse model of human muscular dystrophy, that distinguishes the RAG2<sup>-/-</sup> mouse from other models. The RAG2<sup>-/-</sup> mice also have normal muscle composition, with no evidence of muscle degeneration/regeneration, and no muscle calcifications. CK levels were unchanged by RAG2 genotype (Table 1).

**Table 1.** Histologic and serologic comparisons of RAG2<sup>-/-</sup> versus wildtype mice.

<table>
<thead>
<tr>
<th>Muscle</th>
<th>RAG2&lt;sup&gt;+&lt;/sup&gt; (n = 9)</th>
<th>RAG2&lt;sup&gt;-/-&lt;/sup&gt; (n = 8)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rectus femoris</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fibers with central nuclei (%)</td>
<td>13.9</td>
<td>9.6</td>
<td>0.5</td>
</tr>
<tr>
<td>Type I fibers (%)</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Type 2a fibers (%)</td>
<td>5.5</td>
<td>7.2</td>
<td>0.6</td>
</tr>
<tr>
<td>Type 2b fibers (%)</td>
<td>85.6</td>
<td>80.4</td>
<td>0.4</td>
</tr>
<tr>
<td>Type 2x fibers (%)</td>
<td>8.9</td>
<td>12.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Average type 2b muscle fiber diameter (μM)</td>
<td>50.5</td>
<td>49.9</td>
<td>0.4</td>
</tr>
<tr>
<td>Biceps brachii</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n = 9)</td>
<td>1.7</td>
<td>0.9</td>
<td>0.04</td>
</tr>
<tr>
<td>Serum CK level</td>
<td>3009 (n = 10)</td>
<td>3529 (n = 8)</td>
<td>0.4</td>
</tr>
</tbody>
</table>

RAG2<sup>-/-</sup> mice have the same (rectus femoris) or a slightly lesser (biceps brachii) percentage of fibers with internal nuclei compared to wildtype mice. There is no effect of RAG2 genotype on any muscle fiber type percentage (Student t-test) or serum CK levels (Mann–Whitney test and Wilcoxon signed rank test).
ence of a hypoactive immune system that caused their chronic changes in exercise physiology. This effect has not been investigated by others working with SCID phenotype mice from any cause (RAG1\(^{-/-}\), RAG2\(^{-/-}\), or Prkdc\(^{-/-}\)). The human corollary to these mice are infants afflicted with an SCID phenotype but exercise fatigue, skeletal muscle strength, and pathology have not been examined in these young children. Nonetheless, these findings may be relevant to other more common clinical settings in which the immune system is suppressed.

The chronic, sustained fatigue of RAG2\(^{-/-}\) mice differs from that seen in pure muscle disease models and inflammatory fatigue models. RAG2\(^{-/-}\) mice show no change in running speed, whereas mdx mice show a prominent decrease in VWR speed and running time. Fatigue mouse models due to acute inflammation result in sick behavior, including decreases in grooming and VWR speed and time. Although a defect of neuromuscular transmission cannot be completely excluded by this study, the lack of change in size of the neuromuscular junction and lack of change in running speed argues against this. RAG2\(^{-/-}\) mice do not have FGS fatigue. FGS fatigue, which is a prominent feature in mdx mice and may relate to membrane fragility exacerbated by the eccentric movements that occur with FGS testing. The absence of FGS fatigue or elevations in CK levels in RAG2\(^{-/-}\) mice argues against a role for muscle membrane fragility. Fatigue and change in body weight in the RAG2\(^{-/-}\) mice developed only after 12 weeks of age. This implies developmental regulation and argues against chronic infection in the animals, as infections should occur at random ages. Future testing of in vitro twitch properties, fatigue time course, and force–frequency profiles of wildtype and knockout mouse muscle might further elucidate possible peripheral causes of fatigue.

RAG2\(^{-/-}\) mice have proportionally normal strength per body weight. This differs from both mdx and α-2 laminin-deficient muscle disease models where both absolute FGS and FGS per body weight are significantly lower than wildtype mice from the earliest age tested (3–4 weeks). The RAG2\(^{-/-}\) mouse has preserved muscle histology within the rectus femoris muscle consistent with existing published reports on rodent musculature.

If fatigue were due to changes in muscle fiber make-up, a change to a lower relative percentage of slow fatiguing, oxidative type 1, 2a, or 2d/x fibers might be expected in fatiguing animals compared with an increased percentage of more glycolytic type 2b fibers. This was not observed. The lack of such histologic changes argues against a peripheral cause for fatigue.

The fatigue model reported here is reminiscent of several human conditions with symptomatic fatigue. A causal link between fatigue and immune hypofunction has not been determined for chronic fatigue syndrome, depression, or HIV infection. This model may mimic changes seen in patients with advanced age or undergoing immunosuppressive therapy.

This study has immediate implications for researchers using immunodeficient mice for neuromuscular testing. It is unknown which immune cell type (helper T-cells, cytotoxic T-cells, plasma B-cells, or memory B-cells) or cytokine causes the phenotypic changes. Cytokines including TNF-α, IFN-γ, and IL-6 are known to play a role in some fatigue models. This model for fatigue due to neuromuscular and immune system interactions is amenable to laboratory study.


REFERENCES

ABSTRACT: There is a suprasegmental influence on the masseter reflex (MassR) in animals, which is mediated via the fifth nerve spinal nucleus (5SpN). Corresponding data in humans are lacking. Out of 268 prospectively recruited patients with clinical signs of acute brainstem infarctions, we identified 38 with magnetic resonance imaging (MRI)–documented unilateral infarcts caudal to the levels of the fifth nerve motor and main sensory nuclei. All had biplanar T2- and echo planar diffusion-weighted MRI and MassR testing. Five patients (13%) had ipsilateral MassR abnormalities. In all, the infarcts involved the region of the 5SpN. Patients with medullary infarcts involving the region of the 5SpN may thus have ipsilateral MassR abnormalities. This possibly represents an interruption of an excitatory projection mediated via the 5SpN to masseter motoneurons in the fifth nerve motor nucleus. MassR abnormalities with medullary lesions restrict the topodiagnostic value of the MassR.

MEDULLARY INFARCTS MAY CAUSE IPSILATERAL MASSETER REFLEX ABNORMALITIES

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The masseter reflex (MassR) is a monosynaptically transmitted monophasic (myotatic) stretch reflex of the masseter muscle. Afferents are Ia fibers from the masseter muscle spindles, which run in the masticatory nerve, enter the pons, and ascend in the mesencephalic tract of the trigeminal nerve to the mesencephalic nucleus of the trigeminal nerve. This nucleus contains the cell bodies of the first-order sensory neurons, which send collaterals down to the motor nucleus of the trigeminal nerve in the lower pons, where monosynaptic transmission to masseter muscle motoneurons takes place.2,7,12 According to current knowledge, MassR abnormalities indicate ipsilateral brainstem lesions between the levels of the fifth nerve motor and the third nerve nucleus, provided that trigeminal nerve functions are intact (i.e., normal corneal reflex, trigeminal sensory function, and masseter function).2,7,12 Experimental data from animals indicate a suprasegmental influence on the MassR, especially an excitatory projection from the amygdaloid nucleus via the fifth nerve spinal nucleus (5SpN) to the fifth nerve motor nucleus.5,6,9,11 This raises the question as to whether MassR abnormalities occur with medullary lesions involving the region of the 5SpN or its projection to masseter motoneurons: if they do occur, this would restrict the topodiagnostic value of the MassR. We present data on the existence of MassR abnormalities with such lesions and discuss possible mechanisms.

MATERIALS AND METHODS

We prospectively recruited 268 patients with acute unilateral brainstem infarcts documented by magnetic resonance imaging (MRI), including 180 previously reported patients.3 Among this group, we identified 38 patients with infarcts caudal to the levels of the fifth nerve motor and main sensory nuclei and normal R1-components of the blink reflex, which also excludes functionally relevant lesions in the caudal pons.3 In all patients, MassR testing and diffusion-weighted MRI (DWI-MRI) was done within 48 h after onset of symptoms. High-resolution T1- and T2-weighted MRI was done as...
soon as the patients could tolerate this longer-lasting examination (median, 6.5 days after onset of symptoms). All patients gave their informed consent to these investigations, which were approved by the local ethics committee.

**MRI Acquisition and Postprocessing.** MRI was done with a 1.5 T superconducting system (Magnetom Vision; Siemens, Erlangen, Germany). For DWI-echo planar imaging (TR 4000 ms, TE 103 ms), we measured separately applied diffusion gradients in the three spatial axis (b = 1164 s/mm$^2$, 128 matrix, 250 ms per slice, 20 slices, thickness 3 mm, 8 measurements). Axial and sagittal high-resolution T2-weighted imaging (TR 3810 ms, TE 90 ms, 256 matrix, slice thickness 3 mm) and T1-weighted imaging (TR 600 ms, TE 14 ms, 256 matrix, slice thickness 3 mm) was done before and after intravenous gadolinium. Slice orientation was parallel (sagittal sections) and perpendicular (axial sections) to the sagittal brainstem cuts of the stereotactic anatomical atlas of Schaltenbrand and Wahren.$^{14}$

The area of infarction was identified independently by one neuroradiologist (P.S.) and two neurologist (F.T. and J.M.). We used DWI-MRI to prove the acuity of the infarct and high-resolution T2-weighted MRI to outline the extension of this lesion. Using Unix and NT workstations and Photoshop software (Adobe Systems, San Jose, California) and Photo-Paint (Corel, Ottawa, Canada), the individual slices were normalized and projected onto the corresponding slices of the anatomical atlas. Zero point was set at the pontomesencephalic junction and the number of the given level indicates the distance from zero in millimeters in the caudal direction. Axial slices were used for normalizing the individual slices in plane according to their T2-weighted and T1-weighted brainstem outlines. Sagittal or coronal slices were used for normalizing in the z-axis by determining the best fitting of the anatomical plates with anatomical landmarks like the fourth ventricle or by setting the cranial nerve exit zones in projection to the anatomical plates.

**Three-dimensional Mapping and Statistics.** Given the anatomical–functional right–left symmetry of the brainstem, all left-sided lesions were flipped to the right side, normalized, and imported into a three-dimensional brainstem model$^1$ based on data from topometric and stereotactic atlases.$^{6,13,14}$ This idealized brainstem model was subdivided into 5268 volume elements (“voxels”) ranging from $2 \times 2 \times 2$ mm to $2 \times 2 \times 4$ mm. After MRIs had been imported and normalized into the brainstem model, each voxel of the model was assigned a value of 1 (voxel definitely affected by the MRI-documented lesion), a value of 0 (voxel definitely unaffected), or a value of 0.5 (voxel only partly involved or involvement disagreed by the two raters).

Statistical analysis of the pooled patient data aimed at identifying which voxels of the 5268 were significantly affected by the lesions. For within-group one-sample analysis, the system used Fisher’s exact test. For each voxel the statistical probabilities for a voxel affected in the patient group were calculated against a hypothetical mean value of the probability of a casual lesion, provided by the average number of brainstem voxels affected by ischemia in our population. For two-sample statistical analysis between two patient groups, the Mann–Whitney U test was applied. Statistical significance was set at $P < 0.05$. The significance of the results of any statistical test performed was color-coded in each voxel and displayed at its proper location in the brainstem model, creating a 3D statistical map. From the 3D visualization, 2D slices could be extracted along any of the three main section planes and further elaborated to smooth the boundaries of the areas containing significantly affected voxels.

**Masseter Reflex.** MassR examinations were done with an individually designed recording system. The reflex was elicited by a brisk tap with a reflex hammer on the patient’s jaw. The recording was triggered at the moment of the mechanical tap by a signal from a commercially available piezo-electric element mounted in the hammer. The reflex was recorded simultaneously on both sides with surface electrodes placed over the muscle belly 25 mm above the margin of the mandible (recording electrode), and over the zygoma at the lateral edge of the orbit (reference electrode) with a bandwidth of 20–2000 Hz. Age-related normal values for this self-made recording system were reported elsewhere.$^4$ Latency refers to the onset of the electromyographic response, and mean latencies were calculated from 10 successive events. Criteria of MassR abnormalities were: (1) unilateral or bilateral delay outside the age-related mean + 2.5 standard deviations (SD); (2) unilateral or bilateral loss; (3) right/left differences outside the age-related mean + 2.5 SD.

**Blink Reflex.** Examinations were done with an individually designed recording system. The reflex was elicited by stimulating the supraorbital nerve on either side using rectangular stimuli (duration, 0.1 ms; current, 25 mA; intervals, 10 s). Responses of the orbicularis oculi muscles were recorded simultaneously with surface electrodes inferior to the lower lid, halfway
between the inner and outer edge of the orbit (recording electrode) and at the lateral edge of the orbit (reference electrode) with a bandwidth of 20–2000 Hz. Mean latencies of the R1 and R2 components were calculated from five successive events. Criteria of abnormal R1 or R2 components were: (1) unilateral or bilateral delay outside the age-related mean ± 2.5 SD; (2) unilateral or bilateral loss; (3) right/left differences outside the age-related mean ± 2.5 SD.

RESULTS

Five of 38 patients (13.2%) with unilateral brainstem infarcts caudal to the levels of the fifth nerve motor and main sensory nuclei had ipsilateral MassR abnormalities: two patients had loss of the reflex and three an interside difference outside the normal range (Fig. 1, Table 1). Ipsilateral abnormalities of the R2 or R2c component of the blink reflex occurred equally frequently in both groups and were seen in three of the five patients with ipsilateral MassR abnormalities and in 21 of the other 33 patients with normal MassR findings.

All infarcts involved the usual vascular territory seen with Wallenberg’s syndrome. Patients with and without MassR abnormalities had similar clinical findings, i.e., all had at least four of the following six symptoms characteristic of Wallenberg’s syndrome: (1) ipsilateral Horner’s sign; (2) ipsilateral impairment of facial pain or temperature sensation; (3) palsy of cranial nerve IX or X; (4) contralateral impairment of pain or temperature sensation over the trunk and limbs; (5) ipsilateral limb ataxia; and (6) astasia or gait ataxia. The infarcts of the five patients with ipsilateral MassR abnormalities involved the region of the 5SpN on at least one slice (Fig. 2). Comparing the localization of medullary infarcts in patients with and without MassR abnormalities revealed an area that was shifted more dorsomedially than the infarcts of the dorsolateral medulla typically seen with Wallenberg’s syndrome (Fig. 3). Despite the small number of patients, this difference was significant (P < 0.05). Lateral parts of this area involve the region of the medial 5SpN (Fig. 3).

DISCUSSION

Our data indicate that MassR abnormalities may occur on the side of unilateral brainstem lesions caudal to the levels of the fifth nerve motor and main sensory nuclei, i.e., caudal to the central monosynaptic reflex arc between the levels of the fifth nerve motor and the third nerve nucleus.2,7,12 MRI-documented infarcts in these patients always involved medial parts of the 5SpN and dorsal parts of the medulla medial to the 5SpN. The occurrence of MassR abnormalities always on the side of the lesion argues against a chance occurrence.

A suprasegmental influence on the MassR has been shown in animal studies.5,6,9,11 Stimulation of the supratrigeminal area in rats may cause inhibitory9,11 or excitatory postsynaptic potentials in contralateral masseter motoneurons.11 A number of these supratrigeminal area neurons are activated by ipsilateral stimulation of the central amygdaloid nucleus mediating a disynaptic pathway from that nucleus to the contralateral trigeminal motoneurons.11 Depending on the site of amygdala stimulation, maintained bilateral facilitation or inhibition of the MassR was seen, which ceased after destruction of the 5SpN.5,6 Other, more direct tonic facilitatory

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Abnormal findings are in bold. All patients except Case 5 were male.
FIGURE 2. Lesion sites in five patients with unilateral medullary infarcts caudal to the levels of the fifth nerve motor and main sensory nuclei and ipsilateral masseter reflex abnormalities. The areas of infarction were projected into the corresponding slices of Schaltenbrand and Wahren’s atlas.\textsuperscript{14} Slice levels were between 25 and 39 mm below the pontomesencephalic junction (0 line) as shown in the left figure. Black ovals indicate the region of the spinal nucleus of the trigeminal nerve.

FIGURE 3. Statistical results of the comparison between patients with \((n = 5)\) and without \((n = 33)\) masseter reflex abnormalities on the side of unilateral medullary infarcts caudal to the levels of the fifth nerve motor and main sensory nuclei. Lesions of patients with ipsilateral masseter reflex abnormalities centered more dorsomedially than the infarcts of the patients with normal masseter reflex findings \((P < 0.05)\). The significant area (surrounded by dots) was projected into the brainstem slice of Schaltenbrand and Wahren’s atlas\textsuperscript{14} 27.5 mm below the pontomesencephalic junction. The black oval indicates the region of the spinal nucleus of the trigeminal nerve. DSCT, dorsal spinocerebellar tract; ICP, inferior cerebellar peduncle; ML, medial lemniscus; MLF, medial longitudinal fasciculus; N. IX, glossopharyngeal nerve; PyT, pyramidal tract/medullary pyramid; VSCT, ventral spinocerebellar tract; 5SpN, fifth nerve spinal nucleus.)
afferent influences on the MassR mediated through or originating from the 5SpN seem plausible, but such connections are—to the best of our knowledge—unidentified.

If an excitatory projection to masseter motoneurons exists via the 5SpN in humans, such a connection may be interrupted by a lesion of the 5SpN or its projections to the fifth nerve motor nucleus. The course of such projections may be medial to the 5SpN, as the fifth nerve motor nucleus lies medial and rostral to the 5SpN. Ipsilateral MassR abnormalities occurred in patients with medullary lesions involving medial parts of 5SpN and dorsal parts of the medulla medial to 5SpN. Lesions at that site may impair excitation of masseter motoneurons by damage of the 5SpN or its projections to the fifth nerve motor nucleus (and would also impair as yet unidentified direct tonic facilitatory projections from the 5SpN). This may explain why MassR abnormalities occurred in our patients, ipsilateral to their medullary lesions.

MassR abnormalities with medullary lesions restrict the topodiagnostic value of the MassR. The reflex is highly sensitive in detecting the existence of brainstem lesions, but appears less useful in determining the level of the lesion in the craniocaudal axis. Supported by Deutsche Forschungsgemeinschaft (DFG), Ho 293/10-1.

REFERENCES

THE CaV 1.2 Ca2⁺ CHANNEL IS EXPRESSED IN SARCOLEMMA OF TYPE I AND IIA MYOFIBERS OF ADULT SKELETAL MUSCLE

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Voltage-gated Ca2⁺ channels comprise a multigene family, and individual members serve specific functions within the cell. The L-type Ca2⁺ channels, CaV 1.1–1.4, are closely related to each other and share sensitivity to the dihydropyridines. The CaV 1.1 channel is restricted to skeletal muscle, where it is expressed in the T-tubules and provides the gating charge movement that triggers the ryanodine receptor to release Ca2⁺ from the sarcoplasmic reticulum and initiate muscle contraction. The CaV 1.2 channel is broadly expressed in the surface membranes of cardiac, smooth muscle, nerve, and neuroendocrine cells, where it generates Ca2⁺ action potentials that initiate intracellular signaling events such as excitation–contraction coupling in heart and smooth muscle and downstream signaling cascades through calcineurin and Ca2⁺/calmodulin-dependent kinase (CAMK) pathways in all these cell types. The broad expression and importance of the CaV 1.2 channel was recently demonstrated by a human mutation that causes Timothy syndrome, a disorder characterized by arrhythmia, autism, and other problems. Although the authors of this study looked at CaV 1.2 expression in a number of tissues, they did not directly characterize expression or defects in skeletal muscle.

The other L-type Ca2⁺ channels, CaV 1.3 and 1.4, are expressed in a number of cell types, but especially in neurons and neuroendocrine cells, where mutations in these channels cause deafness and night blindness, respectively. More distantly related voltage-gated Ca2⁺ channels include the CaV 2.1–2.3 channels, which initiate neurotransmitter re-
lease, and the CaV 3.1–3.3 channels, which are responsible for T-type currents.10 Taken together, the voltage-gated Ca$^{2+}$ channel gene family plays critical roles in many aspects of cell physiology.

In this study, we addressed expression of the CaV 1.2 Ca$^{2+}$ channel in skeletal muscle because it is the predominant L-type Ca$^{2+}$ channel expressed in other excitable cell types.10,17,41 In addition, we compared its subcellular distribution to that of the well-known T-tubular Ca$^{2+}$ channel, the CaV 1.1.20,31 Our findings suggest that the CaV 1.2 channel is expressed in the surface membrane of type I and IIA skeletal muscle myofibers, where it may allow entry of Ca$^{2+}$ to activate downstream signaling pathways.

**METHODS**

**Preparation of Membrane Fractions and Western Blot Analysis.** Rat hind-leg skeletal muscle (mixed muscles from the entire hind-leg region), heart, liver, and brain were harvested and membrane fractions prepared as described previously,53 using a comprehensive panel of phosphatase and protease inhibitors (Catalog Nos. 539134, 208733, and 524625; Calbiochem, San Diego, California) effective against acid and alkaline phosphatases and all classes of proteases, including calpains. A Lowry protein assay was used to normalize protein content between samples, with 200 μg of membrane protein used per lane on a 5%–15% gradient sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gel for the CaV 1.1 Ca$^{2+}$ channel and a 10% SDS-PAGE gel for the CaV 1.2 Ca$^{2+}$ channel. Following electrophoretic transfer to nitrocellulose membranes, the CaV 1.1 Ca$^{2+}$ channel was detected with a monoclonal antibody (Abcam, Cambridge, MA) and the CaV 1.1 Ca$^{2+}$ channel was detected with a rabbit polyclonal antibody (Alomone, Jerusalem, Israel). To confirm that the detected CaV 1.2 Ca$^{2+}$ channel signal was specific, a competition assay with the immunizing peptide was carried out using 5 μg peptide/1 μg of antibody. Primary antibodies were visualized using the Western Star detection kit (Tropix, Bedford, Massachusetts).

To prepare membranes from the superficial white vastus lateralis, a muscle enriched in type IIB fibers,36 and tibialis anterior muscles, a muscle that contains type I, type IIA, and type IIB fibers,2 four muscles of each were harvested and pooled for the membrane preparation. Otherwise, the procedure followed was the same as described earlier.

**RNase Protection Assays.** Total RNA from hind-leg muscles, heart, liver, and brain were obtained using standard protocols.13 RNase protection assays were carried out as described previously using a complementary probe specific to nucleotides 3306–3638 of the CaV 1.2 Ca$^{2+}$ channel, which yields an expected band of 333 bp.26 As a control, protection of the 28S RNA was also assessed, using a commercially available complementary probe, pTRI-RNA-28S (Ambion, Austin, Texas). In RNase protection assays with total RNA, this 28S probe yields two closely spaced protection products around 100 bp.

**Preparation and Immunocytochemistry of Muscle Sections.** Tibialis anterior muscles were removed from rats, fixed in 4% paraformaldehyde, cryoprotected in 15% sucrose, and frozen in liquid nitrogen-cooled isopentane. Cross-sections or longitudinal sections (10 μm) were cut on a Microm cryostat and mounted on chilled glass microscope slides. Sections were stained with the same antibodies to the CaV 1.1 or CaV 1.2 Ca$^{2+}$ channels used in the Western blot analyses or with antibodies to caveolin-3 (Catalog No. sc-28828; Santa Cruz Biotechnology, Santa Cruz, California), dystrophin (Catalog No. sc-7461; Santa Cruz), and type I (Catalog No. M8421; Sigma, St. Louis, Missouri) or IIa (Catalog No. N2.261; Iowa Hybridoma Bank, Iowa City, Iowa) myosin. Competitions for the CaV 1.2 Ca$^{2+}$ channel were carried out in the same manner used for the Western analysis. For staining with the antibody to type Iib myosin (Catalog No. BF-F3; German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany), it was necessary to use fresh-frozen sections (5 μm), which were obtained from plantaris muscles as described previously.2 To visualize primary antibodies, the sections were counterstained with fluorescein-isothiocyanate (FITC) or tetramethyl rhodamine-isothiocyanate (TRITC)–labeled secondary antibodies (Jackson ImmunoResearch, West Grove, Pennsylvania). Immunostained sections were analyzed by confocal microscopy using an Olympus Fluoview (20×, air 0.7 NA and 60× oil 1.4 NA objectives; Olympus, Tokyo, Japan). The images shown are from single planes.

To assess changes in fiber types induced by exercise, C57Bl/6J mice were allowed to exercise freely by long-term voluntary running (4 weeks), as described previously.25 Control C57Bl/6J mice were maintained in cages with exercise wheels in locked position. For quantification of fiber types from these exercised or control mice (n = 3 for each group), 200-μm$^2$ regions from plantaris muscle cross-sections were scored for fibers positive to the CaV 1.2 Ca$^{2+}$ channel and type IIA myosin, stained as described above. Data are reported as averages ± SEM.
Data were analyzed by two-way analysis of variance (ANOVA), and a post hoc Tukey’s comparison carried out to determine which samples were statistically different. Different letters are used to indicate samples that were statistically different from each other ($P < 0.05$).

All animal protocols accorded with the NIH Guide for Care and Use of Laboratory Animals, and were approved by the animal care and use committees at the institutions in which the studies were carried out.

RESULTS

**Both CaV 1.1 and CaV 1.2 Ca$^{2+}$ Channels Are Expressed in Skeletal Muscle.** Using isoform-specific antibodies to the L-type voltage-gated calcium channels, CaV 1.1 and CaV 1.2, we examined expression of these channels in brain, heart, liver, and skeletal muscle membranes using Western blot analyses (Fig. 1A). The CaV 1.1 Ca$^{2+}$ channel, also known as the dihydropyridine receptor or $\alpha_1$C, was expressed only in skeletal muscle, consistent with previous reports.$^{10,17,31,41}$ In contrast, the CaV 1.2 Ca$^{2+}$ channel, also known as $\alpha_1$D, was expressed in brain, heart, liver, and skeletal muscle. The slightly different size observed for this channel in different tissues is consistent with a previous finding that different splice variants of the CaV 1.2 Ca$^{2+}$ channel are expressed in different tissues.$^{10,17}$ To confirm the identification of the CaV 1.2 channel in skeletal muscle, the peptide used to generate the CaV 1.2 antibody was used as a competitor to displace binding in both brain and skeletal muscle (Fig. 1B).

To confirm the expression of the CaV 1.2 Ca$^{2+}$ channel with a different approach, RNase protection assays were carried out using RNA from these same tissues. A probe complementary to nucleotides 3506–3638 of the CaV 1.2 channel yielded a band of approximately 300 bp in all of these tissues (Fig. 1C). Surprisingly, the level of CaV 1.2 mRNA was similar in all of these tissues, although there was more robust expression of the protein in brain and skeletal muscle, suggesting that post-transcriptional mechanisms contribute to the level of protein expression. Taken together, these data confirm the novel finding that the CaV 1.2 Ca$^{2+}$ channel is expressed in skeletal muscle.

**Distinct Distribution of CaV 1.1 and CaV 1.2 Ca$^{2+}$ Channels in Skeletal Muscle.** To address the issue of Ca$^{2+}$ channel distribution in skeletal muscle, we analyzed muscle cross-sections and longitudinal sections stained with both antibodies (Fig. 2). In muscle cross-sections, both antibodies exhibited a somewhat mosaic pattern of expression. The CaV 1.1 antibody stained most fibers, although there was some heterogeneity. Because this heterogeneity could arise from the fact that this Ca$^{2+}$ channel is present in the

![FIGURE 1.](image-url)
T-tubule membranes and cross-sections were taken parallel to T-tubules, it was important to analyze staining in longitudinal sections as well. At either low or higher magnification, the CaV 1.1 Ca2⁺/H11001 channel staining in longitudinal sections gave rise to a uniformly striped pattern, identical to that found previously for this channel due to its localization in the T-tubular membranes.³¹ There was little, if any, fiber-to-fiber variation in this pattern.

The staining pattern of the CaV 1.2 antibody was far more complex than that of the CaV 1.1, especially in the longitudinal sections. The mosaic pattern found in the muscle cross-sections with the CaV 1.2 antibody was also found in the longitudinal sections at both low and higher magnification. This antibody gave rise to a robust staining of the outer rim of some muscle fibers, consistent with a sarcolemmal location for this channel.

That the CaV 1.2 staining was present in some fibers and not others suggests that this surface CaV 1.2 Ca2⁺ channel was expressed in some muscle fiber types and not others. However, there was also a striped pattern present in all fibers, consistent with a portion of the CaV 1.2 Ca2⁺ channel being localized within the T-tubular membranes of all muscle fibers. Competition with the peptide used to generate the CaV 1.2 antibody displaced binding to the sections, as shown at high power. The scale bar for the low-power images indicates 50 μm and that for the high power indicates 20 μm.

FIGURE 2. The CaV 1.2 Ca2⁺ channel is only expressed in a subset of skeletal muscle fibers. Muscles analyzed in cross-sections with the two antibodies indicate that there was a mosaic of some fibers stained with the CaV 1.2 antibody, whereas others were not stained. A thick outer membrane staining was found in CaV 1.2-positive fibers. The scale bar in these panels indicates 50 μm. To more fully visualize the T-tubular membrane system, longitudinal sections of muscle were also analyzed, both at low power and high power. Staining with the CaV 1.1 antibody was fairly uniform throughout the muscle at both low and high power, but staining with the CaV 1.2 antibody demonstrated a mosaic, with some fibers having intense surface membrane staining. Competition with the peptides used to generate the CaV 1.2 antibody displaced binding to the sections, as shown at high power. The scale bar for the low-power images indicates 50 μm and that for the high power indicates 20 μm.
**CaV 1.2 Ca\(^{2+}\) Channel Expressed in Surface Membrane of Type I and IIa Fibers, But Not Type IIb Fibers.** To address the issue of fiber type–specific expression of the surface CaV 1.2 Ca\(^{2+}\) channel, muscle cross-sections were analyzed with both the CaV 1.2–specific antibody and antibodies that are specific for myosin heavy chain isoforms (see Methods). Both type I– and type IIa–positive fibers stained with the CaV 1.2 antibody, whereas the type IIb–positive fibers did not stain with the CaV 1.2 antibody. The scale bar in each panel indicates 50 \(\mu\)m. **(B)** Western blot analysis was carried out on membrane proteins prepared from superficial white vastus lateralis muscles (WVL), a muscle type composed of 95% type IIb fibers,\(^3\) and tibialis anterior (TA), the muscle of mixed fiber type used for the immunostaining. Although both muscles expressed the CaV 1.1 robustly, expression of CaV 1.2 was greatly reduced in the muscle enriched in type IIb fibers, consistent with the immunostaining results.

**FIGURE 3.** The CaV 1.2 Ca\(^{2+}\) channel is expressed in type I and IIa fibers, but not IIb fibers. (A) To determine whether specific fiber types expressed the CaV 1.2 Ca\(^{2+}\) channel, muscle cross-sections were analyzed with both the CaV 1.2–specific antibody and antibodies that are specific for myosin heavy chain isoforms (see Methods). Both type I– and type IIa–positive fibers stained with the CaV 1.2 antibody, whereas the type IIb–positive fibers did not stain with the CaV 1.2 antibody. The scale bar in each panel indicates 50 \(\mu\)m. (B) Western blot analysis was carried out on membrane proteins prepared from superficial white vastus lateralis muscles (WVL), a muscle type composed of 95% type IIb fibers,\(^3\) and tibialis anterior (TA), the muscle of mixed fiber type used for the immunostaining. Although both muscles expressed the CaV 1.1 robustly, expression of CaV 1.2 was greatly reduced in the muscle enriched in type IIb fibers, consistent with the immunostaining results.
for the immunostaining (Fig. 3B). Although both muscles express the T-tubular CaV 1.1 channel robustly, there was reduced expression of the CaV 1.2 channel in the type IIb–rich muscle, consistent with the immunostaining results.

Excision of Type IIa fibers and CaV 1.2–Positive Fibers Concomitantly Increased by Exercise. To determine whether there might be a relationship between expression of the CaV 1.2 Ca\(^{2+}\) channel in the surface membrane and fiber type switching, we applied stimuli known to change fiber type specificity and determined whether there was a corresponding change in CaV 1.2 expression (Fig. 4). As our stimulus, we chose exercise, which is known to increase the proportion of type IIa fibers in plantaris muscle. In plantaris muscles from control mice, there were more CaV 1.2–positive fibers than type IIa fibers. Exercise changed this pattern in that both the number of type IIa fibers and CaV 1.2 fibers increased concomitantly, with the end result being that there were the same proportions of type IIa and CaV 1.2–positive fibers. Taken together, these data suggest that some fibers express the CaV 1.2 Ca\(^{2+}\) channel prior to becoming type IIa fibers.

Markers of Sarcolemma Indicate CaV 1.2 Ca\(^{2+}\) Channel Expression in Both Sarcolemma and a Subsarcolemmal Region. To confirm that the CaV 1.2 was expressed in the sarcolemma, co-staining with the CaV 1.2 antibody and an antibody to dystrophin, a marker of the surface membrane, was carried out (Fig. 5, top row). As shown most clearly in the overlay, there was considerable overlap in the staining pattern with the

![FIGURE 4](image-url) There is a concomitant increase in type IIa– and CaV 1.2–positive fibers in the plantaris muscles from animals that exercise. Plantaris muscles from control animals or animals that were allowed to exercise were isolated and stained with antibodies to type IIa MHC and CaV 1.2 Ca\(^{2+}\) channels. The number of positive fibers for each group (type IIa– or CaV 1.2–positive) is indicated per 200 \(\mu\)m\(^2\). Consistent with previous results, the number of type IIa fibers increased in muscle cross-sections taken from animals that were allowed to exercise. In control muscles, there were significantly more CaV 1.2–positive fibers than type IIa fibers and, in the exercised muscles, there was a concomitant increase in the number of both type IIa– and CaV 1.2–positive fibers. Different letters indicate significant differences between numbers of fibers in each sample set (\(P < 0.05\)).

![FIGURE 5](image-url) The CaV 1.2 Ca\(^{2+}\) channel is expressed in both the sarcolemma and a subsarcolemmal region of the muscle fiber. To determine whether the CaV 1.2 channel was expressed in the surface membrane, co-staining with the sarcolemmal marker dystrophin was carried out (top row). As indicated by yellow arrow 1 in the overlay, there is considerable overlap in the staining of the CaV 1.2 and dystrophin antibodies, but as indicated by red arrow 2, the CaV 1.2 staining extends into a subsarcolemmal region. In fibers that are negative for CaV 1.2 staining, only the dystrophin staining is observed, as indicated by green arrow 3. To determine whether the denser staining observed with the CaV 1.2 Ca\(^{2+}\) channel corresponded to lipid rafts, co-staining with a caveolin-3 antibody was also carried out, but the pattern of co-staining was similar to that observed with the dystrophin antibody. The scale bar in each panel indicates 50 \(\mu\)m.
dystrophin and CaV 1.2 antibodies (Fig. 5, yellow arrow 1). However, immediately beneath this region, there was further extension of the CaV 1.2 staining (Fig. 5, red arrow 2). The surface membranes of fibers that were negative for CaV 1.2 channel were stained only by the dystrophin antibody (Fig. 5, green arrow 3).

Recently, it has been reported that the CaV 1.2 channel in cardiac muscle is expressed in lipid rafts, as shown by co-localization with the lipid raft protein caveolin.5 To determine whether the thick staining observed with the CaV 1.2 antibody corresponded to these lipid rafts, we also carried out co-staining with a caveolin-3 antibody. However, the pattern of co-staining with the caveolin-3 and CaV 1.2 antibodies was similar to that observed with the dystrophin and CaV 1.2 staining. Collectively, these data indicate that the CaV 1.2 Ca\(^{2+}\) channel is expressed in both the sarcolemma and a subsarcolemmal region.

**DISCUSSION**

The results of this study present the novel finding that the CaV 1.2 Ca\(^{2+}\) channel is expressed in the surface membrane of type I and IIa fibers in adult skeletal muscle and perhaps also expressed on a subset of fibers that later switch to type IIa as a result of stimuli such as exercise. The apparent molecular weight (Mr) of both the CaV 1.1 and CaV 1.2 Ca\(^{2+}\) channel in our Western blot analysis is similar to the high-Mr, 212-kDa band in previously published reports.15,16,25,35; we attribute the appearance of only this high-Mr band to the utilization of calpain inhibitors, as degradation of this larger Mr band due to calpains is a well-documented phenomenon.16 The differences in Mr of the CaV 1.2 Ca\(^{2+}\) channel in different tissues may well be due to the splice variants expressed in different tissues.10,17 To confirm the expression of the CaV 1.2 Ca\(^{2+}\) channel using an independent method, RNAse protection assays were used. The expression of CaV 1.2 mRNA in all these tissues is consistent with a previous report that this Ca\(^{2+}\) channel is broadly expressed in many cell types, including multiple brain regions, cardiac regions, and liver;31 and now, as shown in this report, in skeletal muscle.

The spatial expression of the CaV 1.2 Ca\(^{2+}\) channel is quite distinct from that of the T-tubular CaV 1.1 Ca\(^{2+}\) channel. Whereas the latter channel is expressed uniformly in the T-tubules of all muscle fibers, consistent with earlier work,31 the CaV 1.2 Ca\(^{2+}\) channel is expressed predominantly in the surface membrane of type I, IIa, and possibly IId/x fibers, but not in IIb fibers.

One important role of L-type Ca\(^{2+}\) channels is as a source of Ca\(^{2+}\) to regulate downstream signaling pathways.6,7,11,27,29,40 One set of pathways are the Ca\(^{2+}\)/calmodulin-dependent kinase (CAMK) pathways. Different isoforms of CAMK are known to regulate muscle plasticity and expression of the oxidative muscle phenotype through transcriptional regulators that include serum response factor (SRF), activator protein-1 (AP-1), and peroxisome proliferators activator gamma (PPAR\(\gamma\)) coactivator-1alpha (PGC-1\(\alpha\)). A second pathway involves the Ca\(^{2+}\)/calmodulin-activated phosphatase, calcineurin. Calcineurin ultimately activates several downstream transcription factors, including Id, myocyte-enhancing factor 2 (MEF2), and nuclear factor of activated T cells (NFATs), to regulate both initial differentiation and subsequent maturation of myofibers.21,22,28,30,32 In adult skeletal muscle, calcineurin is involved in specification of different fiber types.43,45,46

One manifestation of muscle plasticity is switching of fiber types. There are four different fiber types in adult skeletal muscle that are categorized by the predominant expression of different isoforms of MHC.6,7 MHC I is highly expressed in slow-twitch fiber types, which predominate in postural muscles, such as soleus, that are characterized by slow tonic contraction, fatigue resistance, and relatively high levels of resting cytosolic calcium.5,6,7 MHC Ila, IId/x, or IIb are expressed in fast-twitch fiber types with type Ila fibers being oxidative, type IIb fibers being glycolytic, and type IId/x fibers being in between.47 Muscles that are rich in fast-twitch glycolytic fibers are characterized by fast and powerful force generation, greater fatigability, and relatively low levels of resting cytosolic calcium.6–8,18,19 Alterations in neuromuscular activity, such as that induced by nerve stimulation or exercise, can induce a switch from one type to another.2,9,24,48,55 with a concomitant change of other cellular proteins. For example, plantaris muscles from mice have an increased proportion of MHC Ila–positive fibers after long-term voluntary running.2,55 In this study, we have demonstrated that there is a concomitant increase in both Ila and CaV 1.2–positive fibers in response to running, suggesting that calcium influx through this channel is important for specifying fiber type.

One possibility is that the CaV 1.2 Ca\(^{2+}\) channel is an entry pathway in skeletal muscle for the Ca\(^{2+}\) that activates the calcineurin signaling pathway, shown by other investigators to activate fiber type switching and specification.6,7,27 There are several lines of evidence, both in vivo and in vitro, implicating calcineurin signaling in muscle plasticity. First, in vitro experiments have shown that cultured muscle cells
express a mixture of fiber types, with MHC IIa, IId/x, and IIb predominating, but that an electrical stimulation pattern simulating that of a slow fiber type induces an increased proportion of MHC I in these cells.\textsuperscript{35,36,38,39} In these same experiments, addition of the calcium ionophore, A23187, directly induced expression of MHC I and the calcineurin blocker, cyclosporine, blocked this induction. In vivo, transgenic mice that had a constitutively active form of calcineurin driven by the muscle creatine kinase promoter had an increased proportion of MHC I–positive fibers,\textsuperscript{45} whereas mice lacking the catalytic subunit of calcineurin had reduced numbers of MHC I–positive fibers\textsuperscript{45} and those lacking the regulatory subunit of calcineurin had deficiencies in expression of both MHC I– and IIa–positive fibers.\textsuperscript{46} Taken together, these data suggest prominent roles for calcineurin signaling in both type I and IIa fiber types.

The results with the calcineurin knockout mice are consistent with studies focused on analyzing the promoters that drive MHC genes, in that calcineurin signaling pathways stimulate the MHC I promoter\textsuperscript{12,20} and also the fast MHC promoters in the order Ila > IId/x > IIb.\textsuperscript{3,4} Our finding that the Ca\textsubscript{V} 1.2 Ca\textsuperscript{2+} channel is expressed preferentially in the sarcolemma of these same fiber types establishes the possibility that influx of Ca\textsuperscript{2+} through this channel may activate the calcineurin signaling pathway, as it does in other excitable cell types such as neurons and smooth muscle.\textsuperscript{10,23,44,52}

Establishing a direct causal link between influx of Ca\textsuperscript{2+} through surface L-type Ca\textsubscript{V} 1.2 Ca\textsuperscript{2+} channels and activation of the calcineurin signaling pathway will require further work. Current pharmacological agents directed at L-type Ca\textsuperscript{2+} channels do not distinguish between these channel isoforms. However, conditional knockout of the Ca\textsubscript{V} 1.2 Ca\textsuperscript{2+} channel isoform in skeletal muscle may be possible, given that such lines have been established for neuronal specific expression of this channel isoform.\textsuperscript{41,42,49} Nevertheless, the data presented in this study clearly indicate that the Ca\textsubscript{V} 1.2 Ca\textsuperscript{2+} channels are present in selected fiber types and likely play an important and unique role in the biology of adult skeletal muscle.

This work was supported by NIH grants AR46477 (S.D.K.), AG000242 (D.M.J.), and AG024190 and AG027297 (C.M.N.). The authors thank Dr. Eric Blalock for his assistance with statistical analysis and Dr. Philip Landfield for critical reading of the manuscript.

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ABSTRACT: The second lumbrical interossei latency difference test (2-LINT) is a frequently used test for diagnosing carpal tunnel syndrome (CTS). Recently, the premotor potential (2-LUMP) observed with 2-LINT was identified as a median sensory potential. 2-LINT recording therefore not only compares conduction across equidistant median and ulnar motor segments, but also registers median sensory conduction. In 52 CTS and 50 control hands, we tested whether motor and sensory data obtained with 2-LINT help to reduce the number of tests necessary to diagnose CTS. The combined sensitivity of 2-LINT derived parameters (2-LUMP latency, median second lumbrical to ulnar interossei latency difference, ulnar digit 5 sensory to 2-LUMP velocity, and ulnar interossei to 2-LUMP latency difference) was 89%, identical to that of combined non–2-LINT derived parameters (median digit 3 sensory velocity, ulnar digit 5 to median digit 3 sensory velocity difference, median abductor pollicis brevis [APB] latency, median APB to ulnar abductor digiti minimi latency). The 2-LINT technique with its premotor potential may therefore help to reduce the number of nerve conduction tests commonly needed to investigate patients with suspected CTS.

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CAN STUDIES OF THE SECOND LUMBRICAL INTEROSSEI AND ITS PREMOTOR POTENTIAL REDUCE THE NUMBER OF TESTS FOR CARPAL TUNNEL SYNDROME?

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One of the nerve conduction techniques associated with the highest sensitivity and specificity for diagnosing carpal tunnel syndrome (CTS) is the second lumbrical interossei latency difference (2-LINT) test, with sensitivity ranging as high as 97.5%.9 Because of this, 2-LINT is now commonly employed as a standard test of CTS.3,16 It is a fast, easy-to-perform, median–ulnar motor comparative test that has been widely evaluated against other motor and sensory techniques in the diagnosis of CTS and consistently found to be highly sensitive and specific.7,9,12 An additional advantage of 2-LINT is its persistence in concomitant polyneuropathy and severe CTS.3,10

Recently, it has been shown that the premotor potential observed when recording standard 2-LINT represents a near-field median sensory nerve action potential (SNAP) generated by the digital sensory fibers as they cross the recording electrode.14 This adds a further advantage to the 2-LINT recording technique, which can simultaneously provide information concerning both motor and sensory median nerve fibers that have crossed the carpal tunnel, as well as from ulnar motor fibers. We therefore examined whether motor and sensory data obtained with 2-LINT recording could reduce the number of tests used to support a diagnosis of CTS by comparing data derived from 2-LINT recording with data from recording techniques commonly used for the electrodiagnosis of CTS.

MATERIALS AND METHODS

Patients were prospectively considered for inclusion in the study when referred to us for neurophysiolog-
neurological evaluation of hand symptoms suggestive of CTS. After informed consent, patients were consecutively included in the study, which was performed in 2006. Nerve conduction was performed by two examiners (A.K.T. and E.L.) who were blinded to the clinical diagnosis of the patient. A detailed and standardized history and clinical examination was performed for all patients after completion of nerve conduction studies using a structured questionnaire adapted for the local population from a previously verified scored questionnaire. Patient inclusion criteria were a clinical diagnosis of CTS that was established in the presence of at least two of the four primary symptoms of CTS. These were defined as predominantly nocturnal or early morning hand numbness, hand numbness provoked or worsened by sustained wrist position or manual activities, or relief of hand symptoms with trick movements. The other primary CTS criterion of symptom restriction to a median distribution was not required for the diagnosis, as this sign has been shown to be unreliable in our population.

Patients with weakness or wasting of ulnar-innervated muscles, primary neck symptoms, or proximal nerve pathology, and systemic conditions such as diabetes, uremia, and hypothyroidism were excluded. Controls were healthy people with normal physical examination without neurological complaints or risk factors of peripheral neuropathy.

Neurophysiological Tests. All tests were performed in a quiet air-conditioned room (22–24°C) with subjects lying comfortably. Skin temperature was measured over the palmar wrist using an infrared (no-touch) thermometer (Exergen DT-1001, Watertown, Massachusetts), and nerve conduction was performed only if the temperature was above 32°C. Nerve conduction recordings were performed using a 2-channel EMG machine (Medelec Synergy, Hong Kong). Monopolar surface recording electrodes (Teca Accessories, Hong Kong) and bipolar handheld surface stimulating electrodes were used to obtain the sensory nerve action (SNAP) and compound muscle action potentials (CMAP).

Sensory Nerve Conduction. The SNAPs of the median and ulnar nerves at the wrist were elicited using the orthodromic technique. In addition, the second lumbral premotor potential (2-LUMP) was measured and represents an antidromic SNAP derived from median sensory fibers traveling across the carpal tunnel to the digits.

The SNAP of the median digit 3 sensory branches was evoked by ring-electrode stimulation over the third proximal phalanx and recorded 11–14 cm proximally at the wrist. The ulnar nerve orthodromic SNAP was evoked by ring-electrode stimulation over the fifth proximal phalanx and recorded 10–13 cm proximally at the wrist. Because of the varying distances used, the conduction velocity was determined. Equipment settings for median and ulnar orthodromic recordings were: low filter 20 Hz, high filter 2 kHz, sweep duration 15 ms, and sensitivity 10 μV. The 2-LUMP was recorded when stimulating the median nerve at the wrist with the active recording electrode just lateral to the midpoint of the third metacarpal bone over the second lumbral muscle. Median nerve electrical stimulation was performed using a fixed distance of 10 cm proximal to the active recording electrode at the wrist in all cases and the peak latency of the evoked response was used. For the 2-LUMP recordings, the low filter was 3 Hz, high filter 10 kHz, sweep duration 20 ms, and sensitivity 500 μV. The level of gain is less than the usual chosen for sensory recordings since the primary recording is motor, but at this level the 2-LUMP peak latency is easily measureable. The 2-LUMP was taken as absent if there was no discernable deflection from the baseline at a gain of 500 μV; the median digit 3 SNAP was taken as absent when there was no discernable deflection from the baseline at a gain of 10 μV.

Motor Nerve Conduction. The motor tests performed were median conduction to abductor pollicis brevis (APB), ulnar conduction to abductor digit minimi (ADM), and 2-LINT.

The low filter was set at 3 Hz, high filter 10 kHz, sweep duration was 50 ms, and sensitivity 5 mV for median and ulnar recordings. For 2-LINT we followed the details previously given for 2-LUMP. To record the APB and ADM CMAP the median and ulnar nerves were stimulated 6 cm proximal to the active recording electrodes placed over the respective muscle belly. The onset of the recorded CMAPs was measured to determine the distal motor latencies.

For 2-LINT recording the median and ulnar stimulations were performed at the wrist equidistant, 10 cm proximal to the active recording electrode, as previously specified.

Outcome Measures. Outcome variables were categorized into 2-LINT and other derived parameters. The other derived parameters were median digit 3 sensory velocity, ulnar to median sensory velocity difference, median motor latency, and median to ulnar motor latency difference. The 2-LINT parameters consisted of 2-LUMP latency, differences in median second lumbral to ulnar interossei latency...
(MLUI), ulnar digit 5 sensory to 2-LUMP velocity, and ulnar interossei to 2-LUMP latency. The last was performed because test sensitivity is highest when a parameter most likely to be affected by CTS is compared to one least likely to be affected. A neurophysiological diagnosis of CTS was achieved when focal slowing of the transcarpal median motor or sensory nerve fibers was demonstrated in the presence of normal comparator (ulnar) nerve conduction. Sensory comparator nerve was the ulnar sensory nerve conduction; motor comparator was the ulnar motor conduction to the second interosseous and ADM muscles.

Neurophysiological Scales. CTS was graded with the standard scale of Bland: normal (grade 0); very mild (grade 1; abnormal 2-LINT only); mild (grade 2; median sensory nerve conduction <40 m/s); moderate (grade 3; sensory potential preserved, distal motor latency to APB prolonged but <6.5 ms); severe (grade 4; sensory potentials absent, motor latency to APB <6.5 ms); very severe (grade 5; sensory potentials absent, terminal latency to APB >6.5 ms); extremely severe (grade 6); sensory and motor potentials unrecordable.

Table 1. Results of nerve conduction measurements in controls and carpal tunnel syndrome (CTS).

<table>
<thead>
<tr>
<th>Test</th>
<th>Control hands</th>
<th>CTS hands</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median (digit 3) sensory conduction (m/s)</td>
<td>55 (5.1)</td>
<td>40.6 (8.0)*</td>
</tr>
<tr>
<td>Median (digit 3) SNAP (µV)</td>
<td>21.0 (7.5)</td>
<td>11.4 (8.6)*</td>
</tr>
<tr>
<td>Median APB motor distal latency (ms)</td>
<td>3.3 (0.4)</td>
<td>4.7 (1.5)*</td>
</tr>
<tr>
<td>2nd Lumbrical interossei testing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2-LUMP SNAP peak latency (ms)</td>
<td>2.25 (0.23)</td>
<td>2.99 (0.55)*</td>
</tr>
<tr>
<td>2-LUMP SNAP (µV)</td>
<td>88.1 (36.4)</td>
<td>57.4 (43.3)*</td>
</tr>
<tr>
<td>Ulnar interossei motor to 2-LUMP sensory latency comparison (ms)</td>
<td>0.91 (0.27)</td>
<td>0.14 (0.67)*</td>
</tr>
<tr>
<td>Median 2nd lumbrical to ulnar interossei latency difference (ms)</td>
<td>0.19 (0.25)</td>
<td>1.4 (1.3)*</td>
</tr>
</tbody>
</table>

Absent responses were excluded from analysis, P < 0.0001 for all tests performed.

SNAP, sensory nerve action potential; APB, abductor pollicis brevis; 2-LUMP, 2nd lumbrical premotor potential.

*Statistically significant difference using the t-test.

†Statistically significant difference using the Mann–Whitney U-test.

Statistical Analysis. All analyses were performed using SPSS statistical software (v. 14.0, SPSS, Chicago, Illinois) with statistical significance set at P < 0.05. The differences in the nerve conduction measurements between CTS patients and controls were assessed using the two-samples t-test when normality (checked by Komolgorov–Smirnov 1-sample test coupled with histograms) and homogeneity assumptions were satisfied; otherwise the equivalent non-parametric Mann–Whitney U-test was applied. The optimal cutoffs for each nerve conduction measure to differentiate the two groups were determined using the receiver operator curve (ROC) technique, with sensitivity, specificity, and positive and negative predictive values presented.

Linear association between the 2-LUMP and median digit-3 sensory conduction velocity was established. Sensitivity was calculated as the proportion of people with disease (CTS) having a positive test result, and specificity as the proportion of people without disease having a negative test result.

RESULTS

A total of 102 hands were examined. We investigated 52 hands with a clinical diagnosis of CTS from 33 patients aged 34–65 years (mean, 49 years); 21 patients (64%) were women. Control values were obtained from 50 hands of 27 healthy subjects aged 30–54 years (mean, 41 years); 16 subjects (59%) were women. Table 1 shows the results of the nerve conduction studies and Table 2 shows the comparison of two scales of neurophysiological severity. The main difference between the scales is that the new scale devised for 2-LUMP shows a more even spread

Table 2. Neurophysiological scales comparison.

<table>
<thead>
<tr>
<th>Grade</th>
<th>Bland's scale</th>
<th>Premotor-related scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>9</td>
<td>7</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>5</td>
</tr>
<tr>
<td>2</td>
<td>5</td>
<td>14</td>
</tr>
<tr>
<td>3</td>
<td>29</td>
<td>14</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>8</td>
</tr>
<tr>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>

The numbers under each scale represent the number of abnormal hands.
of the severity. There were significant differences between the CTS cases and controls. Table 3 shows the best cutoff values for each nerve conduction parameter and the corresponding maximal combined sensitivity and specificity as calculated using ROC analyses in addition to the negative and positive predictive values. Figure 1 shows the 2-LUMP in a healthy and CTS hand.

The 2-LUMP was recordable in all controls. In the CTS group the median digit 5 sensory conduction was unobtainable in 8 hands and the 2-LUMP was unobtainable in 12 hands. Two hands had absent APB and one absent 2-LINT recording. In 47 (90%) hands with clinical CTS, a neurophysiological diagnosis of CTS was achieved. Five hands (10%) showed no neurophysiological abnormality. In one hand the latency difference between 2-LUMP and the second interossei motor latency was the only abnormality. The maximum sensitivity and specificity of an individual non–2-LINT parameter was 85% and 92%, respectively (median sensory digit 3 and ulnar digit 5 conduction difference), and for a 2-LINT parameter was 79% and 94%, respectively (latency difference between 2-LUMP and second interossei motor latency). Combining all non–2-LINT associated parameters resulted in a sensitivity of 89%; combining all 2-LINT produced parameters in an identical sensitivity of 89%; both 2-LINT and non–2-LINT-associated parameters joined together resulted in 90% sensitivity. Disregarding the ulnar (digit 5) sensory to 2-LUMP velocity comparison did not influence the combined sensitivity of the 2-LINT parameters significantly (87%).

The coefficient of correlation (r) for 2-LUMP peak latency with median (digit 3) sensory conduction was −0.950 (P < 0.0001) and for 2-LUMP amplitude with digit 3 sensory amplitude was 0.721 (P < 0.0001).

**DISCUSSION**

The standard number of nerve conduction tests required to diagnose CTS neurophysiologically is generally taken as four, consisting of one motor and sensory transcarpal median nerve conduction compared with a similar segment of unaffected nerve, usually the ulnar nerve. Reducing the number of nerve conduction tests while maintaining sensitivity

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<table>
<thead>
<tr>
<th>Table 3. Sensitivity, specificity, positive and negative predictive value (PPV, NPV) of the nerve conduction measurements.</th>
</tr>
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<tbody>
<tr>
<td><strong>Test</strong></td>
</tr>
<tr>
<td>Non–2-LINT test</td>
</tr>
<tr>
<td>Median (digit 3) sensory conduction</td>
</tr>
<tr>
<td>Median motor distal latency</td>
</tr>
<tr>
<td>Ulnar (digit 5) and median (digit 3) sensory velocity difference</td>
</tr>
<tr>
<td>Median to ulnar motor difference</td>
</tr>
<tr>
<td>2-LINT test</td>
</tr>
<tr>
<td>2-LUMP SNAP peak latency</td>
</tr>
<tr>
<td>Ulnar interossei motor to 2-LUMP sensory latency comparison</td>
</tr>
<tr>
<td>Median 2nd lumbrical to ulnar interossei latency difference</td>
</tr>
<tr>
<td>Ulnar (digit 5) sensory and 2-LUMP velocity comparison</td>
</tr>
</tbody>
</table>

SNAP, sensory nerve action potential; 2-LUMP, 2nd lumbrical premotor potential; 2-LINT, second lumbrical interossei latency difference test.
and specificity would be useful in reducing time and cost as well as patient discomfort. The premotor potential (2-LUMP) observed when recording standard 2-LINT is a median SNAP produced by fibers en route to the digits; thus, 2-LINT simultaneously provides both motor and sensory median nerve parameters as well as an ulnar motor nerve comparison.\(^\text{14}\) It therefore seems an ideal means to both simplify and reduce the number of nerve conduction techniques for CTS.\(^\text{14}\) The comparative median to ulnar motor conduction sensitivity of the 2-LINT on its own is consistently one of the highest for a single test, generally reported as between 77.5% and 97.5%.\(^\text{9,2,4}\) Median sensory antidromic nerve conduction also has high and similar sensitivity and specificity in diagnosing CTS.\(^\text{15}\) A recent study investigating the utility of eight commonly used nerve conduction techniques for CTS concluded that short-segment, latency-based transcarpal mixed nerve conduction velocity yielded sensitivity among the highest (75%).\(^\text{8}\) The 2-LUMP is such a mixed nerve sensory test, with the difference of recording from a slightly longer stretch of nerve than that described in the referenced article.

We found that combined 2-LINT derived parameters provided the same sensitivity and specificity for diagnosing CTS as obtained with non–2-LINT parameters. Of particular value was the sensory-to-motor comparison of 2-LUMP and the ulnar second interosseus latency, which gave the highest sensitivity and specificity for this group. The ulnar motor second interosseus comparison to the 2-LUMP performs at the same or a slightly better level than the sensory ulnar comparison. This suggests that the additional sensory ulnar nerve comparison is not necessary, again simplifying the use of the 2-LINT method. Since it is common to compare a sensory nerve with another similar sensory nerve, it is advisable to do this until other groups confirm our results.

Our results suggest that in CTS the use of 2-LUMP and other 2-LINT–associated parameters could replace the recording of a conventional median sensory conduction to the digits, and motor studies of the median and ulnar nerves. Our neurophysiological grading scale for the 2-LINT recordings results in a more even spread of severity grades than the Bland scale.

This study has its limitations and problems. As this study was designed to test the practicability of 2-LUMP in normals and those with uncomplicated carpal tunnel, we were not able to test its use in special circumstances, such as CTS complicated by additional ulnar neuropathy or polyneuropathy. Furthermore, the role of sensory testing changes with increasing CTS severity. In our study ~10% of CTS showed absent sensory responses. In these cases additional sensory nerve testing must be performed to ascertain whether the absent responses are due to severe CTS or from comorbidities such as polyneuropathy.

The 2-LUMP was absent slightly more often than the median (digit 3) SNAP, an observation that needs explanation, as antidromic studies yield larger amplitudes. Most likely this is due to the smaller amplification used for measuring 2-LUMP than median sensory conduction (gain of 500 µV vs. 10 µV).

Future studies should aim to establish whether this test should be used as a routine primary step in CTS testing. Careful case selection will be important and should be designed to allow inclusion of both simple CTS, as in this study, as well as CTS complicated by comorbidities such as ulnar nerve pathology and polyneuropathy to evaluate the test’s performance in a more complex context.

REFERENCES

SYNEMIN EXPRESSION IN BRAIN

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β-Synemin,10,21 a novel intermediate filament protein, was originally identified as an α-dystrobrevin-binding protein through a yeast two-hybrid screen using an amino acid sequence derived from exons 1 through 16 of α-dystrobrevin,16,17 a region common to both α-dystrobrevin-1 and -2.10 Although this region is shared between both α-dystrobrevin isoforms, β-synemin preferentially associates with α-dystrobrevin-1 in muscle.12 Based on its expression pattern and location in muscle, it has been postulated that β-synemin predominantly functions in muscle to maintain costameric linkages, although expression has also been observed in muscle neuromuscular junctions,12 suggesting a role for β-synemin in transmitting neural signals to the muscles.

Although Northern blot analysis shows that a single isoform of human synemin is strongly expressed in skeletal muscle and heart, a longer exposure of the same Northern blot shows a faint doublet in brain, suggesting that there are two synemin isoforms expressed in brain tissue.10 Both synemin isoforms derive from differential splicing of the synemin gene on chromosome 15q26.3.11 The α-synemin transcript is larger than β-synemin’s transcript, as the intron between exons 4 and 5 is not removed.11,21 As a result, the human α-synemin protein is 312 amino acids longer at the C-terminus than β-synemin. Previous studies have shown that β-synemin is specifically expressed in skeletal muscle, whereas α- and β-synemin are expressed in brain tissue.6,12 It is not understood why two synemin isoforms are expressed in brain, although α-synemin expression is tissue-specific, suggesting that the two proteins have different functions.

During rat brain development, synemin is expressed only in a subpopulation of astrocytic precursors that are glial fibrillary acidic protein (GFAP) and vimentin positive.20 In the adult rat, astrocytes no longer express synemin in the cerebral hemispheres except in intermingled vascular smooth muscle cells.20 In contrast, only the α-synemin isoform is detected in sections from normal human brain, although astrocytic tumors express both α- and β-synemin.7 Furthermore, the α-synemin protein is highly expressed in astrocytomas compared to expression in unaffected brain.7 Increased synemin expression in astrocytomas is likely a result of increased vascularization within the tumor and the increased numbers of reactive astrocytes.7
Since it is unknown how synemin functions in neural tissue, in situ and immunohistochemical analyses were completed to identify where the synemin transcripts and proteins are normally expressed in brain tissue. To complete this study we examined brain tissue from 8-week-old C57BL/6 mice. Sections from brain were analyzed using a human synemin antibody, which recognizes both the α- and β-synemin proteins, and four different RNA probes, two of which are located in the alternative splicing region specific to α-synemin and two of which are present in a noncoding region common to both synemin isoforms.

**MATERIALS AND METHODS**

**RNA Probe Design and Preparation.** The following four in situ probes were used in this study (Fig. 1): RNA probe-1 [445 bp; position 3524–3968 (3459–3903 bp from the initiation codon)], RNA probe-2 [388 bp; position 3949–4336 (3884–4271 bp from the initiation codon)], RNA probe-3 [479 bp; position 5972–6450 (5907–6385 bp from the initiation codon)], and RNA probe-4 [510 bp; position 7116–7625 (7051–7560 bp from the initiation codon)]. Positions are relative to the mouse muscle cDNA for synemin (GenBank access. no. NM_201639). Each gene fragment was subcloned into the pGEM-T vector (Promega, Madison, Wisconsin) and digoxigenin-RNA probes were expressed from T7 and S6 promoters to generate both the sense and anti-sense RNA probes (Roche, Basel, Switzerland).

**In Situ Hybridization.** All in situ hybridization protocols were performed by Genostaff (Tokyo, Japan) and all results were confirmed by repeating the experiments either two (probes-1-3) or three (probe-4) times. Mouse brains were isolated from 8-week-old C57BL/6 and mdx (dystrophin-deficient) mice, fixed with tissue fixative, and subsequently embedded in paraffin blocks. Dystrophic (mdx) mice were provided by the Central Institute for Experimental Animals (Kanagawa, Japan). Tissue sections (6 μm) were prepared for in situ hybridization. They were dewaxed with xylene and rehydrated through a series of washes with ethanol and phosphate-buffered saline (PBS). Sections were fixed in PBS with 4% paraformaldehyde for 15 min and then washed with PBS. Sections were then treated with 10 μg/ml proteinase K in PBS for 30 min at 37°C, washed with PBS, refixed with 4% paraformaldehyde in PBS, washed again with PBS, and placed in 0.2 M HCl for 10 min. After washing with PBS the sections were acetylated by incubation in 0.1 M triethanolamine-HCl (pH 8.0), 0.25% acetic anhydride for 10 min. The sections were then washed with PBS and dehydrated through a series of ethanol washes. Hybridization was performed with either RNA probe-1, -2, -3, or -4 as indicated in the text at concentrations of 100 ng/ml in probe diluent at 60°C for 16 h. After hybridization, the sections were washed in 5× HybridWash (Genostaff, Tokyo, Japan) at 60°C for 20 min and then in 50% formamide, 2× HybridWash at 60°C for 20 min, followed by RNase treatment in 50 μg/ml RNaseA in 10 mM Tris-HCl (pH 8.0), 1 M NaCl, and 1 mM ethylenediaminetetraacetic acid (EDTA) for 30 min at 37°C. The sections were then washed twice with 2× HybridWash at 60°C for 20 min, twice 0.2× HybridWash at 60°C for 20 min, and once with TBST [0.1% Tween20 in Tris-buffered saline (TBS)]. After treatment with 0.5% blocking reagent (Roche, Basel, Switzerland) in TBST for 30 min the sections were incubated with anti-digoxigenin-AP conjugate (Roche) diluted 1:1,000 with TBST for 2 h. The sections were then washed twice with TBST and then incubated in 100 mM NaCl, 50 mM MgCl₂, 0.1% Tween20, 100 mM Tris-HCl (pH 9.5). Coloring

**FIGURE 1.** Summary of synemin, probes, and antibody. The α-synemin gene is labeled with the positions of the in situ probes and antibody-binding site. The region specific to α-synemin but spliced away in β-synemin is designated in red. Binding sites for in situ probe-1 and -2 are within the spliced region and therefore specific to α-synemin, whereas those for probe-3 and -4 are present in a noncoding region and recognize both synemin isoforms.
labeling patterns for the antisense probes were specific. Using sense probes were negative, suggesting the probes-3 (Fig. 2C) and -4 (Fig. 2D). Control experiments using sense probes were negative, suggesting the antisense probes were specific (data not shown). To better illustrate the parts of the brain being analyzed, a figure from the mouse brain atlas is shown\textsuperscript{15} with brain areas labeled (Fig. 2G).

Synemin expression was also analyzed in other brain regions. Detailed examination of the region of square-1 of Figure 3A and its corresponding region in Fig. 3B identified cells labeled by probe-3 in the septum (Fig. 3E) similar to that seen in serial sections stained with probe-1 (Fig. 3C) and -2 (Fig. 3D), although the levels of intensity with probe-3 were greater. In the dentate gyrus of the hippocampus (square-2 in Fig. 3A and its corresponding region in Fig. 3F), no cells stained with probe-1 (Fig. 3G) and only one cell staining with probe-2 was detected (Fig. 3H). Scattered cells stained by probe-3 were observed in the dentate gyrus (Fig. 3I); however, the number of positive cells was low and the intensities were weak compared to the staining observed in the midbrain and pons. The synemin-positive cells seemed to position outside of the dentate gyrus rather than inside of it. In coronal sections it was possible to identify cells stained by all four probes in the CA3 field of the hippocampus (data not shown). Based on sagittal brain sections hybridized with synemin-specific probes, detectable levels of synemin mRNA are observed in the mouse brain between the pons and midbrain, the hippocampus, and the septum.

**RESULTS**

*Localization of the Synemin Transcript in Mouse Midbrain, Pons, Hippocampus, and Septum.* In order to investigate synemin expression in the brain, in situ analysis was performed on sagittal sections of mouse brain using a general synemin probe (see probe-3, Fig. 1). This analysis showed that synemin transcription was largely localized to the ventral side of the cerebellum (see square, Fig. 2A). Larger magnification of the same area showed more obvious staining scattered at the midbrain to pons region (Fig. 2B), and even further magnification of the square in Figure 2B (see Fig. 2C) makes it possible to discern large clear reactive neurons. As expected, the second general synemin probe (probe-4) showed a similarly labeling pattern (Fig. 2D) to that of probe-3 (Fig. 2C). In order to investigate which synemin isoform was expressed in these tissues, α-synemin specific probes (-1 and -2, Fig. 1) were used for in situ analysis in the same sagittal sections. Both probe-1 and -2 (Fig. 2E,F) in general showed similar labeling patterns as that for the general synemin probes, although the intensities were weak compared to probe-3 (Fig. 2C) and -4 (Fig. 2D). Control experiments using sense probes were negative, suggesting the labeling patterns for the antisense probes were specific.

**Immunohistochemistry.** For immunohistochemistry, tissue sections were dewaxed with xylene, and rehydrated through a series of ethanol and TBS washes. Antigen retrieval was performed by microwaving the samples for 20 min with citrate buffer (pH 6.0). The sections were treated with 3% hydrogen peroxide in methanol for 15 min, and then Protein Block (Dako, Kyoto, Japan) for 10 min. The sections were treated with antihuman synemin rabbit polyclonal antibody\textsuperscript{12} at a final concentration of 0.4 μg/ml, at 2–8°C overnight. The 12 amino acid peptide sequence used to generate the antihuman synemin antibody is conserved in mice (amino acids 857–868 in human and 848–859 in mouse). The sections were treated with Histofine Simplestain mouse MAX-PO (R) (Nichirei Biosciences, Tokyo, Japan) for 30 min, and then incubated with 3,3′-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan). The sections were counterstained with Mayer’s hematoxylin (Muto), dehydrated, and mounted with malinol (Muto).

**Colocalization of the Synemin Transcript and Protein in Brain.** To determine whether synemin is expressed as protein in cells expressing the synemin transcript, we examined mirror sections (sections on either side of an initial cut) of mouse brain using both in situ hybridization (using probe-3) and immunohistochemistry (using an antisynemin antibody). Mirror sections were used to determine colocalization since they provide the greatest resolution when positively labeled structures are small. In mirror sagittal sections, regions 1, 2, and 3 (Fig. 4A) demonstrated convincing colocalization of transcript and its protein. A more detailed alignment of transcript-positive (Fig. 4B,F) and protein-positive (Fig. 4C,G) structures were observed in the midbrain and pons. Higher-resolution analysis of the squares shown in Figure 4B,C,F,G demonstrated that there were large numbers of the neurons expressing both the synemin transcript (Fig. 4D,H) and its protein (Fig. 4E,I). The differences in size of the neurons are likely due to the angle at which individual neurons were sectioned (Fig. 4D,E). In the hippocampus, synemin-positive structures were very small, making them difficult to observe; however, large magnification of squares in Figure 4J,K showed that the same cells were positive with the in situ probe-3 (arrow in Fig. 4L) and the antisynemin antibody (arrow in Fig. 4M). In the...
septum cells stained with probe-3 or the antisynemin antibody, although it was difficult to confirm colocalization even under large magnification (data not shown). Similar results were also obtained using the other general synemin in situ probe-4, and results from both probes were performed twice, confirming the specificity of our results.

**Synemin Expression in Dystrophin-Deficient (mdx) Mice Brain.** To determine whether synemin expression was altered in dystrophin-deficient mouse brain, in situ hybridizations (using probe-3) and immunohistochemical analyses (using an antisynemin antibody) were performed simultaneously on control and mdx mouse brain sections. In control brain synemin transcript was clearly expressed in the neurons of the midbrain to pons (Fig. 5A), while synemin expression was decreased in the mdx brain (Fig. 5C), suggesting that synemin mRNA expression is at least partially dependent on full-length dystrophin expression. In control mice, immunohistochemical analysis of mirror sections (Fig. 5B) shows that the synemin protein was expressed in the same neurons where synemin transcript was detected using in situ analysis (Fig. 5A). Unexpectedly, immunohistochemical analysis shows that the synemin protein was also expressed in mdx mice brain (Fig. 5D), even though in situ analysis detected an overall decrease in synemin mRNA levels (Fig. 5C).
DISCUSSION

Both mouse α- and β-synemin isoforms are structurally identical to their human orthologs with the exception that intron 4 of β-synemin is 906-bp long in mice and 936 bp in humans. In addition, a third synemin isoform exists in mice and this isoform skips exon 4 of β-synemin, thereby connecting exon 3 to exon 5, which, as a result of a frameshift, encodes a unique 36-amino acid C-terminus. Previous studies have investigated the function of synemin in muscle and have shown that this protein positions to the muscle costamere (the region that connects the plasma membrane to the muscle Z-line) and to neuromuscular and myotendinous junctions. These locations suggest that synemin functions in skeletal muscle as a structural protein maintaining muscle cell integrity during repeated cycles of contraction. In addition to its expression in muscle, synemin is also expressed in brain. In this study, we localize the synemin transcript and its protein in mouse brain to begin understanding the characteristics of this protein in neural tissue.

Sultana et al. previously reported that synemin is expressed transiently in radial glial cells, which develop into mature astrocytes in the rat brain cortex during development. Furthermore, Hirako et al. showed that synemin is expressed in astrocytes of the central nervous system (such as adult bovine and rabbit optic nerve) and in non–myelin-forming Schwann cells of the peripheral nervous system. Although synemin is expressed in some astrocytes, it is not expressed in all of them. For example, tissues from progressive multifocal leukoencephalopathy and from glial scars following stroke showed synemin-positive reactive astrocytes, whereas those from epileptic foci were synemin negative. Since the brain tissues we examined were from normal mice, no reactive astrocytes were likely to be found and therefore our results describe synemin expression in an unaffected system. Our results show that synemin expression is limited to neurons or cells in very restricted regions, with the strongest expression in the midbrain and pons, suggesting a role for synemin in those tissues. A mouse brain atlas shows that synemin-positive structures align in the dorsolateral, lateral, and ventrolateral periaqueductal gray of the midbrain or the laterodorsal tegmental nucleus of pons. This pattern of expression suggests that synemin is expressed in some of the cranial nerves.

In mammalian muscle, both α-synemin and β-synemin are incorporated with desmin into heteropolymeric intermediate filaments. Whether synemin in the midbrain and pons colocalizes with...
other intermediate filament proteins or dimerizes with another synemin isoform is not understood. Given that β-synemin is a smaller splice variant of α-synemin, it is impossible to generate a probe specific to the β-isoform. Although synemin expression patterns were very similar using probe-1 and probe-2 (specific to α-synemin) and probe-3 and probe-4 (common to both synemin transcripts), the intensities of the reactions were different, suggesting that β-synemin dimerizes with α-synemin in different molar ratios. In addition, since astrocytes in optic nerves and non-myelin-forming Schwann cells show synemin, GFAP and vimentin-positive reactions and astrocytes of rabbit spinal cord lack synemin but are GFAP-positive, it would be helpful to understand what proteins might interact with synemin in vivo. Based on its role in skeletal muscle, synemin likely plays a role in maintaining mechanical integrity of neural cells and tissues by connecting type III and IV intermediate filament proteins.

It is tempting to speculate why synemin is most strongly expressed in the neurons of the midbrain and pons and whether synemin interacts with the dystrophin-associated proteins in brain as it does in muscle. β-Synemin was originally identified through a yeast two-hybrid screen using a portion of α-dystrobrevin as the “bait.” In muscle, α-dystrobrevin binds to dystrophin on the intracellular portion of the sarcolemma membrane. Since α-dystrobrevin is also expressed in brain tissue, it is possible that this interaction is maintained in brain. According to Blake et al., α-dystrobrevin-1 is expressed in the vasculature of the isocortex, dentate gyrus, astrocytes, and CA2/CA3 in the hippocampus, and the soma and processes of Bergmann astroglial cells in the cerebellum. Because synemin expression does not always coincide with that reported for α-dystro-

**FIGURE 4.** Colocalization of the synemin transcript and protein in mouse sagittal brain. (B,D,F,H,J,L) In situ experiments hybridized with probe-3, whereas (C,E,G,I,K,M) are mirror sections immunostained with an antisynemin antibody. Light purple shows in situ positive structures and orange indicates those immuno-stained with the antibody. The location of the pons is designated with a “1” in (A) and its respective staining patterns are shown in (B–E). The squares in B and C are shown in D and E, respectively, and show clear colocalization of the synemin transcript and protein. The location of midbrain is designated with a “2” in A and its respective staining patterns are shown in F–I. The squares in F and G are shown in H and I, respectively. The CA3 field of hippocampus is designated with a “3” in A and its respective staining patterns are shown in J–M. The squares in J and K are shown in L and M. Arrows indicate structures positively staining with both the in situ probe and the anti-synemin antibody. Scale bar, 50 μm.
brevin-1 in brain, it is likely that synemin forms a different complex in brain from that observed in skeletal muscle. Furthermore, β-dystrobrevin,14,15 a second member of the dystrobrevin family, is expressed predominantly in granule cells and Purkinje cell somata in the cerebellum, a region where synemin was not expressed, suggesting that β-synemin also does not colocalize with β-dystrobrevin in brain. Using an immunohistochemical analysis, Izmiryan et al.6 recently reported the expression of α- and β-synemin in Purkinje cells in mouse adult brain. In addition, they reported synemin expression in the medulla oblongata, but not in the midbrain or pons. Since our study evaluated synemin expression at both the mRNA and protein levels, we cannot fully explain the differences with their immunohistochemical data, although this could be caused by varying specificities of the two different antibodies used in these studies.

In muscle, synemin is connected to dystrophin through its association with α-dystrobrevin and it is possible that the connection between synemin and dystrophin is maintained either directly or indirectly in brain tissue. To determine whether dystrophin expression is required for synemin localization in brain, in situ analysis was performed for synemin in normal and dystrophic (mdx) mouse brain. Synemin mRNA levels were decreased in the brain of mdx mice (Fig. 5A,C), suggesting that changes in dystrophin expression affect synemin mRNA expression. Unexpectedly, though, the synemin protein did not show the same decrease in expression levels as its mRNA transcript (Fig. 5B,D). There are at least two possible explanations for this observation. First, since the causative dystrophin point mutation19 in mdx mice is upstream of the initiation codon for the smaller dystrophin isoforms (<260 kDa),1,3,5,8,9 it is possible that the dystrophin-short isoforms were able to stabilize the synemin protein in the mdx brain tissue. Second, it is also possible that the antibody used in this study recognizes synemin along with another related protein although previous antibody characterization does not support this explanation.12

In summary, we have identified regions where synemin is expressed in the adult mouse brain. These results show that synemin transcript and pro-

**FIGURE 5.** Synemin expression in control and dystrophin-deficient (mdx) mouse brain. (A,C) In situ experiments hybridized with synemin probe-3, whereas (B,D) are mirror sections immunostained with an anti-synemin antibody. A and B are from control mice, whereas C and D are from mdx mice. Light purple shows in situ positive structures and orange indicates those immunostained with the antibody. Arrows indicate structures positively stained with both the in situ probe-3 and the antisynemin antibody. Scale bar, 50 μm.
tein are not widely distributed in the mouse brain but are limited to expression in localized regions such as the midbrain, pons, hippocampus, and septum. Overall, the number of synemin-positive cells was low in the brain except in the midbrain and pons, suggesting a role for synemin in those regions. Further examination of the role of synemin-positive neurons in mammalian brain development and identification of proteins specifically interacting with synemin in those neurons will contribute to our understanding of synemin’s function in the brain and help ascertain whether mutations in this gene contribute to human disease.

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ABSTRACT: In resting skeletal muscle, endotoxemia causes disturbances in energy metabolism that could potentially disturb intracellular pH (pHi) during muscular activity. We tested this hypothesis using in situ 31P-magnetic resonance spectroscopy in contracting rat gastrocnemius muscle. Endotoxemia was induced by injecting rats intraperitoneally at t0 and t0 + 24 h with Klebsiella pneumoniae endotoxin (lipopolysaccharides at 3 mg/kg) or saline vehicle. Muscle function was investigated strictly noninvasively at t0 + 48 h through a transcutaneous electrical stimulation protocol consisting of 5.7 minutes of repeated isometric contraction at 3.3 Hz, and force production was measured with an ergometer. At rest, endotoxin treatment did not affect pHi and adenosine triphosphate concentration, but significantly reduced phosphocreatine and glycogen contents. Endotoxemia produced both a reduction of isometric force production and a marked linear recovery (0.08 ± 0.01 pH unit/min) of pHi during the second part of the stimulation period. This recovery was not due to any phenomenon of fiber inactivation linked to development of muscle fatigue, and was not associated with any change in intracellular proton buffering, net proton efflux from the cell, or proton turnovers through creatine kinase reaction and oxidative phosphorylation. This paradoxical pH recovery in exercising rat skeletal muscle under endotoxemia is likely due to slowing of glycolytic flux following the reduction in intramuscular glycogen content. These findings may be useful in the follow-up of septic patients and in the assessment of therapies.
induced acid load. These discrepancies might be linked to the extent of the sepsis-induced acid load according to the experimental model parameters such as intensity and duration of septic shock.

In order to further characterize the exact metabolic effects of endotoxemia at the muscle level, we aimed to determine noninvasively, using $^{31}$P-MRS, whether the septic state affects pH$_i$ regulation in contracting rat gastrocnemius muscle, that is, when acid load is greatly increased because of the acceleration of energy metabolism.

**MATERIALS AND METHODS**

**Animal Care and Feeding.** Twenty-six virus-free male Wistar rats (Charles River Laboratories, L’Arbresle, France), weighing 300–325 g, were used for these experiments in accordance with national guidelines. Rats were housed in an environmentally controlled facility (12–12 h light–dark cycle, 22°C), and received water and standard food ad libitum until the time of experiment.

**Experimental Protocol.** Experiment 1 was designed to measure in vitro phosphorylated compound concentration and intramuscular contents of protein and glycogen in resting skeletal muscle using analytical procedures. Animals were randomly assigned to two groups. The first group (endotoxemic, $n = 5$) received two intraperitoneal injections of lipopolysaccharides (LPS) suspension at 3 mg/kg body weight, at $t_0$ and after 24 h ($t_0 + 24$ h). For this purpose, lyophilized LPS from *Klebsiella pneumoniae* (ref. L4268; Sigma-Aldrich Chemie GmbH, Schnell-dorf, Germany) was dissolved in a sterile physiological saline [0.9% NaCl (w/v)]. The second group (control, $n = 5$) received intraperitoneally equivalent volumes of saline vehicle and served as control.

Experiment 2 was designed to investigate muscle function noninvasively in 16 rats randomly assigned to endotoxemic ($n = 8$) or control ($n = 8$) groups, which received LPS or saline vehicle, respectively, at $t_0$ and $t_0 + 24$ h as in Experiment 1.

**Experiment 1: In Vitro Analytical Procedures.** Rats were initially anesthetized in an induction chamber (Equipement Vétérinaire Minerve, Esterenay, France) with 4% isoflurane (Forene; Abbott France, Rungis, France) mixed in 33% O$_2$ (0.5 L/min) and 66% N$_2$O (1 L/min), and were killed by cervical dislocation. Gastrocnemius muscles were immediately dissected free of collagen tissue and surrounding fat and freeze-clamped with liquid nitrogen–chilled metal tongs. These tissues were used for measuring ATP concentration, protein, and glycogen contents in resting muscle.

A first muscle sample (10–20 mg) was homogenized in 1.2 ml of ice-cold 0.6 M perchloric acid with a Polytron PT2100 device (Kinematica AG, Luzern, Switzerland). After incubation for 15 min at 4°C, the homogenate was centrifuged (15 min, 2000 × g, 4°C) and the pellet was dissolved into 1 ml NaOH (1N) for protein calculation according to Lowry et al.$^{38}$ The supernatant was neutralized with K$_2$CO$_3$, placed for 30 min at 4°C, and used for determination of ATP concentration by ion-pairing reverse-phase high-performance liquid chromatography (HPLC). HPLC was carried on an RP$_{18}$ column (5-μm particle size, 250 mm × 4.6 mm i.d.; Merck, Darmstadt, Germany) according to Ally and Park.$^2$ The HPLC system consisted of a Merck-Hitachi L-6200A pump (Merck, Darmstadt, Germany) equipped with a Rheodyne valve and a Merck-Hitachi L-7400 UV-visible detector. Thymidine monophosphate (Sigma, Poole, UK) was used as an internal standard.

Intramuscular glycogen content was determined in another part (350–400 mg) of the freeze-clamped muscle, which was homogenized in cold perchloric acid as mentioned previously. One aliquot (0.2 ml) was neutralized with KHCO$_3$ and glycogen was degraded to glucose with amyloglucosidase [E.C.3.2.1.3] (59.9 U/mg, ref. 10115; Fluka, Buchs, Switzerland) for 2 h at 40°C. The remaining homogenate was centrifuged (15 min, 2000 × g, 4°C) and used for the determination of free glucose. Glycosyl units obtained from glycogen and free glucose were determined using a glucose kit (ref. GL2623; Randox Laboratories, Ltd., Crumlin, UK).

**Experiment 2: In Vivo Investigation of Muscle Function.** Muscle function was investigated 24 h after the second injection (at $t_0 + 48$ h). Body weight was measured at rest before the investigation.

**Preparation.** Rats were initially anesthetized as for Experiment 1. The right lower hindlimb was shaved, and electrode cream was applied at knee and heel levels in order to optimize electrical stimulation. The anesthetized rat was placed in a supine position in a home-built cradle that was specifically designed for the strictly noninvasive functional investigation of the right gastrocnemius muscle.$^{18,19}$ This cradle integrates a hydraulic ergometer and two rod-shaped transcutaneous electrodes connected to an electrical stimulator (Type 215/T; Hugo Sachs Elektronik–Harvard Apparatus GmbH, March-Hugstetten, Germany), so that, when the rat is placed inside, one electrode is located above the knee and
the other at the heel. The foot was positioned on the pedal of the ergometer and the hind limb was immobilized in the cradle. In this position, the belly of the gastrocnemius muscle was located above an elliptic (10 × 16 mm) 31P-MRS surface coil. The gastrocnemius muscle was passively stretched at rest by adjusting the pedal position so as to modify the angle between the foot and the lower hind limb in order to give maximum isometric twitch tension in response to supramaximal square-wave pulses (6–8 mA, 1 ms duration). Throughout the experiment, anesthesia was maintained by gas inhalation with a facemask, which was continuously supplied with 2.5% isoflurane in 33% O2 (0.4 L/min) and 66% N2O (0.8 L/min). Corneas were protected from drying by application of ophthalmic cream (Lacrigel; Europhtha, Monaco). The facemask was connected to an open-circuit gas anesthesia machine (Isotec 3; Ohmeda Medical, Herts, UK). Exhaled and excess gases were removed through a canister filled with activated charcoal (Smiths Industries Medical System, Shefield, UK) mounted on an electrical pump extractor (Equipement Vétérinaire Minerve, Eternany, France). During anesthesia, the animal’s body temperature was maintained using an electric heating blanket (Prang+Partner AG, Pfungen, Switzerland) in a feedback loop with a temperature-control unit (ref. 507137; Harvard Apparatus, Holliston, Massachusetts) connected to a rectal probe (ref. 507145; Harvard Apparatus).

Stimulation Protocol and Force Measurement. The stimulation protocol consisted of repeated isometric contractions for 5.7 min at 3.3 Hz, electrically induced with square-wave pulses (6–8 mA, 1 ms duration). Electrical signal from the pressure transducer was amplified, converted to a digital signal, and processed on a personal computer using ATS software (Sysma, Aix-en-Provence, France). Isometric force was calculated every 14.25 s of stimulation by integrating isometric tension (in N) relative to time, and was expressed as tension–time integral (in N.s). Isometric force production per twitch (N.s/twitch) was calculated by scaling isometric force to the stimulation frequency.

MR Spectroscopy and Data Processing. Investigations were performed in a 4.7-Tesla horizontal superconducting magnet (47/30 Biospec Avance; Bruker, Germany). 31P-MRS spectra (30-μs rectangular pulse, 16 accumulations, 1.8-s repetition time, 8-KHz spectral width, 512 data points) from the gastrocnemius muscle region were continuously acquired in 28.5-s blocks throughout the experimental protocol, which consisted of 5.7 min of rest, 5.7 min of stimulation, and 16.6 min of recovery. Magnetic resonance data acquisition was synchronized to muscle stimulation in order to reduce motion artifacts due to contraction. MR data were processed using a proprietary software developed using IDL (Interactive Data Language, Research System, Inc., Boulder, Colorado). Relative concentrations of PCr and P i were obtained by a time-domain fitting routine using the AMARES-MRUI Fortran code.45 Signal areas were corrected for magnetic saturation effects using fully relaxed spectra collected at rest with a repetition time of 20 s. Intramuscular pH (pHi) was calculated from the chemical shift of P i relative to PCr,6 and cytosolic free [Mg2+] was assessed from the chemical shift of β-ATP.24 Absolute concentrations of phosphorylated compounds were expressed relative to a resting ATP concentration measured in extracts of freeze-clamped gastrocnemius muscles from control and endotoxemic animals (see Experiment 1). Time-points for the time-course of pH i and phosphorylated metabolite concentrations were assigned to the midpoint of the acquisition interval.

Calculations. The apparent buffering capacity (βtotal in slykes, millimoles of acid added per unit change in pH i) was calculated during the stimulation period taking account the buffering capacities of P i (βp i) and of tissue (βtissue): βtotal = βp i + βtissue. Calculation of βp i was done using [P i] with a pKα of 6.7550: βp i = 2.3[P i]/((1 + 10(pH i − 6.75))(1 + 10(6.75 − pH i))). It has been demonstrated that βtissue varies linearly between pH 7 (16 slykes) and pH 6 (37 slykes) in rat gastrocnemius muscle.1 According to these data, βtissue was calculated as follows: βtissue = −21pH i + 163.

The rate of net proton efflux (Veff, in mM/min) during the stimulation period was calculated with the following relationship: Veff = λΔpHi. The proportionality constant, λ [in mM/min · (pH unit)−1], which is the apparent proton efflux capacity (proton removal out of the cell by passive flux and transport systems) relating V eff to pH i, was calculated at the start of the poststimulation recovery period: λ = −V eff/ΔpHi, as previously described.16,31,48 During this period, PCr is regenerated throughout the creatine kinase (CK) reaction as a consequence of oxidative ATP synthesis in mitochondria, and Veff can be calculated from proton turnover linked to CK reaction (HCr in mM/min) and mitochondrial ATP synthesis (H Ox in mM/min), considering lactate production as negligible: Veff = HCr + H Ox + βtotaldpHi/ dt. HCr was calculated from the stoichiometric coefficient, ϕ = 1/(1 + 10(pH i − 6.75)), representing the number of protons associated with PCr degradation and resynthesis, and from the time-dependent changes in [PCr]: HCr = ϕdPCr/dt. H Ox
was calculated from the factor $m = 0.16/(1 + 10^{(6.1 - \text{pH})})$, which is the amount of protons produced in association with oxidative phosphorylation, and from the rate of oxidative ATP synthesis assuming to be equal to the initial rate of PCr resynthesis ($\text{VPCr}_{\text{rec}}$ in mM/min) at the start of the poststimulation period\(^3\): $H_{\text{ox}} = m\text{VPCr}_{\text{rec}}$. $\text{VPCr}_{\text{rec}}$ was calculated as $\text{VPCr}_{\text{rec}} = k[\text{PCr}]_{\text{cons}}$. The pseudo–first-order rate-constant of PCr recovery ($k$) was estimated by fitting the PCr recovery profile to a monoexponential function described by the equation: $[\text{PCr}]_i = [\text{PCr}]_{\text{rest}} + [\text{PCr}]_{\text{cons}} e^{-kt}$, where $[\text{PCr}]_{\text{rest}}$ is the concentration of PCr measured at rest and $[\text{PCr}]_{\text{cons}}$ indicates the amount of PCr consumed at the end of the stimulation period.

**Statistics.** For variables evolving with time during the stimulation period (force production, metabolite concentration, and pH), the effect of endotoxemia on the overall time-course was tested with one-way repeated-measures analyses of variance (ANOVAs) using JMP software (SAS Institute, Inc., Cary, North Carolina) with post hoc testing (Fisher’s test) to localize significant differences at each time-point. Other variables were compared using two-tailed Student’s $t$-test. Values are presented as mean $\pm$ SEM. $P < 0.05$ was considered statistically significant.

**RESULTS**

**Behavior and Physiological Changes.** Endotoxin treatment induced a number of pathological features such as decreased sensitivity to touch, piloerection, behavioral depression, lethargy, and diarrhea. Endotoxemia further reduced muscle protein content by around 34% (Table 1) and body mass by 9.0$\pm$1.8%. Body mass was 303$\pm$5 g at $t_0 - 48$ h and 323$\pm$4 g at $t_0 + 48$ h for the control group, and 315$\pm$3 g at $t_0 - 48$ h and 288$\pm$3 g at $t_0 + 48$ h for the endotoxic group.

**Glycogen Content.** Resting gastrocnemius glycogen content was significantly reduced by 54% in endotoxic animals when compared with controls (Table 1).

**Muscle Performance.** In both groups, isometric force per twitch decreased progressively throughout the stimulation period as a sign of muscle fatigue development (Fig. 1). At the end of the stimulation period, the extent of isometric force decrease did not differ between the two groups, reaching 43.5 $\pm$ 3.1% and 40.1 $\pm$ 4.6% of the initial value for control and endotoxic groups, respectively. However, when compared with control, one-way repeated-measures ANOVAs showed that endotoxin treatment significantly reduced isometric force production throughout the stimulation period.

**Intramuscular pH.** Endotoxemia did not disturb pH in resting muscle. During this period, pH averaged 7.12 $\pm$ 0.01 in the control group and 7.08 $\pm$ 0.02 in the endotoxic group. By contrast, endotoxemia strongly affected the pH time-course during stimulation and poststimulation periods (Fig. 2). In both groups, pH remained unchanged during the first 15 s of stimulation and declined rapidly afterward. For control animals, pH time-course reached a steady-state after stimulation for 3 min. This steady state remained fairly constant until the end of the stimulation period, when pH was 6.47 $\pm$ 0.03. For the endotoxemic group, the pH time-course was characterized between 2 and 3 min of stimulation by a transient steady-state (average: pH 6.55) that was followed by a phase with a paradoxical net pH recovery. This recovery occurred linearly at a rate of 0.08 $\pm$ 0.01 pH unit/min until the end of the stim-

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**Table 1.** In vitro measurements in resting gastrocnemius muscle.

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<th>Control</th>
<th>Endotoxemia</th>
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<tr>
<td>Muscular protein (mg/ml wet wt)</td>
<td>62 $\pm$ 6</td>
<td>41 $\pm$ 7*</td>
</tr>
<tr>
<td>Glycogen (µmol/g)</td>
<td>20.5 $\pm$ 2.0</td>
<td>9.5 $\pm$ 0.5*</td>
</tr>
<tr>
<td>Intramuscular ATP (mM)</td>
<td>5.12 $\pm$ 0.17</td>
<td>5.46 $\pm$ 0.23</td>
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Values are presented as mean $\pm$ SEM. *Significantly different from control value ($P < 0.05$).
ulation period, when pH was 6.81 ± 0.02 and the extent of pH recovery was around 48%. During the poststimulation period, pH returned progressively to its basal value in both groups. For the endotoxemic group, this return was preceded by a transient phase of net acidosis at the start of the poststimulation period.

Cytosolic free [Mg^{2+}]. The time-course of cytosolic free [Mg^{2+}] was not affected by endotoxin treatment (Fig. 3A). In both groups, cytosolic free [Mg^{2+}] remained fairly constant throughout the stimulation protocol. The paradoxical and net pH recovery in the second part of the stimulation period was still observed in endotoxemic animals when intramuscular pH was calculated according to the equation taking into account [Mg^{2+}] (Fig. 3B).

Proton Efflux and Buffer Capacities. For control animals, $\beta_p$, $\beta_{tissue}$, and $\beta_{total}$ increased in the first part of the stimulation period to reach a steady state during the second part, which was maintained until the end of the stimulation period (Fig. 4). Endotoxemia did not affect $\beta_p$ (Fig. 4A), but significantly disturbed the time-course of both $\beta_{tissue}$ and $\beta_{total}$. When compared with control, $\beta_{tissue}$ and $\beta_{total}$ were higher in the first part of the stimulation and lower in the second part (Fig. 4B and C). Endotoxemia did alter the proton efflux capacity (Table 2).

Phosphorylated Compounds. At rest, ATP concentration measured in vitro using HPLC did not differ between the two groups (Table 1). These concentrations were used for calculation of absolute concentrations of phosphorylated compounds measured with $^{31}$P-MRS. In the control group, concentrations were 15.6 ± 0.5 mM for PCr (Fig. 5A) and 1.0 ± 0.1 mM for $P_i$ (Fig. 5B); endotoxemia caused a significant decrease in PCr concentration, which was 13.8 ± 0.5 mM (Fig. 5A), whereas $P_i$ content ($[P_i]$ = 0.9 ± 0.2 mM) remained unchanged (Fig. 5B). Similarly, during the stimulation period, endotoxemia altered the PCr concentration time-course (Fig. 5A), but did not affect that of $[P_i]$ (Fig. 5B). Under control and endotoxemic conditions, PCr was rapidly consumed at the onset of the stimulation period and reached a steady state after stimulation for 1.5 min. This steady state was maintained until the end of the stimulation period, when PCr levels were 42.3 ± 2.2% and 39.4 ± 1.6% of their basal values under control and endotoxemic conditions, respec-

**FIGURE 2.** Changes in intramuscular pH during 5.7 min of repeated isometric contractions induced electrically at 3.3 Hz and during poststimulation recovery in gastrocnemius muscle of control and endotoxin-injected animals. The first time-point (t = 0) indicates the resting value. Values are presented as mean ± SEM. $P$-value indicates the result of one-way repeated-measures ANOVA used to test the effect of infection on the overall time-course. *Significant difference between control and endotoxemia values at the same time ($P < 0.05$).

**FIGURE 3.** Changes in cytosolic free [Mg^{2+}] (A) and cytosolic pH (B) during 5.7 min of repeated isometric contractions induced electrically at 3.3 Hz in gastrocnemius muscle of control and endotoxin-injected animals. Cytosolic pH was calculated according to the equation that takes into account [Mg^{2+}]. The first time-point (t = 0) indicates the resting value. Values are presented as mean ± SEM. $P$-value indicates the result of one-way repeated-measures ANOVA used to test the effect of infection on the overall time-course. *Significant difference between control and endotoxemia values at the same time ($P < 0.05$).
tively. Further, the changes in PCr turnover (dPCr/dt) throughout the stimulation period were strongly similar under both conditions (Fig. 5C). During the poststimulation period, endotoxemia significantly increased both the initial rate and the constant rate of PCr recovery (Table 2). In both conditions, the time-course of [Pi] exhibited an initial phase of rapid and massive accumulation followed by a steady-state phase (Fig. 5B).

DISCUSSION

Our major finding is that endotoxemia causes a paradoxical intracellular pH recovery during repeated isometric contractions. Endotoxin, which constitutes the outer membrane of Gram-negative bacteria, is known to play a central role in the development of septic shock, and has been widely used in animal models of sepsis. As expected, 24 hours after the second injection, each rat displayed clinical signs of sepsis, including decreased sensitivity to touch, lethargy, and marked loss of body mass. Endotoxin treatment further induced a reduction in force production throughout the stimulation period, which is consistent with previous studies showing that injection of *Klebsiella pneumoniae* endotoxin reduces force-generating capacity in isolated rat epitrochlearis muscle and shortens running distance in rats undergoing treadmill tests.

In resting muscle, endotoxemia caused a depletion in PCr store, but did not affect the basal ATP concentration. These findings accord with previous biochemical and in vivo 31P-MRS studies, and indicate that muscle ATP level is maintained at the expense of PCr stores in the septic state. A decline in PCr content is usually observed in the early stage of muscle ischemia when it is associated with intracellular acidosis. Cellular hypoxia and abnormal microvascular control of oxygenation have been reported in endotoxemic rats, suggesting that the net endotoxemia-induced PCr store depletion could be due to a lack of oxygen. This hypothesis can be dismissed, however, because we did not find any concomitant intramuscular acidosis in endotoxemic animals. Sepsis-induced mitochondrial

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**Table 2.** Proton efflux capacity and PCr resynthesis during the poststimulation recovery period.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Endotoxemia</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda$ [mM/min · (pH unit)$^{-1}$]</td>
<td>3.82 ± 0.68</td>
<td>2.72 ± 1.45</td>
</tr>
<tr>
<td>$V_{PCr_{rec}}$ [mM/min]</td>
<td>0.33 ± 0.01</td>
<td>0.48 ± 0.05 *</td>
</tr>
<tr>
<td>$k_{rec}$ [min$^{-1}$]</td>
<td>2.3 ± 0.2</td>
<td>3.5 ± 0.4 *</td>
</tr>
</tbody>
</table>

$\lambda$, proton efflux rate constant calculated as the ratio between proton efflux and the extent of intracellular acidosis measured at end of exercise; $V_{PCr_{rec}}$, initial rate of PCr resynthesis at the start of the poststimulation period; $k_{rec}$, rate constant of PCr recovery. Values are presented as mean ± SEM. * Significantly different from control value ($P < 0.05$).
dysfunction could account for this basal PCr content
duction, but given that the rate of PCr resynthesis
during the poststimulation period (an index of
oxidative phosphorylation activity) was increased
during endotoxemia, this explanation is unlikely.
It remains possible that this PCr reduction could be
linked to the acceleration of the basal glycolytic
flux. In addition, considering that both the
rate constant and the initial rate of PCr recovery
were increased with endotoxemia, we suggest an
upward shift of the relationship between the mito-
chondrial rate of ATP production and adenosine
diphosphate (ADP; the error signal in the feedback
loop controlling aerobic ATP production) rather
than a simple increase in activation.

Resting pHi is strongly dependent on the balance
between the acid load resulting from cellular energy
metabolism and buffering processes such as P;
accumulation and PCr degradation. In addition to PCr
store depletion, the septic state has been character-
ized by an acceleration of basal glycolytic flux in
response to the enhancement of sarcolemmal Na–
K–ATPase activity due to increased release of epi-
nephrine. This acceleration of glycolysis
would lead to an increased accumulation of protons
in the cytosol given that, when coupled with ATP
hydrolysis, glycolytic ATP production is linked to a
net proton generation with a stoichiometry of 1.5
moles of ATP per proton. Indeed, the degrada-
tion of each mole of glycosyl unit generates 3 moles
of ATP that, when hydrolyzed by cell ATPase, causes
a net yield of 2 moles of protons. The absence of a
pHi disturbance in endotoxemic resting muscle is
consistent with previous in vivo 31P-MRS studies in
septic rats and indicates that the mechanisms
implied in pH regulation at rest, specifically the
continuous activity of membrane-bound transporters
ejeciting protons out of the cell, are either not
affected or can adapt to a sepsis-induced increase in
acid load.

In the early stage of the stimulation period, pH
was significantly lower in endotoxemic animals, sug-
gesting an initial increase in glycolysis flux. This
increase could result from greater activation of gly-
cogenolysis and glycolytic pathways as a conse-
quence of the septic-induced acceleration of basal glycolytic
flux. This transient increase was followed by a net
recovery of pHi at a linear rate of 0.08 pH
unit/min. It might be that this paradoxical recovery
phenomenon is linked to the reduction in muscle
performance that we measured throughout the stim-
ulation period. During muscle activity under normal
conditions, pHi decreases to reach a steady state
after a few minutes, and the acidosis of this steady

FIGURE 5. Changes in [PCr] (A), [P] (B), and PCr turnover rate
throughout the creatine kinase reaction (C) during 5.7 min of
repeated isometric contractions induced electrically at 3.3 Hz in
gastrocnemius muscle of control and endotoxin-injected animals.
The first time-point (t = 0) indicates the resting value. Values are
presented as mean ± SEM. P-value indicates the result of one-
way repeated-measures ANOVA used to test the effect of infec-
tion on the overall time-course. *Significant difference between
control and endotoxin values at the same time (P < 0.05).
state increases with force production.\textsuperscript{15,49} Thus, considering the lesser force generation during endotoxemia, a reduced acidosis would be expected, but not a net paradoxical pH\textsubscript{i} recovery.

Net pH\textsubscript{i} recovery is commonly observed during the postactivity period, when muscle ceases to produce mechanical work.\textsuperscript{6,17} In that case, the return of pH\textsubscript{i} toward basal values is mediated by proton expulsion from the cell, mainly through lactate/proton cotransport and the Na\textsuperscript{+}/H\textsuperscript{+} exchange system.\textsuperscript{28} Paradoxical pH\textsubscript{i} recovery in contracting muscle has been previously observed in rat gastrocnemius muscle and has been linked to a partial inactivation of muscle fibers.\textsuperscript{17,35} Rat gastrocnemius muscle is composed of fast- and slow-twitch fibers; during intense or prolonged muscular activity, some of the fast-twitch fibers, which are less fatigue-resistant than slow-twitch fibers, could be inactivated as the consequence of fatigue development and thus shift to the recovery phase as they are unable to contribute further to force production.\textsuperscript{17,35} Nevertheless, it is noteworthy that the arrest of fiber contraction is accompanied by PCr replenishment through mitochondrial ATP production.\textsuperscript{5} In the present study, the net paradoxical pH\textsubscript{i} recovery during the stimulation period was not associated with any significant PCr resynthesis, suggesting that it may not be linked to fiber inactivation.

A possible difference in [Mg\textsuperscript{2+}] between the two rat population could affect the pattern of intramuscular pH given the relationship between Mg\textsuperscript{2+} and pH.\textsuperscript{24} This possibility can, however, be excluded in the present study because the time-course of [Mg\textsuperscript{2+}] did not differ significantly between groups. In fact, in the endotoxemic animals, the paradoxical and net pH recovery in the second part of the stimulation period was still present when intramuscular pH\textsubscript{i} was calculated according to the equation taking [Mg\textsuperscript{2+}] into account.

In contracting muscle, pH\textsubscript{i} is influenced by the balance between various processes accounting for proton removal, proton consumption, and proton production. Protons are removed from the cytosol via intracellular buffers and net proton efflux out of the cell\textsuperscript{13,29} and are consumed by PCr degradation throughout the CK reaction: PCr + ADP + H\textsuperscript{+} → ATP + creatine. Proton-producing reactions are PCr resynthesis throughout the CK reaction (if any during the stimulation procedure), oxidative phosphorylation, and anaerobic glycolysis. Alteration in pH\textsubscript{i} regulation under the septic state in contracting gastrocnemius muscle should then result from an imbalance among these processes.

Any possible effect of endotoxemia on proton removal can be discarded directly. For most of the stimulation period, the apparent buffer capacity was lower in the endotoxemic group and not higher, as would be required to explain the net pH recovery. We have calculated the apparent buffer capacity taking into account the buffer capacities of P\textsubscript{i} and tissue. Whereas the buffering capacity of P\textsubscript{i} was directly calculated from the P\textsubscript{i} concentration and pH\textsubscript{i},\textsuperscript{31} the buffering capacity of tissue was estimated from the data of previous homogenate titrations in gastrocnemius muscle from normal rats.\textsuperscript{1} Tissue buffering capacity is mainly handled by the imidazole groups of histidine residues in proteins.\textsuperscript{13} The septic state causes an enhancement of protein catabolism leading to net protein breakdown,\textsuperscript{9} and we found that the muscle protein content was decreased in endotoxemic animals by $\sim 30\%$ compared with control. Accordingly, our estimation of the tissue buffering capacity during endotoxemia could be distorted because of the reduction in muscle protein content and the concomitant liberation of amino acids in the cytosol. Protein content reduction should indeed cause a decrease in the available imidazole groups of histidine, thus leading to a reduction in tissue buffering capacity that could not account for the net pH\textsubscript{i} recovery. Besides, it is unlikely that liberated amino acids play a role in the buffering capacity given that they are rapidly released into the bloodstream.\textsuperscript{22,39} Consequently, although it cannot be excluded, a massive and physicochemically bizarre change in the non-P\textsubscript{i} cytosolic buffer capacity at rest and during the stimulation period is unlikely to have occurred during endotoxemia.

Previous results suggest that sepsis affects proton efflux. In contracting muscle, lactate is exported into the bloodstream through lactate/proton cotransport and passive diffusion of undissociated lactic acid.\textsuperscript{28–30} In this context, increased lactate production during sepsis could affect pH\textsubscript{i} regulation. Further, septic shock causes widespread damage to the myofiber membrane,\textsuperscript{14} which could cause proton leak across the membrane. We have estimated the proton efflux capacity of the gastrocnemius muscle from metabolic data, as done previously.\textsuperscript{16,31,48} Considering that the proton efflux rate constant ($\lambda$) did not differ significantly between control and endotoxemic conditions, we can exclude a direct effect of endotoxemia on the proton efflux capacity. Therefore, net pH\textsubscript{i} recovery in contracting muscle during endotoxemia is not linked to changes in proton efflux.

Considering that endotoxemia did not alter proton removal, net pH\textsubscript{i} recovery during the stimulation...
period should be due to an imbalance between proton production and consumption coupled with energy production. Throughout the stimulation period, changes in PCr turnover did not differ significantly between the two groups, thereby indicating that proton consumption through the CK reaction did not account for the net pH recovery. Besides, mitochondrial ATP production is a proton-generating process and its slowing would limit acid load during muscle stimulation, thus contributing to pH recovery. In any event, such a possibility can be dismissed as we found an upward setting of mitochondrial function in endotoxemic animals.

By exclusion, the net pH recovery in contracting muscle during endotoxemia should be due to decreased proton production related to a slowing of glycolytic ATP production. We found that basal gastrocnemius glycogen content was significantly reduced by 54% in endotoxemic animals compared with controls. Our finding is consistent with previous studies in patients and animals reporting that the septic state decreases basal glycogen stores by around twofold.\textsuperscript{3,47} This depletion plausibly results from the similar rates of PCr degradation. The later period should be due to an imbalance between proton production and consumption coupled with energy production. Throughout the stimulation period, changes in PCr turnover did not differ significantly between the two groups, thereby indicating that proton consumption through the CK reaction did not account for the net pH recovery. In any event, such a possibility can be dismissed as we found an upward setting of mitochondrial function in endotoxemic animals.

Another point of interest in the present study is that force production measured at the end of the stimulation period was similar, whereas intramuscular acidosis was significantly lower in endotoxemic animals compared with controls. These results support the hypothesis that acidosis has no or little direct effect on the development of muscle fatigue.\textsuperscript{41,49} In addition, it also illustrates the absence of any abnormality in contractile efficiency considering the similar rates of PCr degradation. The later fall in tension in the endotoxemic animals is consistent with a later fall in total ATP turnover.

In conclusion, endotoxin-induced sepsis state causes a paradoxical recovery of intracellular pH in contracting rat skeletal muscle. This net recovery is very likely due to a decrease in anaerobic glycolysis activity following the reduction in intramuscular glycogen stores. Given that we found no alteration in proton transport or buffering capacity, this slowed glycolytic ATP production could be physiologically consistent with either a decrease in ATP demand or an increase in aerobic ATP synthesis. We suggest that this glycolytic energy reduction is compensated by an upward setting of mitochondrial function rather than simply increased activation. This work was supported by grants from CNRS (UMR 6612), from AFM (Association Francaise contre les Myopathies), from the French Interdisciplinary Small Animal Imaging Program (CEA-CNRS), and from the ACI’s “Plates-Formes d’Explorations Fonctionnelles Thématisées” Program.

REFERENCES

ABSTRACT: The magnitude of failure in voluntary drive after fatiguing contractions of different intensities in men and women is not known. The purpose of this study was to compare the time to task failure and voluntary activation of men and women for a sustained isometric contraction performed at a low and high intensity with the elbow flexor muscles. Nine men and nine women sustained an isometric contraction at 20% and 80% of maximal voluntary contraction (MVC) force until task failure during separate sessions. The men had a shorter time to failure than women for the 20% but not the 80% MVC task. Voluntary activation was reduced to similar levels for the men and women at the end of the fatiguing contractions but was reduced less after the 80% MVC task than the 20% MVC contraction. Twitch amplitude was reduced similarly at task failure for both sexes and to similar levels at termination of the 20% and 80% MVC tasks. The rate of change in mean arterial pressure was the main predictor of time to failure for the low-force sustained contraction. These results suggest that women experienced greater muscle perfusion, less peripheral fatigue, and a longer time to task failure than men during the low-force fatiguing contraction. However, the low-force task induced greater central fatigue than the high-force contraction for both men and women. Thus, low-force, long-duration fatiguing contractions can be used in rehabilitation to induce significant fatigue within the central nervous system and potentially greater neural adaptations in men and women.

MECHANISMS OF FATIGUE DIFFER AFTER LOW- AND HIGH-FORCE FATIGUING CONTRACTIONS IN MEN AND WOMEN

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When an individual maintains a submaximal fatiguing contraction, failure of the task occurs due to a reduced force capacity of the muscle that involves both neural and muscular mechanisms.14,44 The processes that contribute to task failure depend on the details of the task performed including the type and intensity of contraction and the sex of the individual.8,14,18,24 For example, low-force contractions can be sustained longer than high-intensity contractions with greater contributions of neural mechanisms for the low-force tasks.3,30 Neural mechanisms and the magnitude of central fatigue can be quantified as a reduction in voluntary activation. However, few studies have directly compared the magnitude of change in voluntary activation for a high- and low-intensity contraction when sustained to failure. Voluntary activation is often quantified as the extra force evoked when the motor nerve is stimulated during maximal contractions. This extra force implies a failure of voluntary drive at one or more sites proximal to the motor nerve and therefore within the central nervous system. A failure in voluntary activation during maximal efforts indicates that the level of neural drive to the muscle is suboptimal14 because either the motor units are not all recruited voluntarily or they are discharging at an insufficient rate to produce full fusion of force.17

Women can maintain steady low-force fatiguing contractions longer than men.5,20,26,48 However, the sex difference in time to failure and muscle fatiga-
bility is task-dependent, such that the magnitude and mechanism for sex differences varies according to the muscle being assessed and the contraction type and its intensity.5,22,23,37 For the elbow flexor muscles, reduction in voluntary activation was similar for men and women during maximal contractions when assessed by stimulating the motor cortex despite less fatigue experienced by the women.25 The site of failure of voluntary drive therefore was at or above the level of motor cortical output41,44,45 and was similar for men and women.25 It is unknown, however, whether there are sex differences in voluntary activation that originate between the motor cortex and neuromuscular junction and contribute to the sex differences in muscle fatigue.

One purpose of this study, therefore, was to compare the time to task failure and voluntary activation assessed at the peripheral motor nerve and muscle for men and women after a fatiguing contraction performed with the elbow flexor muscles. We examined these effects for both a low-force and high-force fatiguing contraction because the relative contribution of failure in voluntary drive for the different tasks is not known for men and women. We hypothesized that the time to task failure would be greater for women than men for the low-force contraction but similar for the sexes for the high-force contraction and that the reduction in voluntary drive would be similar at task failure for both sexes and contraction intensities. Additional measures including torque fluctuations, electromyographic activity (EMG), mean arterial pressure (MAP), heart rate, and rating of perceived exertion (RPE) were recorded to determine the physiological adjustments and provide evidence for any difference in rate of change in central and peripheral mechanisms during the fatiguing tasks.

**MATERIALS AND METHODS**

Eighteen young adults (nine women, nine men; 21–33 years of age) were recruited for this study. All subjects were healthy, with no known neurological or cardiovascular diseases, and were naive to the protocol. Prior to participation in the study each subject provided informed consent and the protocol was approved by Institutional Review Board.

The physical activity level for each subject was assessed with a questionnaire that estimated the relative kiloccalorie expenditure of energy per week.28 All subjects were right-handed (0.71 ± 0.1 vs. 0.72 ± 0.2 for men and women, respectively, with a ratio of 1 indicating complete right-handedness) as estimated with the Edinburgh Handedness Inventory.

Subjects reported to the laboratory on three occasions: once for a familiarization session and then for two experimental sessions that were 7–10 days apart to perform a protocol that focused on a fatiguing contraction with the elbow flexor muscles of the nondominant arm. In each experimental session the fatiguing contraction involved maintaining a force that was equivalent to 20% or 80% of the maximal voluntary contraction (MVC) force for as long as possible. The order of the experimental sessions was randomized for each subject. Five men and three women performed the 20% MVC task during their first experimental session.

**Mechanical Recording.** Subjects were seated upright in an adjustable chair with the nondominant arm abducted slightly and the elbow resting on a padded support. The elbow joint was flexed to 90° so that the forearm was horizontal to the ground and the force at the wrist was directed upward when the elbow flexor muscles were activated during a voluntary contraction. Two nylon straps were placed vertically over each shoulder to restrain the subject and minimize shoulder movement. The hand and forearm were placed in a modified wrist-hand-thumb orthosis (Orthomerica, Newport Beach, California) and the forearm was placed midway between pronation and supination. The force exerted by the wrist in the vertical direction was measured with a transducer (JR-3 Force-Moment Sensor; JR-3 Inc., Woodland, California) that was mounted on a custom-designed, adjustable support. The orthosis was attached to the force transducer. The force detected by the transducer was recorded online using a Power 1401 A-D converter and Spike2 software (CED, Cambridge, UK). The force exerted was displayed on a 19-inch monitor with an oscilloscope display located at eye level and 1.5 m in front of the subject. The force was adjusted for each subject so a horizontal cursor that represented the required target force was displayed at ~60% the height of the screen. Each subject was asked to trace the horizontal cursor with the force signal for as long as possible. The force signal appeared on the screen from the right side of the monitor at 2.5 cm/s.

In addition, the force exerted under the elbow joint was measured with a load cell (YB6; Sentran, Ontario, California) placed under the padded elbow support. The force under the elbow joint was monitored to ensure that each subject placed and maintained the elbow on the padded support. The force was displayed on an oscilloscope and recorded online with Spike2 software (CED, Cambridge, UK) at 500 samples/s.
**Electrical Recordings.** EMG signals were recorded with bipolar surface electrodes (Ag–AgCl, 8-mm diameter; 16 mm between electrodes) that were placed over biceps brachii, brachioradialis, and triceps brachii muscles. Reference electrodes were placed on a bony prominence at the elbow. The EMG signal was amplified (×100) and bandpass-filtered (13–1000 Hz) with Coulbourn modules (Coulbourn Instruments, Allentown, Pennsylvania) prior to being recorded directly to computer with the Power 1401 A-D converter and Spike2 software (CED). The EMG signals were digitized at 2000 samples/s.

**Cardiovascular Measurements.** Heart rate and blood pressure were monitored during the fatiguing contractions because these adjustments involve central and peripheral processes. Both heart rate and blood pressure were monitored with an automated beat-by-beat blood pressure monitor (Finapres 2300; Ohmeda, Madison, Wisconsin). The blood pressure cuff was placed around the middle finger of the relaxed, dominant hand with the arm placed on a table adjacent to the subject at heart level. The blood pressure signal was recorded online to computer at 500 samples/s.

**Electrical Stimulation.** Electrical stimulation of muscle was used to evoke force in the biceps brachii muscle to assess voluntary activation during an MVC. The stimulating cathode was placed over the biceps brachii (midway between the anterior edge of the deltidoid and antecubital fossa) and an anode was placed over the bicipital tendon (2 cm proximal to the elbow). Activation of the muscle was achieved by a constant-current stimulator (Digitimer DS7AH, Welwyn Garden City, UK) that delivered a rectangular pulse of 100-μs duration and at a maximal amplitude of 400 V. The stimulation intensity (∼200 mA to 500 mA) was set to 10% above the level required to produce a resting twitch of maximal amplitude so that the level of stimulation was supramaximal. This level of stimulation was used for the remainder of the protocol. Control twitches were evoked at rest and when the muscle was potentiated with an MVC task. To assess voluntary activation, a single twitch was interpolated during the plateau of the MVC by the constant-current stimulator as determined by the investigator. This occurred during the MVCs performed before and immediately after the fatiguing contraction.

**Experimental Protocol.** The protocol for each experimental session comprised the following procedures: (1) determination of supramaximal levels of electrical stimulation, (2) assessment of MVC torque and voluntary activation for the elbow flexor muscles, (3) performance of an MVC of the elbow extensor muscles, (4) brief submaximal isometric contractions of the elbow flexor muscles to determine the EMG–force and voluntary activation–torque relations, (5) performance of a fatiguing contraction at either 20% or 80% MVC force, immediately followed by (6) a twitch contraction, a recovery MVC with the elbow flexor muscles, and another twitch contraction.

**MVC Torque and Voluntary Activation.** Each subject performed four MVC trials with the elbow flexors, followed by two trials with the elbow extensor muscles. The MVC task involved a gradual increase in force from zero to maximum over ∼2 s, with the maximal force held for 2–3 s. The force exerted by the wrist was displayed on a monitor and each subject was verbally encouraged to achieve maximal force. MVCs with the elbow extensor muscles were performed so that maximal EMG could be recorded and used to normalize the triceps EMG activity during the fatiguing contractions. For the elbow flexor muscles, a single electrical stimulus at the predetermined supramaximal level was delivered once the force was at a plateau and also 3 s after termination of the MVC while the muscle was at rest. Pilot data on subjects indicated that there was no difference in voluntary activation levels when using a single or paired pulse (10 ms apart). These results are consistent with those previously reported. There was a 60-s rest between MVC trials. When the peak forces from two of the three trials were not within 5% of each other, additional trials were performed until this was accomplished. The greatest torque achieved by the subject was taken as the MVC torque and used as the reference to calculate the target level for the constant-force and fatiguing contractions of the elbow flexor muscles.

**Brief Submaximal Contractions.** The EMG activity of the involved muscles was recorded in standardized tasks so that the force–EMG relation could be compared across experimental days. These relations for the men and women were examined to ensure that changes in EMG during the fatiguing contractions and at task failure represented physiological adjustments and were not due to differences in recording conditions across the experimental days. The subjects performed a sustained constant-force contraction with the elbow flexor muscles for 6 s at target values of 20%, 40%, 60%, and 80% MVC force with 60-s rest between each contraction. A single electrical stimulus was delivered to the biceps brachii muscle during and after the brief contractions to assess
voluntary activation levels during the submaximal tasks. The order of the contractions was randomized across subjects, but remained constant for each subject on the two experimental days.

**Fatiguing Contraction.** A fatiguing contraction was performed with the elbow flexor muscles in each experimental session at a target value of either 20% or 80% MVC force. The subject was required to match the vertical target force as displayed on the monitor and was verbally encouraged to sustain the force for as long as possible. The fatiguing contraction was terminated when the force declined by 10% of the target value for 3 of 5 s despite strong verbal encouragement to maintain the force. This time was recorded as the time to task failure. Subjects were not informed of their time to task failure until completion of the second session. Neither the subject nor the investigator who terminated the task knew the time during the tasks.

An index of perceived effort, the rating of perceived exertion (RPE), was assessed with the modified Borg 10-point scale. Subjects were instructed to focus the assessment of effort on the arm muscles performing the fatiguing task. The scale was anchored so that 0 represented the resting state and 10 corresponded to the strongest contraction that the arm muscles could perform. The RPE was recorded at the beginning of the contraction and every minute thereafter until task failure for the 20% MVC fatiguing contraction. Because of the brevity of the 80% MVC task, subjects were asked their RPE at the beginning of the contraction and at task failure.

**Data Analysis.** The torque for the MVC and submaximal contractions was calculated as the product of force and the distance between the elbow joint and the point at which the wrist was attached to the force transducer.

Voluntary activation was quantified by measurement of the force responses to stimulation of the muscle. Any increment in elbow flexion force evoked during a contraction (superimposed twitch) was expressed as a fraction of the amplitude of the control twitch evoked 3 s after the MVC. The level of voluntary activation was derived by the formula: Voluntary activation = 100 × (1 − T interpolated/Tcontrol), where T interpolated was the size of the interpolated twitch and T control was the amplitude of the control twitch produced by stimulation of the peripheral nerve in a relaxed but potentiated muscle.

The MVC torque was quantified as the average value over a 0.5-s interval that was centered about the peak. The maximal EMG for each muscle was determined as the root mean squared (RMS) value over a 0.5-s interval about the same interval of the MVC torque measurement. The RMS EMG value of the constant-force contractions for the elbow flexors performed at 20%, 40%, 60%, and 80% of MVC torque was averaged over the 2 s period prior to electrical stimulation during the 6-s contraction.

The fluctuations in force and the RMS of the EMG signal of the elbow flexor muscles and triceps brachii muscles were quantified during the fatiguing contraction performed at 20% of MVC at the following time intervals: the first and last 60 s of task duration, and 30 s either side of 25%, 50%, and 75% of time to task failure. The fluctuations in force and RMS EMG during the high force (80% MVC) task were quantified at five continuous intervals equivalent to 20% of the task duration. The EMG activity of the elbow flexor and extensor muscles during the fatiguing contraction was normalized to the RMS EMG value obtained during the MVC for each muscle. The amplitude of the force fluctuations was quantified as the coefficient of variation (CV = SD/mean × 100).

Heart rate and MAP recorded during the fatiguing contraction were analyzed by comparing −15-s averages at 25% intervals throughout the low-force fatiguing contraction. For each interval the blood pressure signal was analyzed for the mean peaks [systolic blood pressure (SBP)], mean troughs [diastolic blood pressure (DBP)], and number of pulses per second (multiplied by 60 to determine heart rate). MAP was calculated for each epoch with the following equation: MAP = DBP + 1/3(SBP − DBP). Heart rate and blood pressure were only analyzed for the 20% MVC fatiguing contraction due to a poor signal during recording of many of the 80% MVC tasks.

**Statistical Analysis.** Data are reported as means ± SD within the text and displayed as means ± SE in the figures. ANOVAs with repeated measures on a combination of variables including contraction intensity (20%, 80% MVC), time (0, 25%, 50%, 75%, 100% of time to failure), fatigue (precontraction, postcontraction), and force (20%, 40%, 60%, and 80% MVC) with sex (men, women) as a between-subject factor were used to compare the various dependent variables. Specifically, the statistical designs were as follows for the dependent variables: (1) contraction intensity × sex for time to task failure, the relative change of MVC torque, relative change in voluntary activation and the relative change in control twitch amplitude; (2) contraction intensity × time × sex for comparison of torque fluctuations, RPE, and RMS EMG for each muscle during the

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fatiguing contractions; (3) contraction intensity ×
fatigue × sex for comparison of voluntary activation,
MVC and twitch amplitude; (4) contraction intensity ×
force × sex for comparison of voluntary activi-
tion and RMS EMG during the brief constant force
contractions performed prior to each of the fatigu-
ing contractions; and (5) time × sex for comparison
of heart rate and MAP during the low-force sus-
tained contraction. Multiple comparisons with
Tukey post-hoc tests were used to determine differ-
ences among pairs of means. A significance level of
\( P < 0.05 \) was used to identify statistical significance.

The contribution of several variables to the time
to task failure were analyzed using multiple linear
regressions and the associated partial correlations
\((r)\). These variables included the change in EMG
activity of the biceps brachii and brachioradialis,
MAP (20% MVC task only), voluntary activation,
MVC force, twitch amplitudes, and physical activity
levels. The associated partial correlation coefficients
were used to identify the contribution of each inde-
pendent variable to the time to task failure for each
of the contraction intensities. The strength of an
association is reported as the squared Pearson prod-
uct-moment correlation coefficient \((r^2)\).

RESULTS

The men and women were similar in age (22.4 ± 4.4
years vs. 22.2 ± 4.3 years, respectively) but differed
in height (181.3 ± 5.2 cm vs. 168.5 ± 7.6 cm, \( P <
0.05 \)) and mass (80.9 ± 11.4 kg vs. 63.1 ± 10.9 kg,
\( P < 0.05 \)). The estimated physical activity levels
were similar for the men and women (51.9 ± 33.7
Met.hour/week vs. 59.5 ± 28.3 Met.hour/week, re-
spectively).

Time to Task Failure and MVC Torque. The men had
a shorter time to task failure than the women for the
20% MVC fatiguing contraction (10.6 ± 2.0 min vs.
17.0 ± 8.7 min, respectively) but a similar time for
the 80% MVC task (25.0 ± 6.5 s vs. 24.3 ± 6.6 s,
contraction intensity × sex, \( P < 0.05 \)). The range
in time to failure for the 20% MVC task was 11.3–38.8
min for the women and 6.5–13.0 min for the men.
The range in time to failure for the 80% MVC task
was 13–32 s for the women and 17–34 s for the men.
Correlation analysis showed no association between
the order of testing and the time to task failure for
men and women analyzed separately (women: \( r =
0.38, P = 0.32 \); men: \( r = 0.16, P = 0.69 \)) or pooled
\( r = 0.20, P = 0.42 \).

The men were stronger than the women \( (P <
0.05) \) on both days of testing (88.0 ± 22.1 N.m vs.
45.3 ± 7.2 N.m; prefatigue values) before and after
the fatiguing contractions. MVC torque was reduced
from the initial MVC at the end of the fatiguing
contractions (main effect of fatigue, \( P < 0.05 \)).
These reductions were greater \( (P < 0.05) \) for the
20% MVC task \( (24.1 ± 12.1\% \) than the 80% MVC
task \( (15.8 ± 4.3\% \) ). The decline in MVC torque was
greater for the women than men \( (P < 0.05) \) at the
end of the 20% MVC fatiguing contraction: \( 31.6 ±
11.1\% vs. 16.7 ± 8.1\% \) but was similarly reduced at
the end of the 80% MVC task \( (15.4 ± 5.3\% vs.
16.1 ± 3.2\% \) ). The range in relative reduction in
MVC torque after the 20% MVC task was 15%–51%
for the women and 9%–35% for the men. The
greater reduction in MVC force for the women was
due to two subjects who had a large relative decrease
in their MVC torque \( (44\% \) and \( 51\% \) ). However,
when these two data points were excluded the con-
traction intensity × sex interaction for the relative
reduction in MVC torque was not significant and the
time to task failure remained significantly different
between the sexes for the low-force task \( (17.9 ± 9.8
min for the women, \( n = 7 \) ).

Voluntary Activation. The size of the twitch evoked
during each of the brief submaximal constant-force
tasks decreased and voluntary activation increased as
the intensity of contraction increased between 20%
and 80% of MVC force \( (P < 0.05) \) similarly for the
men and women. The increase in voluntary activa-
tion was similar for each experimental day with no
interactions.

Voluntary activation assessed during the MVC
before the fatiguing contractions was similar for the
men and women and there was no difference be-
tween experimental sessions. Voluntary activation
was reduced \( (P < 0.05) \) at the end of the 20% MVC
fatiguing contraction similarly for the men \( (95.6 ±
4.3\% \) to \( 85.2 ± 10.2\% \) ) and women \( (97.7 ± 1.0\% \) to
\( 80.6 ± 10.0\% \) ). Voluntary activation also declined
after the 80% MVC task \( (P < 0.05) \) similarly for the
men \( (97.6 ± 2.2\% \) to \( 93.1 ± 5.6\% \) ) and women
\( (98.2 ± 1.1\% \) to \( 92.7 ± 5.4\% \) ). However, the reduc-
tion in voluntary activation was greater after the 20%
MVC task \( (14.3 ± 9.9\% \), men and women pooled
) compared with the 80% MVC fatiguing task \( (5.1 ±
4.3\% \), contraction intensity × fatigue, \( P < 0.05 \) ).
There were no other significant interactions for con-
traction intensity, sex, and fatigue.

Twitch Amplitude. The amplitude of the poten-
tiated control twitch torque was greater for the men
than women \( (8.2 ± 2.2 \text{ N.m vs. } 5.9 ± 1.1 \text{ N.m, } P\
< 0.05 \) before the fatiguing contractions and was sim-

Task and Sex Differences in Muscle Fatigue
ilar for each of the experimental days. Twitch torque was reduced at the end of both fatiguing contractions ($P < 0.05$) for both men and women and there were no interactions. The relative decline of twitch amplitude evoked immediately after the 20% MVC fatiguing task was similar for the men ($23 \pm 24\%$) and women ($33 \pm 9\%$). Twitch torque was also reduced similarly after the 80% MVC fatiguing task for the men ($37 \pm 17\%$) and women ($29 \pm 13\%$), indicating a similar magnitude of peripheral fatigue at the termination of both tasks in both sexes.

**Fluctuations in Torque during the Fatiguing Contractions.** The amplitude ($CV$) of the vertical fluctuations in force increased during the 20% and 80% MVC fatiguing contractions ($P < 0.05$, Fig. 1). The increase in fluctuations during the low-intensity fatiguing task was similar for men and women and represented over a 3-fold increase. At the start of the fatiguing task the range of torque fluctuations was 1.0%–2.6% for the women and 1.0%–3.9% for the men. At the end of the low-intensity task the ranges were similar for the women (4.1%–7.5%) and men (4.0%–8.6%). However, the rate of change in fluctuations for men was greater than women ($P < 0.05$) during the 20% MVC force fatiguing contraction. Furthermore, the increase in fluctuations during the 80% MVC fatiguing task was similar for men and women with no interaction for sex $\times$ time. At the start of the 80% MVC fatiguing task the range of torque fluctuations were 0.8%–3.2% for the women and 0.7%–4.1% for the men. At the end of the high-intensity task the ranges were 3.0%–9.9% and 2.5%–7.4%, respectively.

**EMG Activity.** **EMG–Force Relation.** EMG activity increased ($P < 0.05$) with contraction intensity on both days similarly for the men and women during the brief submaximal contractions of the biceps brachii and brachioradialis muscles. There were no differences in the EMG activity across experimental days, nor any interactions of sex $\times$ contraction intensity.

**RMS EMG during the Fatiguing Contraction.** The amplitude of the RMS EMG (% MVC) for each of the elbow flexor muscles increased ($P < 0.05$) during the 20% and 80% MVC force fatiguing contractions (Fig. 2). RMS EMG of biceps brachii for the women was greater than the men at the end of the 20% MVC fatiguing contraction but not for the 80% contraction intensity $\times$ time $\times$ sex, $P < 0.05$). The amplitude of RMS EMG for brachioradialis was less for the women compared with the men for the 20% MVC fatiguing contraction but similar for the sexes for the 80% MVC fatiguing contraction (contraction intensity $\times$ sex, $P < 0.05$). Furthermore, RMS EMG for triceps brachii of the women was greater compared with the men ($P < 0.05$) for the 80% MVC fatiguing contraction throughout the task.

**Mean Arterial Blood Pressure (MAP) and Heart Rate.** MAP increased during the low-force fatiguing contraction for the men and women (main effect of time, $P < 0.05$) (Fig. 3A). MAP increased more for the men than the women during the 20% MVC task (interaction of time $\times$ sex, $P = 0.05$) so that at task failure the men had a greater MAP than the women. Consequently, the rate of increase was greater for
the men compared with the women (6.37 ± 1.52 and 3.15 ± 1.42 mmHg/min, respectively, \( P < 0.05 \)). MAP and heart rate was not able to be analyzed for the 80% MVC task due to poor signal recordings. Heart rate also increased during the low-force fatiguing contraction (\( P < 0.05 \)) and was similar for the men and women. The men and women had similar heart rate values at the start (70 ± 8 beats/min and 74 ± 12 beats/min, respectively) and end (99 ± 18 beats/min and 104 ± 19 beats/min, respectively) of the 20% MVC task. There was no interaction of time × sex and no difference in the rate of increase in the heart rate between men and women (2.87 ± 1.98 beats/min and 2.00 ± 1.13 beats/min).

**Perceived Exertion during the Fatiguing Contractions.**

RPE increased during both fatiguing contractions (\( P < 0.05 \)). RPE was similar for the men and women at the beginning (2.2 ± 1.2 vs. 1.8 ± 1.0) and end (9.7 ± 0.7 vs. 10.0 ± 0.0) of the fatiguing contraction for the 20% MVC task. However, the rate of increase in the RPE was more gradual for the women than the men (0.95 ± 0.2/min vs. 0.68 ± 0.2/min, \( P < 0.05 \)) for the 20% MVC task. All subjects reported an RPE of 10 at task failure of the 80% MVC contraction.
Factors that Contributed to Time to Failure: Regression Analysis. Regression analysis showed the rate of change in MAP ($r^2 = 0.39, P < 0.05$) was the single significant predictor of time to failure for the 20% MVC task. For the 80% MVC task there were no significant predictors of time to task failure. Further analysis indicated that the relation between rate of change in MAP and the time to task failure was best described as an exponential decay ($r = 0.77, r^2 = 0.60, P < 0.05$, Fig. 3B). Those individuals who had the greatest rate of change in MAP had the shortest time to failure.

DISCUSSION

The main findings of this study were that (1) men had a shorter time to task failure than women for the 20% MVC force fatiguing contraction but a similar time for the 80% MVC task; (2) voluntary activation was reduced to a greater magnitude after the 20% MVC task compared with the 80% MVC task for both men and women; (3) voluntary activation was reduced similarly for both sexes after the fatiguing contractions; (4) the reduction in the amplitude of the potentiated control twitch was similar for men and women after the 20% and 80% MVC fatiguing tasks; and (5) the rate of change in MAP was the single predictor of time to failure for the 20% MVC fatiguing contraction.

Sex Difference in Time to Task Failure Was Intensity-Dependent. The women were weaker than the men, but they had a longer time to task failure for the low-intensity fatiguing contraction, which is consistent with other studies.$^{5,20,26,37,48}$ In contrast, the time to failure was similar between men and women for the 80% MVC task. Muscle perfusion may explain the sex difference in time to failure for the sustained contractions at low forces. First, there was no sex difference in time to task failure for the sustained 80% MVC task, when blood flow is usually occluded,$^{4,35}$ and this has been shown for other muscle groups including the finger flexors and quadriceps.$^{31,48}$ The difference in muscle fatigue between men and women was also eliminated when the sexes were matched for strength for a low-force sustained contraction$^{16,22,20}$ and when perfusion was occluded with a cuff and the muscle made ischemic.$^{6,37}$ Furthermore, the sex difference persists for moderate-to-high intensity intermittent contractions when blood flow is periodically restored and is therefore not a limitation to task duration.$^{6,12,23,37}$

Second, the rate of increase in MAP was the main predictor of time to failure for the 20% MVC sustained contraction. The rate of increase in MAP was less for the women than men during the low-force task. The increase in MAP (pressor response) during an isometric contraction is due to peripheral reflexes, mainly the metaboreflex, which is driven by an increase in metabolites in a progressively occluded muscle.$^{32,36}$ The pressor response is also influenced by central command. However, the peripheral reflex appeared to have more influence during these low-force contractions because the increase in heart rate, which is due to central command,$^{32,36}$ did not differ between the men and women. Consequently, mechanically altered muscle perfusion was likely less for women than men during the 20% MVC task and is consistent with sex differences in forearm blood flow after isometric contractions of the handgrip muscles.$^{36}$ Other sex-related mechanisms that influence perfusion, however, may also mediate the task-dependent difference in muscle fatigue between men and women including differences in: (1) fiber type composition$^{34,40,47}$; (2) rates of glycolytic metabolism$^{38,42}$; (3) metabolic vasodilators of the muscle$^7$ mediated by chronic exposure to sex hormones$^{19,53}$; and (4) sympathetic activation, such that vasoconstriction is less for women than men during static exercise.$^9$

Voluntary activation was reduced to similar magnitudes for men and women after the fatiguing contractions, indicating similar magnitudes of impairment to optimally drive the muscle during a maximal effort at termination of both tasks. Either the motor units were not all recruited voluntarily or they were discharging at rates that were insufficient to produce full fusion of force$^{17}$ after the fatiguing contractions. However, the change in voluntary activation was not a predictor of the time to failure for the 20% MVC contractions, and therefore secondary to fatigue within the muscle. An increased accumulation of metabolites in the muscle of men compared with women likely increased afferent feedback to spinal and supraspinal centers$^{11}$ influencing voluntary activation after the low-force task. Supraspinal fatigue contributes significantly to the reduction in voluntary activation and muscle fatigue during low-force and high-force fatiguing tasks and is attributable to suboptimal output from the motor cortex.$^{15,25,41,43}$ Supraspinal fatigue was similar for men and women during repeated sustained maximal contractions of the elbow flexor muscles but the men fatigued more than the women because the women experienced less peripheral fatigue.$^{25}$ The present study, therefore, confirms previous findings for the elbow flexor muscles.
Men and women utilized different activation patterns and strategies among the elbow flexor muscles during the low-force sustained contraction. The men showed greater activation of the brachioradialis and the women utilized the biceps brachii to greater magnitudes than the men toward task failure of the low-force task. These differences in EMG activity were not due to any sex differences in the recording conditions because the EMG activity was similar across experimental days and the sexes during brief submaximal contractions at varying intensities that were recorded before the fatiguing contractions. The increase in EMG activity during a low-force submaximal sustained contraction is largely due to the recruitment of larger motor units as the muscle becomes progressively fatigued.\(^{10,15}\) Differences in activation patterns and recruitment patterns within a muscle and among agonist muscles will alter time to task failure for a low-force contraction.\(^{21,27,39}\) However, there was no association between changes in EMG activity for either of the elbow flexor muscles and time to failure. Although activation patterns differed between men and women, the influence on the sex differences in time to failure was small.

In contrast, the elbow flexor muscles of the men and women were similar in their activation patterns during the 80% MVC task, which is an intensity at which the majority of motor units are recruited.\(^{29}\) The women had greater activation of the triceps brachii during the high-force task than the men, but the magnitude of this difference was small and therefore unlikely to contribute to differences in time to failure.

**Mechanisms of Fatigue Vary with Contraction Intensity.** The magnitude of reduction in voluntary activation was greater after the low-intensity task (14%) than the high-force, short-duration task (5%). The reduction in voluntary drive was in part due to fatigue at or processes that occur prior to the motor cortex for the low-force task.\(^{41}\) Consistent with these results, the EMG activity increased during both tasks but was less than maximal after the 20% MVC task but maximal at task failure for the 80% MVC contraction. The differences in EMG activity between high- and low-force contractions at task failure was also shown for a small muscle of the hand.\(^{11,30}\) These differences in EMG activity and voluntary activation likely originated from a decrease in descending drive and reduced excitability of the motor neuron pool at spinal sources for the low-force task with some contribution from feedback via afferents.\(^{14}\)

The similar reduction in twitch amplitude after the two fatiguing contractions suggests the average rate of peripheral fatigue was much greater during the 80% MVC task than the 20% MVC contraction. Taken together with the observation of the small reduction in voluntary activation after the high-intensity task, failure of the 80% MVC task can be attributed primarily to fatigue within the muscle.

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SHORT REPORT

ABSTRACT: Dysferlinopathies exhibit marked heterogeneity in the initial distribution of muscle involvement at the onset of the disease. We describe a Japanese patient with dysferlinopathy who exhibited distal anterior compartment myopathy (DACM) with early contractures of the ankle, whose pedigree included patients with two other types of dysferlinopathy. The existence of three phenotypes of dysferlinopathy in one pedigree is reported, indicating the involvement of molecules other than dysferlin in the pathogenesis.

DISTAL ANTERIOR COMPARTMENT MYOPATHY WITH EARLY ANKLE CONTRACTURES

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Dysferlinopathies exhibit marked heterogeneity in the initial distribution of muscle involvement at the onset of the disease. Because dysferlinopathies include Miyoshi myopathy (MM) and limb-girdle muscular dystrophy type 2B (LGMD2B), both of which have been observed within the same family, some additional factors distinct from dysferlin are likely to be involved in the pathogenesis.15,16 Distal anterior compartment myopathy (DACM) is a relatively new phenotype of autosomal-recessive muscular dystrophy caused by a dysferlin mutation first described by Illa and colleagues.8 We describe a Japanese patient who exhibited the clinical features of DACM and whose pedigree included two other types of dysferlinopathy.

CASE REPORTS

A 42-year-old man (Fig. 1, IV-9, arrow), who had been noted to stumble frequently at 15 years of age, subsequently developed a gait disturbance with transient swelling of the muscles in the lower legs. By 18 years of age, lower-limb weakness had progressed and he could only walk on tip-toes due to an ankle deformity. He also had difficulty in extending his fingers. At age 29 years, he could not extend his wrists and fingers fully nor stand without support. His grip strength was about 10 kg. The weakness of the lower limbs was pronounced in the anterior tibial muscles, and motor testing revealed grade 2 strength in the anterior tibial muscles on the Medical Research Council (MRC) scale, whereas the iliopsoas, gluteus, quadriceps, hamstrings, and plantar flexors showed moderate weakness (MRC 3). Severe muscle atrophy was present in the peroneal areas of both legs. His serum creatine kinase level was 950–1400 U/L. He lost the ability to walk at 34 years of age. At age 42, all the muscles in the lower extremities (MRC 1–2) and the deltoid, biceps, triceps, and wrist and finger extensors were severely affected (MRC 2), whereas wrist and finger flexors were mildly affected (MRC 4). Tendon reflexes were absent. There was no facial, bulbar, or cardiac muscle involvement.

His first cousin (Fig. 1, IV-2) and older sister (Fig. 1, IV-5) exhibited the typical features of Miyoshi myopathy, characterized by predominant involvement of the posterior compartment of the legs, with onset in the late teens.15 His cousin’s father (Fig. 1, III-1) and cousin’s aunt (Fig. 1, III-3) presented with features of limb-girdle muscular dystrophy with juvenile-onset pelvic-femoral weakness.12,17 They also presented with congenital amblyopia, which was not observed in other members of the pedigree. This...
patient’s older brother (Fig. 1, IV-6) exhibited myopathy affecting the distal part of the lower limbs. We found degenerative and regenerative changes without rimmed vacuoles or myofibrillar formations in biopsied muscles of IV-2 and III-1. Sensory function was intact in all four patients (III-1, IV-2, IV-5, and IV-9). All ancestors of the family members originated from the same small village. Affected members of the family (III-1, IV-2, IV-5, and IV-9) were examined by the same physician (S.H., T.T., or A.M.).

We tested for the presence of the dysferlin mutation in the patients using the single-strand conformation polymorphism technique and automated DNA sequencing for each of 55 exons of the gene. Gene analysis confirmed that all four patients shared the same homozygous dysferlin 4870delT mutation predicted to lead to a stop codon by causing a frameshift.

DISCUSSION

In the present case, both the site of onset in the tibialis anterior muscle and the rapid progression of the lower-limb weakness were similar to the findings in the DACM cases described by Illa and colleagues. However, our case had weakness of the wrist and finger extensor rather than flexor muscles, and early contractures of the ankle deformities were a prominent and unique feature. In cases presenting early ankle contracture, DACM should be considered in addition to X-linked Emery–Dreifuss muscular dystrophy. Muscular dystrophy due to the dysferlin mutation has been shown to have at least three distinct phenotypes (MM, LGMD2B, and DACM); the family studied in this report included all the phenotypes in one pedigree, which further emphasizes the clinical heterogeneity of dysferlinopathies. We are unaware of other cases of DACM in a non-European ethnic group.

Identical mutations in the dysferlin gene can produce different myopathy phenotypes even in the same pedigree as described in the present and other reports. Why can different phenotypes be produced from the same mutation and in the same family? Dysferlin has a role in membrane repair during muscle damage. Many molecules are known to interact with dysferlin. We speculate that the expression levels of these molecules may modify the severity and distribution of disease progress in dysferlinopathy. The pedigree described herein may provide clues in elucidating the causes of the heterogeneity of dysferlinopathy.

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REFERENCES


SHORT REPORT

ABSTRACT: Although surgical division of the transverse carpal ligament is the operative treatment of choice for carpal tunnel syndrome (CTS), controversy exists about the immediate postoperative treatment regimen. Splinting for up to 6 weeks after surgery is recommended by some investigators. We therefore evaluated effectiveness of splinting after open carpal tunnel surgery by a randomized, controlled trial. Fifty consecutive patients with clinically and electrophysiologically confirmed idiopathic CTS were assigned to open carpal tunnel release and randomized to receiving a light bandage (25 patients) or a bulky dressing with a volar splint (25 patients) for 2 days each. All patients were followed up at 3 months. Parameters retrieved were pain as measured using a visual analog scale, two-point discrimination, and grip strength, and nerve conduction studies. At follow-up, all patients reported definite improvement of symptoms, but there was no statistically significant difference between the two groups for any of our outcome measures. Thus, postoperative splinting after open carpal tunnel release does not yield any benefit to eventual outcome. In fact, it adds to the overall operating time and can safely be abandoned.


POSTOPERATIVE SPLINTING AFTER OPEN CARPAL TUNNEL RELEASE DOES NOT IMPROVE FUNCTIONAL AND NEUROLOGICAL OUTCOME

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Carpal tunnel syndrome (CTS) is the most frequently encountered peripheral compression neuropathy, with a prevalence of approximately 1% in the general population. Open carpal tunnel release is the standard of care for the treatment of CTS due to the extensive anatomical exposure that results in efficacious and safe release of the flexor retinaculum. Numerous studies have compared different types of treatment strategies such as endoscopic carpal tunnel release,19,20 steroid injection,9 and various other surgical techniques6,11,12 with the open approach in order to find the most effective way to treat this entity. However, few articles have compared postoperative treatment and rehabilitation, such as postoperative splinting,3,4,7 postoperative physiotherapy,8,18 and postoperative mobilization.16 Postoperative discomfort, such as pain, and objective parameters, such as electrophysiological studies, are generally not considered in this context. The goal of this study is to provide information about the effectiveness of postoperative splinting as determined in a prospective, randomized study.

PATIENTS AND METHODS

All patients who presented to our department with isolated, idiopathic CTS between January and May 2006 were included in this study. Approval was obtained from our institutional ethics committee and informed consent was obtained from all enrolled patients after explanation about the diagnosis of CTS and the treatment options. The diagnosis of CTS was based on the following criteria: history of sensory disturbances along the distribution of the median nerve with dysesthesia and pain; and an abnormal electrodiagnostic study according to published practice parameters for electrodagnosis of CTS,1,2,10 including distal motor latencies (conduction distance 6.5 cm; abnormal value >4.5 ms) and median sensory conduction velocity (between wrist and index finger; abnormal value <46 m/s).

Abbreviations: CTS, carpal tunnel syndrome; 2-PD, two-point discrimination; VAS, visual analog scale
Key words: carpal tunnel syndrome; postoperative therapy; splinting; surgery; treatment
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Preoperatively, patient demographics, such as age, body mass index, smoking habits, and medication intake (including anticoagulants), were recorded as well as objective physical findings such as two-point discrimination (2-PD), the pick-up test,17 pain as measured on a visual analog scale (VAS), and grip strength14 measured with a baseline hydraulic hand dynamometer (Fabrication Enterprises, White Plains, New York).

Intraoperatively, a standard open carpal tunnel release was performed after induction of anesthesia (general or regional anesthesia, depending on the choice of the patient) and application of a tourniquet to the upper arm. A gently curved incision was made in the thenar crease and deepened through subcutaneous fat and palmar aponeurosis down to the transverse carpal ligament. The ligament was transected on the ulnar side while the median nerve was directly visible. After release of the tourniquet and meticulous hemostasis, the wound was closed in a single superficial layer using 4/0 nylon sutures over a 8-French drain. Postoperatively, patients were randomized to receive either a light bandage (group 1; Fig. 1A) or a bulky dressing with a volar splint left in place for 48 hours with the wrist in a neutral position (group 2; Fig. 1B). The randomization was accomplished by applying a volar splint to every even-numbered patient in the consecutive list of 50 patients in this study. Approximately 48 hours after the surgical intervention, all bandages were removed, the collected drainage fluid was recorded after drain removal, and all patients we given a light protective dressing. Sutures were removed after 2 weeks. All patients were seen again after 3 months for electrophysiological testing and functional evaluation.

**Statistical Analysis.** To compare pre- and postoperative values of all patients, regardless of postoperative treatment, a t-test for paired samples was used. In order to compare any differences between the postoperative values between the two groups, a t-test for independent samples was applied. \( P < 0.05 \) was considered statistically significant. All analyses were performed using SPSS version 12.0 statistical software (SPSS, Inc., Chicago, Illinois).

**RESULTS**

All recruited patients completed the study with no dropouts in either group. Fifty patients with 50 affected hands were included and both study groups were of equal size (\( n = 25 \)). There were no median nerve, digital nerve, vascular, or tendon complications; delayed wound healing was not observed.

Table 1 lists average pain scores according to the VAS between the two groups, and scar tenderness, which was divided into no perceptible pain, pain during active motion, and pain even at rest. After carpal tunnel release, all patients reported almost complete resolution of their symptoms, which con-

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<th>Table 1. Summary of evaluated parameters between the two groups.</th>
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<td><strong>Splinted group</strong></td>
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<td>Pain (VAS, mean)</td>
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<td>Postoperative day 2</td>
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<td>3-month follow-up</td>
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<td>Grip strength (mean)</td>
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<td>Pick-up test (mean)</td>
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<td>Two-point discrimination (mean)</td>
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<td>Distal motor latency (improvement; mean)</td>
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<td>3-month follow-up</td>
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VAS, visual analog scale.
sisted of numbness, especially at night, as well as pain and tingling.

Table 1 also summarizes the results of the functional hand evaluation (2-PD, grip strength and pick-up test times) and electrophysiological outcomes (i.e., distal motor latency).

Statistical analysis revealed significant improvement in both groups after the surgical intervention regardless of postoperative management (light bandage vs. bulky dressing with volar splint) for the following parameters: pain; static 2-PD; and distal motor latency. Interestingly, in both groups grip strength deteriorated significantly. When comparing postoperative values for each parameter between the two groups, we found no significant difference at 3-month follow-up.

DISCUSSION

We found that postoperative splinting yields no benefit in outcome as judged by subjective parameters, such as pain, and objective parameters, such as functional hand and electrophysiological tests.

Although numerous studies have dealt with various surgical and non-surgical methods for treating CTS, few have examined the optimal postoperative therapy regimen. Several groups of investigators focused on postoperative physiotherapy and it is concluded that, although most hand surgeons have incorporated an active, controlled hand rehabilitation program in their overall treatment regimen, most use splinting for a variable amount of time after an open carpal tunnel release. One of the main reasons for this approach may be to relieve immediate postoperative pain through immobilization, which led us to look at the efficacy of a volar splint with regard to postoperative comfort. The pain after surgery (second postoperative day) was perceived as low in both treatment groups, with a median score of 2 as measured by VAS. Although all patients were allowed to receive non-steroidal anti-inflammatory agents for pain control, the majority in each group did not request any medication. Similarly, scar tenderness was comparable and equally low between the two groups at 3-month follow-up. Only 2 patients in the splinting group reported persisting scar pain. These findings indicate that splinting yields no advantage in terms of patient comfort.

Regarding functional hand evaluation tests, all parameters improved almost equally in both groups except grip strength, as detected previously after carpal tunnel release. Nerve conduction studies, in particular distal motor latency, improved to an equal extent in the two groups.

Although the results of this study indicate that a postoperative splint is not required after open surgical release of the carpal tunnel, there are some limitations to our study design: the length of splinting was chosen arbitrarily and was only 2 days. However, the current available body of literature is in favor of limiting prolonged immobilization after carpal tunnel release because it is of no advantage or even detrimental due to delayed mobilization and physical therapy. Therefore, we evaluated the potential beneficial effect of short-term splinting in ameliorating postoperative pain or hematoma formation. The type of anesthesia (general vs. axillary block) was also arbitrarily chosen, as requested by the patient during routine preoperative counseling. Pain relief through axillary block was several hours longer, but patients were asked to give a statement of overall pain perception, which was comparable between the two groups. One main additional variable is the postoperative compliance of patients. Immediate forceful use of the operated hand is known to worsen the outcome. Although all patients in the study group were advised to treat the affected hand with care and to avoid hard work, implementation of this recommendation depended on the individual. Five different surgeons performed the operations to an almost equal extent. They were all experienced hand surgeons who used the same operative technique, making it unlikely that the postoperative results were biased.

This study was presented at the 44th Congress of the Austrian Society of Plastic Surgery and the 14th International Congress of the International Confederation for Plastic, Reconstructive and Aesthetic Surgery.

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ABSTRACT: This study was designed to determine the prevalence of definite vitamin B12 deficiency (defined as ≤240 pg/ml) and possible vitamin B12 deficiency (defined as >240 pg/ml and a methylmalonic acid [MMA] level >243 nmol/L) in patients with polyneuropathy and to determine whether patients in both groups respond to vitamin B12 repletion. We performed a retrospective cohort study of 581 patients presenting with polyneuropathy over a 2-year period; 4% had definite vitamin B12 deficiency and 32% had possible deficiency as the sole or contributing cause for their polyneuropathy. For those who received treatment with vitamin B12, subjective improvement was seen in 87% with definite and in 43% with possible deficiency. Possible vitamin B12 deficiency, defined as an elevated MMA level, is a common finding in patients with polyneuropathy and treatment of these patients with vitamin B12 may lead to clinical improvement.

VITAMIN B12 AND METHYLMALONIC ACID LEVELS IN PATIENTS PRESENTING WITH POLYNEUROPATHY

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Vitamin B12 deficiency is an important cause of polyneuropathy because it is treatable. Clinical features, such as early involvement of the hands and preferential involvement of large-fiber sensations, are characteristic of the polyneuropathy of vitamin B12 deficiency, but this picture overlaps with the presentation of polyneuropathy from many other causes. Thus, the diagnosis of vitamin B12 deficiency as a cause of polyneuropathy relies on laboratory tests. The diagnosis is usually based on the finding of a low serum vitamin B12 level (<200 pg/ml). Recently, there has been much interest in the serum levels of methylmalonic acid (MMA), which rise in the setting of vitamin B12 deficiency and have been shown to be a sensitive and specific indicator of such deficiency. MMA levels are elevated, in the setting of a normal vitamin B12 level, in patients with neurologic symptoms responsive to vitamin B12 repletion, suggesting that MMA may be a more sensitive marker for early, mild vitamin B12 deficiency than the vitamin B12 level itself.

Hvas et al. found that 80% of a group of 140 individuals with elevated plasma MMA levels had more than one neurologic symptom (muscle weakness, sensory disturbance, or autonomic disturbance), suggesting that the prevalence of plasma MMA elevation in patients with polyneuropathy is high. However, there are limited data on the prevalence of MMA elevations in patients with polyneuropathy alone.

The response of neurologic symptoms to treatment with vitamin B12 in the setting of definite vitamin B12 deficiency, defined as a vitamin B12 level <200 pg/ml, appears to be good. It is less clear whether patients with polyneuropathy and normal vitamin B12 but elevated MMA levels will respond clinically to vitamin B12 repletion.

We thus set out to examine whether possible vitamin B12 deficiency, defined as an elevated MMA in the setting of a normal vitamin B12 level, is common in patients with polyneuropathy and whether patients with definite or possible B12 deficiency both respond to vitamin B12 repletion.

MATERIALS AND METHODS

We undertook a retrospective record review of all patients presenting with polyneuropathy to the neuromuscular and general neurology clinics at our institution from December 1, 2003, to December 1, 2005. The study was approved by our Institutional
Review Board. We identified potential subjects by screening all billing records for the included clinics and pulling records coded with a diagnosis of polyneuropathy. These records were reviewed by a neuromuscular specialist to identify those patients presenting with polyneuropathy (rather than being seen in follow-up) who clearly had polyneuropathy as a cause for their symptoms. The diagnosis of polyneuropathy was based on the presence of each of the following:

1. A consistent clinical history of numbness, paresthesias, pain, or weakness in the distal extremities.
2. Examination findings of distal sensory loss and absent or reduced stretch reflexes distally or generally, with or without distal or generalized weakness.
3. Confirmation of polyneuropathy, by electrodiagnostic testing, nerve biopsy, or skin biopsy for determination of epidermal nerve fiber density, as the cause for the patient’s symptoms.

Patients who did not have electrodiagnostic testing or biopsy results were included only if the clinical features were felt to be unequivocally consistent with polyneuropathy and other likely causes had been excluded.

We reviewed each record for standardized information, including age, gender, date of initial presentation, symptom duration, comorbid illnesses, medications, number of alcoholic drinks daily, family history of polyneuropathy, laboratory data (vitamin B12, MMA, and homocysteine blood levels; hematocrit and mean corpuscular volume; serum protein electrophoresis and immuno-electrophoresis; random and fasting blood glucose, 2-hour glucose tolerance test, and glycosylated hemoglobin; serum creatinine; liver function tests; and serologic testing for Lyme disease and human immunodeficiency virus infection) and electrodiagnostic testing or biopsy results. All these elements were not always available because the work-up for each patient was nonstandardized and based on the individual treating clinician’s discretion. Based on the available data, experienced neuromuscular specialists assigned a primary cause of polyneuropathy to each patient; where only a single cause was identified, this was considered the primary etiology. When more than one possible cause was identified the investigator assigned the cause most strongly associated with polyneuropathy as primary and recorded the other possible causes as secondary. Cases in which no cause could be identified on record review were classified as cryptogenic.

To assess the effects of vitamin B12 repletion on polyneuropathy we examined the subset of patients with vitamin B12 deficiency treated with vitamin B12 who had follow-up clinic visits documented in the medical record between 5 and 24 months after onset of treatment. For these patients we recorded route and dose of vitamin B12 replacement, their subjective response to treatment, and repeat vitamin B12 and MMA levels after treatment, if available.

We considered subjects to have definite vitamin B12 deficiency if their serum vitamin B12 level was \( \leq 240 \, \text{pg/ml} \). We chose 240 pg/ml because this is the lower limit of normal in our laboratory, representing the 2.5 percentile of a group of patients in the United States. Those with normal levels (>240 pg/ml) but elevated levels of MMA (>243 nmol/L, Quest Diagnostics Nichols Institute, Chantilly, Virginia) were considered to have possible vitamin B12 deficiency.

RESULTS

During the 2-year screening period we identified a total of 581 patients presenting with polyneuropathy. Of these, 375 (65%) had a single cause for the polyneuropathy, 100 (17%) were cryptogenic, and the remaining 106 (18%) had more than one potential etiology.

Of the 581 patients with polyneuropathy, a serum vitamin B12 level was measured in 428 (74%). Nineteen patients (4%) had definite vitamin B12 deficiency. Of these, the vitamin B12 deficiency was felt to be the sole cause of polyneuropathy in 9 (47%). In the remaining 10 patients (53%), vitamin B12 deficiency was found along with another potential cause for the polyneuropathy.

Of the 581 patients with polyneuropathy, an MMA level was measured in 163 (28%), with a mean value of 667 nmol/L (range, 0.18 to 25,845 nmol/L). Comparing patients with normal MMA levels and those with elevated MMA levels, using the Wilcoxon rank sum test, we found no significant differences in age (W = 3458, P < 0.13), serum creatinine level (W = 3510, P < 0.08), or the ratio of blood urea nitrogen to creatinine (W = 3138.5, P < 0.98). Among these 163 patients, 52 (32%) had possible vitamin B12 deficiency, and this was felt to be the sole cause of polyneuropathy in 31 of the 52 (60%). There were 21 patients (40%) who had one or more additional potential causes for polyneuropathy.

MMA levels were obtained in 13 of the 19 patients with definite vitamin B12 deficiency and were abnormal in 8. The average MMA level was greater in those patients with definite vitamin B12 deficiency...
(4,103 nmol/L) than in those patients with possible deficiency (725 nmol/L) and the MMA values were significantly higher in those patients with definite than possible B12 deficiency (Wilcoxon rank sum test, W = 1506.5, P < 0.003).

There were 263 patients with diabetic neuropathy; vitamin B12 levels were measured in 158 and MMA levels in 36. Definite vitamin B12 deficiency was found in one patient (0.6%) and possible deficiency in two (5.5%). Of 35 patients with alcohol as a cause of their polyneuropathy, vitamin B12 levels were measured in 31 and MMA levels in 19. Three patients (10%) had definite deficiency and four (21%) had possible deficiency.

Thirteen of the 19 patients with definite vitamin B12 deficiency had repletion therapy. Six received intramuscular repletion and one received oral repletion; in the remaining six, the exact route and dose of repletion was not documented. Adequate follow-up information was available for 8 of these 13. Seven (87%) improved with repletion. Twenty-eight of the 52 patients with possible vitamin B12 deficiency had repletion therapy. Eleven received intramuscular repletion, five received oral repletion, and the remaining 12 did not have the route and dose of repletion documented. Adequate follow-up information was available for 23 of these 28; 10 of these 23 patients (43%) improved.

**DISCUSSION**

Although homocysteine also appears to be a sensitive indicator of vitamin B12 deficiency, we chose to use plasma MMA levels as a marker for possible vitamin B12 deficiency because there are more confounding conditions that increase homocysteine than increase MMA levels. MMA can be elevated by increasing age, renal insufficiency, hypovolemia, and genetic disorders, whereas homocysteine levels are affected by these and additionally by hypothyroidism, carcinoma, treatment with methotrexate or phenytoin, and deficiency of folate or vitamin B6.6 We found no significant difference in age, serum creatinine (indicating renal insufficiency), or the ratio of blood urea nitrogen to creatinine (indicating hypovolemia) between subjects with elevated MMA and subjects with normal MMA levels, suggesting that the MMA elevations likely reflect vitamin B12 deficiency.

Although vitamin B12 and MMA levels were not measured in every patient, the prevalence of definite vitamin B12 deficiency in those patients in whom it was measured was 4%, and the prevalence of possible vitamin B12 deficiency in those in whom MMA was measured was 32%. Previously published data has shown that the prevalence of vitamin B12 deficiency–associated polyneuropathy ranges from 2.2%–8%.1,7 The prevalence of definite vitamin B12 deficiency in our population is consistent with these data, but our study suggests that possible vitamin B12 deficiency is more prevalent than previously noted; if clinically significant, this substantially increases the number of patients who may benefit from vitamin B12 repletion.

Our data suggest that vitamin B12 and MMA levels are not checked in a substantial minority of polyneuropathy patients evaluated in general neurology and neuromuscular clinics, especially when another cause for the polyneuropathy is readily identified. We found that 26% of all patients in our study, and 40% of those with diabetes, did not have a vitamin B12 level checked. Although limited numbers of subjects with diabetes had MMA levels determined, our data suggest that checking vitamin B12 and MMA levels in all patients presenting with polyneuropathy may be valuable for identifying this potentially treatable contributor; we documented vitamin B12 deficiency in 6.1% of diabetics and 31% of alcoholics.

Although many investigators regard increases in MMA to be an early and specific indicator of functional vitamin B12 deficiency, this opinion is not unanimous. Several authors have documented a lack of clear responsiveness in symptoms of vitamin B12 deficiency to repletion therapy in patients with elevated serum MMA levels. A placebo-controlled study of 140 people with elevated MMA levels treated with vitamin B12 showed improvement in the serum markers of MMA and homocysteine, but there was no change in hematologic, neurologic, or gastroenterologic symptoms.3 Solomon,9 in a retrospective review of 456 ambulatory patients, also found discordance between serum markers of vitamin B12 deficiency and symptom improvement with vitamin B12 repletion.

As noted, 87% of our patients with definite vitamin B12 deficiency, and 43% of those with possible vitamin B12 deficiency, improved subjectively with repletion. Because this was not a controlled trial the route and dosage of repletion varied among patients. This variation in repletion strategy, and the absence of consistent objective criteria in the patient records by which to confirm subjective reports of improvement, limits the accuracy of our estimates of improvement. Despite these limitations, the subjective improvement rate of 87% in patients with definite vitamin B12 deficiency is likely significant. The significance of the 43% improvement rate in patients with possible vitamin B12 deficiency is less clear.
In the absence of a gold standard for diagnosing vitamin B<sub>12</sub> deficiency, the only way to know that elevated serum MMA levels indicate vitamin B<sub>12</sub> deficiency is to show that patients with clinical syndromes consistent with vitamin B<sub>12</sub> deficiency have improvement in both their clinical findings and MMA levels when treated with vitamin B<sub>12</sub>. A prior retrospective study also suggested, but could not prove, a better outcome in treated polyneuropathy patients with definite and possible vitamin B<sub>12</sub> deficiency compared to patients with untreated cryptogenic polyneuropathy. Our data support the findings in the literature thus far that elevated MMA levels in patients with polyneuropathy may indicate a vitamin B<sub>12</sub>-responsive disorder, but many questions remain. Given the cost of MMA assays, a prospective, placebo-controlled treatment study of patients with polyneuropathy and possible vitamin B<sub>12</sub> deficiency is warranted.

REFERENCES

ABSTRACT: In spite of extensive studies it is unclear whether impaired fasting glucose (IFG) or impaired glucose tolerance (IGT), i.e., impaired glucose metabolism (IGM), causes diabetic sensorimotor polyneuropathy (DSPN) or chronic idiopathic axonal polyneuropathy (CIAP); the results and conclusions vary considerably in different studies. Some studies suggest that IGM is a common and important cause of CIAP, whereas others do not. On reviewing these data, we judge that a considerable degree of this disparity may relate to differences in selection of patients, choice of controls, assessment of chronic glycemic exposure and of diabetic complications, and statistical power. Here we review previous studies, list the reasons that the issue needs further study, and outline a study now in progress to address the question more definitively.

THERE is now strong interest in the subjects of “pre-diabetes” and the closely related “metabolic syndrome” because these conditions are thought to be highly prevalent and their occurrence increases the risk of diabetes (DM) and cardiovascular, cerebrovascular, and peripheral vascular disease. The expected increased prevalence of DM, postulated to be due to overweight, sedentary lifestyle, and lack of sufficient physical activity, might also be expected to increase the prevalence of polyneuropathy, retinopathy, and nephropathy. Whether obesity, hyperlipidemia, and a sedentary lifestyle (characteristic of the metabolic syndrome), and prediabetes cause polyneuropathy (and retinopathy and nephropathy) is therefore a topic of considerable current interest. The subject of the “metabolic syndrome” was critically reviewed in a recent joint statement from the American Diabetes Association and the European Association for the Study of Diabetes. This report defines the metabolic syndrome as “a clustering of specific cardiovascular disease risk factors whose underlying pathophysiology is thought to relate to insulin resistance.” The report concludes, “While there is no question that certain cardiovascular risk factors are prone to cluster, we found that the metabolic syndrome has been imprecisely defined, there is lack of certainty regarding its pathogenesis, and there is important information missing to warrant its designation as a syndrome.”

Here we focus on the association of impaired fasting glucose (IFG) or impaired glucose tolerance (IGT), here referred to as impaired glucose metabolism (IGM), and diabetic complications of distal sensorimotor polyneuropathy (DSPN). We also consider the nonneurological complications of retinopathy and nephropathy because strong frequency and severity associations link them. We first describe previously published studies, especially associations of IFG or IGT and DSPN or chronic idiopathic axonal polyneuropathy (CIAP), list the possible reasons that there is considerable discrepancy in the data about the frequency of DSPN or CIAP and IGM,
and then describe the design of our prospective, controlled, double-masked (to the degree possible), population-based study, the Rochester Diabetic Neuropathy Study of IGM patients (RDNS-IGM), assessing the prevalence of DSPN, retinopathy, and nephropathy and their severities in IGM and non-IGM patients.

DEFINITIONS AND EARLIER STUDIES

Although it is known that both major types of DM represent more than a state of chronic hyperglycemia, the diagnosis of DM rests critically on elevation of the fasting plasma glucose (FPG) level (≥126 mg/dl). Impaired fasting glucose is usually defined as FPG values ≥100 mg/dl and <126 mg/dl. Impaired glucose tolerance is defined as plasma glucose levels ≥140 mg/dl to <200 mg/dl at 2 hours after a standard oral 75-g glucose load (oral glucose tolerance test), the patient having followed dietary recommendations for several days.

Long ago, Ellenberg9 expressed the view that polyneuropathy might begin before onset of DM, suggesting that factors other than chronic hyperglycemia are implicated in its cause. Because chronic hyperglycemia is known to be involved in the cause of diabetic complications and the lowest level of chronic hyperglycemia, which just causes complications, is the basis of the diagnosis of DM, we take the view that patients without chronic hyperglycemia who develop polyneuropathy may have another cause than DM for their polyneuropathy. That chronic hyperglycemia is implicated in diabetic complications rests on three lines of evidence—controlled single medical center or multicenter treatment trials, epidemiologic studies, and mechanistic studies. In our RDNS, a cross-sectional and longitudinal survey, ~40% of the variability of the severity of polyneuropathy and retinopathy endpoint measurements was explained by a chronic glycemic exposure index [a variable of glycated hemoglobin (A1C), duration of DM, and age of onset of DM]. In the Eurodiab Study of 1,172 patients with type 1 DM, vascular risk factors contributed to the occurrence of neuropathy. However, the usual assumption is that complications are not directly from chronic hyperglycemia itself but from various metabolic derangements secondary to chronic hyperglycemia. It is therefore not unreasonable to question whether a lower level of chronic hyperglycemia than considered diagnostic of DM might cause complications similar to those found in DM, but presumably at a lower frequency and severity than occurs in DM.

The frequency of DSPN was studied in a large cohort of type 2 DM patients in the San Luis Valley Diabetes Study. Age-adjusted prevalence of neuropathy in controls, impaired glucose tolerance, and DM were 3.9%, 11.2%, and 25.8%, respectively. Prevalence odds ratios were 3.5 and 10.6 for the latter two groups as compared to the first group, respectively. Because this was a screening study with minimal evaluations and mainly by nurses, the criteria for the diagnosis of polyneuropathy is a key issue.

An initial study had been done on 38 patients comparing the screening evaluation of nurses with that of a neurologist’s examination. A 90% agreement was reported. The subsets of the examination most sensitive in detecting neuropathy were neurologic symptoms and decreased or absent deep tendon reflexes. Neuropathy correlated with age, duration of diabetes, male sex, and glycemic control, but not with Anglo/Hispanic status.

Rezende et al.24 used heart pulse variations with deep breathing and the Valsalva maneuver as well as the postural blood pressure response to look for evidence of autonomic neuropathy. They reported a higher frequency in patients with IGT than in controls.

Singleton et al.29 prospectively studied 107 sequentially referred patients to the Neuromuscular Division of the University of Utah with CIAP. They reported that 13 of the 107 patients had DM, whereas 36 (34%) had IGT—nearly three times the prevalence of age-matched controls (P < 0.01). They suggested that IGT may cause or contribute to small-fiber neuropathy, similar in phenotype to painful sensory neuropathy. They also noted that the 2-h glucose tolerance test is more sensitive for detection of IGT than other measures. The prevalence of abnormal glucose handling of their patients had been compared to United States adults published by Harris et al.15 In a second report by the same authors, presumably of a nonoverlapping group of 89 patients with idiopathic polyneuropathy in which glucose handling was studied, a similar high frequency (25%) of IGT was found. Lower frequencies were found if IFG or elevated A1C% levels were used as the criteria for study. Although the authors acknowledge that “bias due to referral pattern, body weight, or genetics” might have affected the observed difference in prevalence, they state that an association between IGT and “the painful polyneuropathy of DM has been shown.”

Novella et al.23 studied the frequency of undiagnosed abnormal glucose metabolism in 48 patients with idiopathic sensory neuropathy. Patients with painful polyneuropathy had a higher frequency of
abnormal glucose handling than “literature-based controls.” Likewise, in the study of Sumner et al., oral glucose tolerance tests were given to 73 patients with idiopathic peripheral neuropathy. An oral glucose tolerance test was abnormal in 41 patients, 26 of whom had IGT, and 15 had DM. The authors concluded that the polyneuropathy associated with IGT is milder than that associated with DM. They suggested that small-fiber polyneuropathy is typical of “glucose dysmetabolism.”

By contrast, studying 50 consecutive cases of CIAP and 50 consecutive subjects without polyneuropathy from the same region, Hughes et al. reported that 32% of patients and 14% of controls had IGT or IFG. After adjusting for age and sex, the observed differences were not found to be significantly different (P = 0.45) even in painful neuropathy. By contrast, significantly higher serum triglycerides were found in the polyneuropathy group (P = 0.02). The authors concluded that “environmental toxin exposure and hypertriglyceridemia but not glucose intolerance or alcohol overuse are significant risk factors that deserve further investigation as possible causes of CIAP.”

Focusing on the value of the oral glucose tolerance test as compared to the FPG level but also on the prevalence of abnormal IFG and IGT, Hoffman-Snyder et al. studied a cohort of patients with CIAP. Of 100 consecutive retrospectively analyzed patients, the authors found that IFG “was found to be nearly two-fold higher in CIAP (62%) than in controls previously published by other authors (33%).” When IGT was used as the criterion, an even higher abnormal frequency was found for CIAP. The authors concluded that these results add to previous evidence that IGM may be a risk factor for CIAP.

Epidermal nerve fiber counts have been used as endpoints to assess occurrence and change of DSPN with treatment in IGM and DM. In the earlier study of six patients with IGT, eight patients with early DM, and five controls, it was concluded that fiber counts were abnormal (reduced) in all neuropathy subjects, but correlated poorly with symptoms. It was further observed that “Neuropathy associated with IGT primarily affects small fibers and is similar to early diabetes-associated neuropathy.” In a later study, nerve fiber counts were performed on 3-mm skin biopsies from distal leg and proximal thigh at baseline and after 1 year in 32 patients with IGT. All patients then received intensive diet and exercise instruction. On average after this intervention, nerve counts were improved both in leg and in thigh—the latter improvement being statistically significant (P < 0.004). It was suggested that improvement in diet and exercise “results in cutaneous reinnervation and improved pain.” It was also concluded that epidermal nerve fiber counts were the most sensitive measure of neuropathy change over a period of 1 year. They further concluded that such counts should be used as endpoints in future neuropathy trials.

**PREVIOUS REVIEWS ON THE ASSOCIATIONS**

As a basis for their review, Russell and Feldman emphasize that: (1) chronic hyperglycemia is implicated as a cause of diabetic polyneuropathy, citing the DCCT studies; (2) there is a known association of IFG or IGT with increased risk of coronary and carotid disease; and (3) the severity of chronic hyperglycemia appears to relate to severity of polyneuropathy. With respect to the issue of glucose impairment and polyneuropathy, they urge caution because of their concern that results of a contemporary study may not be truly comparable to that of an earlier reference group. They note, “The Harris data (the healthy subject controls used), however, were obtained in a United States population study with subjects ranging in age from 20 to 74 years and cannot readily be compared with subjects obtained from a selected referral-based cohort.” Referring to the Singleton et al. and Novella et al. studies, they state, “One cannot determine whether the prevalence of neuropathy is greater in a patient with IGT.” However, they do allow for the possibility that IGT may be associated with increased prevalence of DSPN.

Singleton et al. reviewed the evidence (including that of their own studies) that “impaired glucose tolerance serves as a marker for insulin resistance and predicts both large and small vessel complications, independent of a patient’s progression to diabetes.” They also suggest that IGT is independently predictive of diabetic complications (i.e., retinopathy, nephropathy, and polyneuropathy).

In an editorial, Kissel reviewed the strengths and weaknesses of the association between IFG or IGT and DSPN. His pun in the subtitle followed by a question mark, “A Sweet Smell of Success?”, reflects uncertainty regarding the association between IGT and DSPN. His main concern appears to be the “retrospective and uncontrolled” nature of the study and that the only prospective study (referring to the Hughes et al. study) showed an association with triglycerides and not with IGT.

**OUR CRITIQUE OF THE ASSOCIATION**

Although we are open to the possibility that IGM is a covariate of DSPN or CIAP (the two diagnoses not
necessarily being the same), the issue still remains unsettled because results of previous studies are quite variable, with some not showing an association and others showing a definite association and a large effect of IGM on the prevalence of DSPN or CIAP. There are other concerns: (1) the disorders DSPN and CIAP may have overlapping symptoms but probably should be distinguished; (2) results of the two studies\textsuperscript{12,17} using contemporary controls came to different conclusions about the causative role of IGM; (3) the referral bias of patients with DSPN or CIAP attending tertiary medical centers may be great and, in any case, was unascertained; (4) the reference frequency of IGM was assessed at an earlier time\textsuperscript{7} and may now be different; (5) retinopathy and nephropathy, known also to be associated with DSPN, were not included in assessments; (6) only prevalence of occurrence (heavily dependent on definition of minimal criteria) of polyneuropathy was assessed, without taking severity of complications into account; (7) other causes of polyneuropathy were generally not adequately considered or set aside; (8) associations were based only on IFG or IGT, usually not on A1C, but A1C correlates and predicts diabetic complications better than FPG;\textsuperscript{6,7} and (9) epidermal nerve fiber counts still need further study.\textsuperscript{32,33}

We accept that chronic hyperglycemic exposure is a known and major risk covariate for atherosclerotic complications\textsuperscript{13,18} and for the three diabetic complications of neuropathy, retinopathy, and nephropathy. In the RDNS cohort, a glycemic exposure index combining A1C\%, duration of DM, and age of onset of DM correlated and predicted these complications, explaining about 40\% of the variability of the data.\textsuperscript{6,7} The DCCT studies showed that rigorous as compared to conventional control of hyperglycemia prevented or may even have improved neuropathy, retinopathy, and nephropathy.\textsuperscript{1,2} Allowing that IGM may be a risk covariate, we need to know more. What is the frequency, severity, and kind of neuropathy associated with IGM? How soon after onset of IGM and at what level of IGM does neuropathy at various stages of severity develop? Do focal and multifocal neuropathies occur at increased frequencies in IGM? What is needed are population-based controlled cohort studies of diabetic complication in patients with and without IGM studied concurrently and over time.

The claims that epidermal nerve fiber counts are most sensitive among tests and that diet and exercise improve reinnervation\textsuperscript{33} is a claim that awaits confirmation. We note that significant improvement was from thigh skin biopsies—a region that presumably was not symptomatic. We question whether reinnervation has been demonstrated. With loss of weight, surface area might decrease, accounting for some or all of the increase in density of nerve fibers. Therefore, for this and future studies of epidermal nerve fiber counts, corrections for decreased surface areas from weight loss need to be made. Also, a meaningful degree of improvement of epidermal nerve fiber counts will need to be set. It may be too early to decide whether this approach should be used for all future trials; more information on sensitivity, specificity, accuracy, meaningfulness, generalizability, and monotonicity is needed.

What type of study is needed to know whether IGM causes or is a risk covariate for DSPN or CIAP? We suggest cohort studies of complications (polyneuropathy, retinopathy, and nephropathy) comparing patients with known degrees of IGM for known periods of time to comparable cohorts who do not have IGM but whose chronic glycemic exposure is known for the same period of time. Both groups of patients should be drawn without preselection from the same general population, and ideally should be alike except for the degree of chronic glycemic exposure, a random sample of a geographic area being ideal. We accept the criteria for IFG or IGT previously defined. Although difficult, it may be possible to abstract the duration of IGM from the patient’s medical record. Assuming unbiased selection of both cohorts, the next issue is to assess the frequency and severity of complications using objective quantitative and meaningful measures. Generally, neuropathic clinical signs and symptoms, electrophysiologic tests, and specialized sensation and autonomic tests have been used for these purposes. To avoid bias, observations should be based on observers not knowing the final glucose handling status of patients. Counts of fibers in biopsied nerve have been used as endpoints, but generally, now are considered to be too invasive. Epidermal nerve fiber counts might be considered also, but as we note above, further studies are needed.

**DESCRIPTION OF THE ROCHELLE DIABETIC NEUROPATHY STUDY OF IGM PATIENTS (RDNS-IGM)**

With the intent of definitively answering the question as to whether DSPN, retinopathy, and nephropathy are more prevalent and more severe in patients with IGM compared to non-IGM controls, we initiated the RDNS-IGM study on April 1, 2004 (NS36797).

The trial was designed to be prospective, controlled, masked (to the degree possible), and population-based. A patient’s metabolic handling of glu-
cose was to be assessed by currently accepted measures and over prolonged times. Complications of polyneuropathy, retinopathy, and nephropathy were to be assessed quantitatively, comprehensively, and without knowledge of a patient’s glucose handling status.

Our RDNS-IGM study has some of the design features of a prospective, double-blind controlled trial. Although attempts are made to have observers masked as to the IGM status of patients, the physical appearance of patients and the patients’ own knowledge of her/his IGM status prevents true double masking. This comparative study of IGM and non-IGM patients from the Olmsted County population is possible because ~80% of persons ≥18 years old living in the county are medically evaluated at Mayo Clinic Rochester, Minnesota, in a 3-year period and frequently have FPG values determined at such visits, and this information is retrievable. It is therefore possible to identify and invite a cohort of representative persons from the community who have IFG and matched control persons. At present we have evaluated ~200 persons with a goal of evaluating 600. In the following paragraphs we describe our selection of IGM and non-IGM patients for study, recruitment, assessment of glucose handling and of diabetic complications, and planned analysis.

Letters are sent to all persons 18–84 years old having an Olmsted County, Minnesota address, three FPG values ≥110 mg/dl and <126 mg/dl obtained between January 1, 1999, to December 1, 2005, persons having given consent to have their medical records used for research purposes, and without a diagnosis of DM, inviting them to participate in a prospective study. The consenting patients are tentatively selected as the IGM group. Concurrently letters are sent to each of six persons identified as a match for an IGM patient by the criteria of age, gender, and Olmsted County address, and having three FPG values <100 mg/dl for the dates listed above. The first person (of six) who volunteers for study is the healthy subject control for an IGM patient. Informed consent to perform prospective evaluation of glucose handling and of complications is obtained.

Patients are randomly assigned for assessment of nerve, eye, and kidney complications and of glucose handling status without examiners being told whether they are in the IGM or non-IGM cohort. The criteria used to diagnose and stage DSPN have been published. As assessed for this purpose are neuropathic signs, symptoms, attributes of nerve conduction, and quantitative sensation and autonomic function. Where possible, test results are expressed as percentiles and normal deviates using reference values drawn from healthy persons without neurologic disease recruited from Olmsted County. Retinopathy is staged based on seven stereoscopic photographs of each eye and as assessed against standard photographs in the Wisconsin Eye Reading Center. Nephropathy is staged by published criteria. For nerve, eye, and kidney complications, investigators attempt to identify whether the complication is explained by another cause than IGM.

To determine whether complications typical of DM are more prevalent in IGM than in non-IGM, what frequency differences are needed to be statistically significant? Assessing 300 persons per group and doing a one-sided test at 0.05 level, power is 84% of distinguishing 5% versus 1% and 93% for 5% and 0.5% prevalence of complications. Assuming that complications are significantly more prevalent in IGM patients, it will be important to know whether IGM causes symptomatic and meaningful complications and of which kind. It is of course possible that IGM needs to be present for long times (e.g., beyond the 10 years of the present study) to exhibit a measurable or large effect on complications. Therefore, a further study at 15 and 20 years might be needed.

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CASE OF THE MONTH

ABSTRACT: A 74-year-old man presented with progressive weakness involving only his back muscles. Serum protein electrophoresis revealed the presence of a monoclonal gammopathy. Muscle biopsy of affected muscles demonstrated amyloid light chain deposition surrounding individual muscle fibers as well as in the walls of blood vessels in association with vasculitis. Selective involvement of axial muscles in isolated amyloid myopathy has not been previously described. We report the occurrence of axial myopathy with associated vasculitis as a presenting feature of primary (AL) amyloidosis. Amyloidosis should be considered in the differential diagnosis of focal myopathies, since the condition may be responsive to chemotherapy.


AXIAL MYOPATHY DUE TO PRIMARY AMYLOIDOSIS

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Amyloidosis is a generic term that refers to the extracellular tissue deposition of insoluble amyloid fibrils.11 A variety of human protein precursors of amyloid fibrils have been identified. The type of precursor protein, tissue distribution, and amount of amyloid deposition largely determine the clinical manifestations. The most common types of systemic amyloidosis include primary systemic amyloidosis (AL) related to immunocyte dyscrasia; secondary amyloidosis (AA), which usually occurs as a result of chronic infections or inflammatory conditions; and familial forms of amyloidosis.11,13

Primary amyloidosis is a disorder in which insoluble immunoglobulin light chains (AL) form amyloid fibrils that are subsequently deposited into the extracellular spaces in various organs, especially the kidneys, heart, liver, and skin, as well as the peripheral nerves. The most frequently encountered neurological manifestations include carpal tunnel syndrome, sensorimotor polyneuropathy, and autonomic neuropathy resulting in bowel or bladder dysfunction, and orthostatic hypotension.3–5,7,10–13

Although amyloid deposition into skeletal muscle does occur, myopathy is an uncommon presenting feature of AL. Patients with amyloid myopathy usually have nonspecific symptoms of proximal muscle weakness and fatigue. We are unaware of any reports of preferential involvement of axial muscles in amyloid myopathy. We present a case of axial myopathy as the initial manifestation of AL amyloidosis.

CASE REPORT

A 74-year-old previously healthy man sought medical attention for a 6-month history of weakness of his back muscles. He first noted difficulties with bending during golf games. This progressed until he felt that he might collapse while bending over the sink to brush his teeth. He could not tolerate walking or standing for prolonged periods of time because his back was weak and he tended to flex at the spine when standing. He had pain in the upper back, neck, and shoulders. He denied dysphagia or diplopia. There was no history of bowel and bladder dysfunction or of upper- or lower-extremity weakness or numbness. There was no change in appetite or weight loss.
His past medical history was notable for hypothyroidism, hypertension, colitis, and an inguinal herniorrhaphy. His medications included telmisartan, hydrochlorothiazide, levothyroxine, aspirin, mesalamine, glucosamine, and multivitamins.

General physical examination revealed mild upper-thoracic kyphosis and increased lumbar lordosis. There was no head drop. Neurological examination revealed intact cranial nerves II–XII. Motor examination did not reveal any fasciculations, muscle atrophy, or pseudohypertrophy. Tone and power were normal in all muscle groups including neck extensors and flexors. Deep tendon reflexes were normal except for absent ankle reflexes. Sensory examination was normal to all modalities.

Laboratory investigations including complete blood count, serum calcium level and creatinine, thyroid function tests, and erythrocyte sedimentation rate were all within normal limits. Serum creatine kinase (CK) level was elevated at 867 U/L (normal 225 U/L). Serum lactate dehydrogenase was slightly increased at 259 U/L (normal 110–215 U/L). Serum protein electrophoresis revealed a monoclonal spike in the gamma-globulin region, which was identified as lambda free light chain. Quantitative immunoelectrophoresis detected an IgG level of 14.8 g/L (normal 5.83–17.6 g/L). Urine immunoelectrophoresis revealed an elevated protein at 0.2 g/L (normal <0.12 g/L) and the presence of Bence Jones protein. Bone marrow biopsy showed 15% plasmacytosis. Some of the cells displayed lymphoplasmacytic features. Skeletal survey did not detect any lytic lesions. Plain X-rays of the cervical and lumbar spine revealed moderate arthritic changes. Magnetic resonance imaging of the spine demonstrated muscle atrophy and fat infiltration of the paraspinal muscles.

Nerve conduction studies were normal in the upper and lower extremities (Table 1). Repetitive nerve stimulation of the facial nerve at 3 Hz was done to exclude a postjunctional disorder and no decrement was observed. Stimulation of the median nerve with recording from the thenar muscle was done before and after 30 s of exercise to exclude a prejunctional disorder and no increment was observed. Needle electrode examination of the deltoid, triceps, infraspinatus, vastus lateralis, and tibialis anterior muscles was normal. However, throughout the length of the paraspinal muscles as well as the trapezius muscle, positive sharp waves and fibrillation potentials and short-duration, low-amplitude motor unit potentials were identified. Recruitment was early. This pattern was consistent with myopathic changes in these muscles.

Muscle biopsies obtained from the left trapezius and left lumbar paraspinal muscles showed similar findings. Both paraffin-embedded and cryostat sections showed patchy expansion of the endomysium (Fig. 1A,B) due to the deposition of amorphous eosinophilic material that stained with Congo red and demonstrated apple-green birefringence on examination with polarized light (Fig. 1C). These amyloid deposits often surrounded individual muscle fibers, not only by thickening the endomysium but also by infiltrating the space between the basement lamina and the sarcolemma, a feature best appreciated in plastic-embedded sections (Fig. 2A). The amyloid deposits were stained by lambda, but not kappa light chain antibodies (Fig. 1D). Electron microscopy demonstrated a markedly irregular outline of the muscle fibers, as narrow, branching, irregular folds of sarcolemma projected into the surrounding deposits of amyloid, made up of 7–9 nm diameter randomly arranged fibrils (Fig. 2B,C). The muscle fibers showed marked variation in fiber diameter, vacuolar changes, disrupted cytoarchitecture, and scattered necrosis with phagocytosis by macrophages. Numerous regenerating fibers were charac-

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Normal values in parentheses.
characterized by their basophilia and large round nuclei. In addition, evidence of denervation included group atrophy, nuclear knots, type grouping, and focally abundant target fibers, best seen on NADH-tetrazolium reductase stain. However, amyloid deposits were not identified on intramuscular nerves.

Amyloid deposits were prominent in the walls of blood vessels and were often accompanied by inflammatory cell infiltrates made up of lymphocytes, histiocytes, plasma cells, and polymorphonuclear cells involving the vascular wall and surrounding tissue (Fig. 1E). Fibrinoid necrosis of the vessel wall was demonstrated with Martius scarlet blue stain (Fig. 1F).

**DISCUSSION**

This patient presented with isolated weakness of his axial muscles. Neurophysiological tests revealed a
myopathic process affecting only the trapezius and paraspinal muscles. Amyloid (lambda light chain) deposition in relation to muscle fibers and blood vessels in association with vasculitis was demonstrated on muscle biopsy.

Although it has been recognized that amyloid can be deposited into skeletal muscle, the clinical features of amyloid myopathy are often not recognized, and hence it remains underdiagnosed. Two series reviewing the clinical and presenting features of patients with muscle weakness and biopsy evidence of amyloid deposition within skeletal muscle have been published. The larger of the two studies reviewed 79 patients and found that proximal muscle weakness was the most common finding, present in 90% of the patients. Other frequent manifestations included fatigue, autonomic symptoms, weight loss / decrease in appetite, and macroglossia or muscle pseudohypertrophy. An atrophic form of amyloid myopathy exists whereby patients present with muscle weakness and atrophy without pseudohypertrophy and macroglossia. A small proportion of the patients (10%) had distal predominant weakness. A case of amyloid myopathy presenting with distal weakness and atrophy of finger flexors has been described previously.

The second series looking at the clinicopathological features of 16 patients with confirmed amyloid myopathy found that there was a frequent association with plasma cell dyscrasias. In that series, five patients had evidence of malignancy, most frequently multiple myeloma, and four patients had a monoclonal spike detected on serum protein electrophoresis. Histopathologically, amyloid deposition was noted to be perivascular or perimysial in 14 of the patients and endomysial in 7 patients. Neurogenic atrophy was the other major pathological finding present in at least half of the muscle biopsy specimens.

Our patient presents with some typical and some atypical features of amyloid myopathy. His predominant symptom was that of back weakness. Electrophysiological tests confirmed the myopathic changes only in the trapezius and paraspinal muscles. In all the other cases of amyloid myopathy that have been described to our knowledge, patients have had limb weakness with a predilection for proximal muscles. One case of amyloid myopathy involving the diaphragm and resulting in respiratory failure has been reported.

Our patient did not have any autonomic symptoms, dysphagia, weight loss, decrease in appetite, or other symptoms to suggest a more systemic process. He did have an elevated serum CK level. Typically in amyloid myopathy, serum CK levels can be several-fold greater than normal. However, CK levels have been found to be elevated in only one-third of patients in the series reviewing 79 patients with amyloid myopathy. In contrast, the presence of a monoclonal protein in serum or urine was found in the majority (70%) of the patients. This was the only clue to suggest amyloidosis in our patient prior to the muscle biopsy.

The findings of lambda light chain deposits in the walls of blood vessels and in the endomysium as well as neurogenic atrophy in our patient’s muscle biopsy are consistent with other reported findings in patients with amyloid myopathy. What is unusual in our case is the finding of vasculitis. Inflammatory cells were identified in only a minority of muscle biopsies and these were described as small amounts of mononuclear inflammatory cells in the endomysium or perivascular spaces. In none of the other cases of amyloid myopathy was the presence of fi-
brinoid necrosis of vessel walls demonstrated. Although vasculitis (including granulomatous angiitis) in association with cerebral amyloid angiopathy has been well described in the literature, the association in muscle has not. The mechanism by which amyloid deposits cause injury and subsequent necrosis of muscle fibers remains unknown. Several mechanisms have been postulated, including altered electrical conduction, and ischemia as well as amyloid mechanically interfering with muscle function. Our finding of amyloid deposition between the basal lamina and sarcoclemma lends support to the hypothesis that amyloid interferes with the mechanical functions of muscle cells. The encasement of individual muscle fibers with amyloid leads to a disruption of the delivery of nutrients and removal of waste products from muscle cells, thereby resulting in ischemia. Although we did not find amyloid deposition in intramuscular nerves, evidence of neurogenic atrophy was demonstrated. This is in keeping with other reported findings in amyloid myopathy and may relate to an effect of amyloid on more proximal nerves.

The involvement of only axial muscles in this case raises the diagnostic possibility of axial myopathy as a separate clinical entity. Axial myopathy has been described as a disorder whereby a selective involvement of spinal muscles occurs, resulting in a progressive stooped posture (“bent spine”) or dropped head. Electromyography shows a pattern of myopathic changes and the serum CK is elevated. The differential diagnosis is broad and includes dystrophies, metabolic and inflammatory myopathies such as focal myositis, and neuromuscular junction and motor neuron disorders. Our case demonstrates that amyloidosis, which has not been included in the differential of axial myopathy, should also be considered.

This case highlights the difficulties that exist when evaluating patients with atypical forms of muscle weakness, particularly when the weakness is so localized to one group of muscles. These symptoms can often be overlooked as they are nonspecific and can mimic those seen in other inflammatory myopathies. In addition, serum CK levels can be elevated, as is the case in various other myopathies. Thus, the clinician needs to be rigorous in the assessment of patients with progressive muscle weakness and consider the diagnosis of amyloid myopathy even in the absence of macroglossia, muscle pseudohypertrophy, peripheral neuropathy, autonomic symptoms, dysphagia, or a known plasma cell dyscrasia. Serum and urine electrophoresis are good initial screening tests to assess for the presence of a monoclonal protein, which if detected is a clue to the presence of systemic amyloidosis. Ultimately, the utility of muscle biopsy with the appropriate Congo red staining and immunohistochemistry for lambda and kappa light chains cannot be understated in the evaluation of patients with suspected amyloid myopathy.

Amyloid myopathy, with its various phenotypic expressions including focal presentations should be considered in the differential diagnosis of progressive myopathy. It can be the first manifestation of systemic amyloidosis, as was the case with our patient. A timely diagnosis may potentially affect treatment initiation with melphalan, although the prognosis for amyloid myopathy is generally poor.

REFERENCES

ABSTRACT: A 30-year-old man with essential cryoglobulinemia presented with an axonal neuropathy and was found to have vasculitis at nerve biopsy. After 44 months, in accord with clinical deterioration, motor conduction studies showed excessive temporal dispersion multifocally, with partial conduction block persisting for 3 years. Antibody testing showed the presence of IgM anti-GM1, anti-GD1a, and anti-GM2 antibodies. Transitory conduction block has been reported occasionally in patients with vasculitis. The persistent multifocal conduction abnormalities found in this patient were more likely due to a superimposed immunomediated demyelination rather than to chronic nerve ischemia secondary to vasculitis.

PERSISTENT MULTIFOCAL CONDUCTION BLOCK IN VASCULITIC NEUROPATHY WITH IgM ANTI-GANGLIOSIDES

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The association of cryoglobulinemia and peripheral neuropathy, with or without hepatitis C virus infection, is well known.1,3,8 Patients usually present with a predominantly sensory axonal neuropathy or an axonal mononeuritis multiplex due to vasculitis.1,8 Conduction block (CB) and excessive temporal dispersion (TD) are the electrophysiological correlates of demyelination and remyelination.35,37 However, CB has been reported occasionally in vasculitis, although its reality and significance have been questioned.6,12,17,20,31

We report a patient with cryoglobulinemia and biopsy-proven vasculitic neuropathy who developed, during a period of clinical worsening, excessive TD of proximal compound muscle action potentials (CMAP) and partial CB in multiple nerves. These abnormalities persisted for 3 years and were associated with IgM anti-ganglioside antibodies.

CASE REPORT

A 30-year-old man with a history of autoimmune thyroiditis presented with intermittent fever and asthenia. He was treated with antibiotics and, after developing hemolytic anemia, with steroids for 3 months. During treatment, fever remitted and asthenia diminished, but 1 week after steroid discontinuation his fever relapsed. Laboratory testing revealed increased erythrocyte sedimentation rate (109 mm/h; normal, <12) and C-reactive protein (6.79 mg/dl; normal, <0.5), positive rheumatoid factor (189 IU/mL; normal, <15), and cryoglobulinemia (2%). Serum protein electrophoresis showed a monoclonal component in the gamma region (γ globulins 23.8%; normal, 10–22) and light chain kappa-IgG type on immunofixation. Complement levels decreased at onset (C3 89.6 mg/dl, normal 90–180; C4 2.9 mg/dl, normal 10–40) but returned to normal after 1 month. Serology for human immunodeficiency virus, hepatitis C and B, hepatitis C virus RNA, autoantibody screening for connective tissue diseases, and antineutrophil cytoplasmic antibodies were negative. Bence–Jones protein was absent. Bone marrow biopsy excluded a lymphoproliferative disorder and skeletal investigation for myeloma was negative. Reinstitution of steroids (methylprednisolone 32 mg/day, tapered and stopped over 4 months) induced clinical improve-
ment, but at steroid withdrawal fever and asthenia relapsed along with the development of numbness and tingling in the legs. Neurological examination showed difficulty in walking on the heels and toes, more on the left; absent ankle and knee deep tendon reflexes; and loss of touch and pinprick sensation distally in the lower limbs, more evident on the left. Strength in the upper limbs was normal.

Nerve conduction studies were performed with a Viking IV system (Nicolet Biomedical, Madison, Wisconsin). Motor nerves were stimulated at conventional sites (wrist and elbow for median and ulnar nerves; ankle and knee for peroneal nerve) and CMAPs recorded by surface electrodes. To evaluate CB and TD, the amplitude and duration of the negative phase of the CMAP were measured. Excessive TD and partial CB were defined according to the criteria of the American Association of Electrodiagnostic Medicine. The first electrophysiologic study showed slightly slowed conduction velocities of the peroneal nerves, with reduced amplitude of CMAPs (right: 0.8 mV, 41 m/s; left: 0.4 mV, 39.5 m/s). Right and left sural sensory nerve action potentials (SNAPs) were not recordable. Nerves conduction in upper limbs were normal. Sural nerve biopsy showed severe loss of myelinated fibers and signs of epineurial vasculitis with intramural mononuclear infiltration. Some vessels showed fibrinoid necrosis, whereas others showed intimal thickening with almost total occlusion (Fig. 1). Neither thinly myelinated fibers nor naked axons were present. Immunostaining for immunoglobulins was negative.

Steroids were reintroduced (methylprednisolone 8 mg/day for 2 months, then prednisone 75 mg/day that was slowly tapered and stopped over 1 year) and the patient remained stable with improvement of the paresthesias in his lower extremities. Eighteen months after onset, immediately after steroid suspension, the patient began to have tingling and numbness of the hands. Cryoglobulins were increased (8%). Protein immunofixation electrophoresis showed a monoclonal component of light chain kappa-IgG type, without polyclonal or oligoclonal components. C4 level was decreased (5 mg/dl), whereas C3 was normal. Type 1 cryoglobulinemia was confirmed and a follow-up screening for lymphoproliferative disorder was negative.

The electrophysiological studies were repeated 18 months after the onset of neurological symptoms. Median CMAPs were reduced with normal conduction velocities (right: 3.5 mV, 54 m/s, and left: 1.5 mV, 53 m/s), whereas right ulnar CMAP and conduction velocity was normal (10.3 mV, 53 m/s) (Fig.

FIGURE 1. Sural nerve biopsy. (A,B) Serial transverse cryostat sections; scale bar, 50 μm. (A) Epineurial vasculitic lesion with obliteration of lumen by fibrinoid necrosis (hematoxylin–eosin). (B) Intramural infiltration of CD4+ lymphocytes (indirect immunoperoxidase). (C,D) Epon-embedded semithin transverse sections. Toluidine blue; scale bar, 50 μm. (C) Marked loss of myelinated fibers with normal myelin thickness in the few residual small-diameter fibers. (D) Thrombosed endoneurial microvessels (arrow).
There was neither excessive TD of proximal CMAPs nor evidence of CB. Right median SNAP was not recordable. Amplitude of right ulnar SNAP was reduced (1.3 mV, 56 m/s). The patient was treated with eight plasma exchanges, prednisone (25 mg/day) for 7 months, and azathioprine (100 mg/day) and slowly improved, with partial recovery of strength in hand muscles. Two months after treatment, cryoglobulins were 5%. Forty-four months after onset the patient had a worsening of hand muscle weakness and paresthesias involving the cutaneous territories of the median and ulnar nerves bilaterally. Motor conduction showed excessive TD and partial CB in the forearm segment of both median and the right ulnar nerves (Fig. 2). Peroneal conduction showed reduced CMAP amplitudes with normal conduction velocities (right: 0.6 mV, 42 m/s; left: 0.6 mV, 40 m/s). These findings persisted almost unchanged at sequential recordings for 3 years (Fig. 2). At month 55, cryoglobulins were 6%. Seventy-one months after onset, enzyme–linked immunosorbent assay for antibodies to gangliosides was performed as previously described and positivity was assigned at a titer ≥1:200.\(^4\) Enzyme–linked immunosorbent assay showed high titer of IgM anti-GM1 (1:3200) and anti-GD1a (1:3200) and a lower titer of IgG anti-GM2 (1:800). There were neither IgM nor IgG anti-GD1b. Cryoglobulins were 15%. At month 76 azathioprine was increased (150 mg/day) and the patient was treated again with six plasma exchanges. After 3 weeks he reported a slight improvement of weakness of intrinsic hand muscles. At this time cryoglobulins were decreased (5%). Electrophysiological testing at month 80 showed persistence of TD and partial CB in both median and right ulnar nerves (Fig. 2).

At month 86 protein immunofixation electrophoresis showed a persistent IgG monoclonal component along with an IgM oligoclonal component consistent with a type II cryoglobulinemia.

**DISCUSSION**

The patient we report presented with an asymmetric sensory-motor axonal neuropathy and nerve biopsy showed vasculitis with severe axonal loss without demyelinating features.

Fourty-four months after onset, when there was worsening of hand muscles weakness, motor conduction studies showed excessive TD of proximal...
CMAPs and partial CB in nerves of the upper limbs. CB and excessive TD are the electrophysiological hallmarks of demyelination and remyelination\textsuperscript{35,37} and when persistent are characteristic of Lewis–Sumner syndrome and multifocal motor neuropathy (MMN).\textsuperscript{16,21,29,33} CB has been described occasionally in vasculitis. Ropert and Metral\textsuperscript{31} reported five examples of what they called CB in 32 patients with a mononeuropathy multiplex associated with necrotizing vasculitis. In all nerves the CMAP proximal amplitude dropped by at least 50% but in four of five nerves distal CMAP amplitude was markedly reduced (0.2–0.4 mV). The fifth case was a tibial nerve with a distal amplitude of 1.5 mV. Follow-up was available in four cases: in two nerves there was complete or near-complete denervation that did not allow CB assessment. In the other two nerves CB disappeared during treatment, in one without evident change of distal CMAP amplitude. These findings are questionable, as the decline in amplitude of proximal CMAP could be alternatively ascribed to: (1) submaximal stimulation at proximal sites where the nerve is deeply located, as in the tibial nerve; (2) interphase shift and cancellation between the few remaining motor units after stimulation at a proximal site, resulting in a further amplitude reduction of proximal CMAP compared to distal CMAP\textsuperscript{16,30}; and (3) pseudo-CB due to ischemic focal axonal damage occurring in the nerve segment between the proximal and distal stimulation sites within a few days preceding the electrophysiological study. In the latter setting, proximal stimulation evokes a response with smaller amplitude than distal stimulation. Sequential studies over the following days would show a progressive drop in the distal CMAP amplitude because of advancing Wallerian degeneration.\textsuperscript{6,17,18}

Jamieson et al.\textsuperscript{12} reported a patient with necrotizing angiopathy who had in the ulnar nerve a distal CMAP of 6.0 mV and a proximal CMAP of 0.5 mV; 21 days later, corresponding values were 4.2 mV and 3.6 mV, respectively. There was no excessive TD in any response and the remaining electrophysiological findings reflected axonal loss. Although there was a reduction of distal CMAP amplitude between the two recordings, the authors speculated that preservation of distal CMAP amplitude implied that this block was not simply due to conduction failure in a nerve undergoing Wallerian degeneration. More recently, Mohamed et al.\textsuperscript{20} reported a patient with a 2-year history of symmetrical sensory-motor neuropathy who showed CB in two nerves that persisted for 1 month. Sural nerve biopsy demonstrated severe fiber loss and a teased-fiber study demonstrated 45% of fibers with segmental demyelination but only 4% undergoing axonal degeneration. No evidence of fascicular involvement, a feature that may suggest vasculitis, was reported. Epineurial vessels were surrounded by mononuclear cells and some vessels were reported to have focal necrosis of walls with invasion of polymorphonuclear cells. However, necrotizing vasculitis was not adequately shown in the published figure.\textsuperscript{20} CBs markedly improved after treatment with steroids and azathioprine for 6 months. We agree with McCluskey et al.\textsuperscript{17,18} that in this patient the clinical, electrodiagnostic, and pathological findings are more consistent with an acquired demyelinating neuropathy rather than a vasculitic neuropathy.

Reversible CB has been described in ischemic (nonvasculitic) neuropathies.\textsuperscript{11,14} In these cases the prompt resolution of CB without development of excessive TD has been thought to be incompatible with segmental demyelination.\textsuperscript{14}

Transient (hours) or prolonged (days) CB has been reported following experimental acute occlusion of vasa nervorum by injecting low and high doses of arachidonic acid.\textsuperscript{27,28} Morphological examination did not reveal evidence of segmental demyelination, suggesting that in this experimental setting CB has a metabolic nature. Parry and Linn\textsuperscript{28} suggested that hypoperfusion of the region surrounding the infarct, through which surviving axons pass, may be sufficient to block impulse transmission without inducing morphological changes. Fowler and Gilliatt,\textsuperscript{7} after ligation of iliac arteries in rabbits, observed evidence of axonal degeneration and CB in a minority of surviving fibers lasting as long as 5–11 days. They also reported a small amount of paranodal and segmental demyelination compared to Wallerian degeneration.\textsuperscript{7} Nukada et al.\textsuperscript{22} suggested that the sequence of pathological alterations of acute experimental nerve ischemia induced by microsphere injection was axonal swelling and attenuation with secondary demyelination. Therefore, although few human cases are reported, it is likely that CB, either metabolic or due to segmental demyelination, is present in some axons around the infarcted area in vasculitic neuropathy. CB does not seem to last more than few days and does not modify substantially the overall axonal picture in patients with nerve vasculitis.

In the patient we report, excessive TD and CB appeared 44 months after the diagnosis of an axonal neuropathy due to vasculitis and persisted at sequential recordings for 3 years. Such long-lasting focal conduction abnormalities have never been described in vasculitis or ischemic neuropathies, at least to our knowledge. This prompted us to test for
anti-ganglioside antibodies. We found high titers of IgM anti-GM1 and anti-GD1a and a lower titer of IgM anti-GM2. This might be a mere epiphenomenon without pathogenetic significance consequent to the primary nerve damage due to vasculitis with the exposure of normally sequestered neural epitopes. Alternatively, these antibodies may play a role in the induction and persistence of focal conduction abnormalities. Monoclonal or polyclonal IgM anti-GM2 antibodies have been reported in rare patients with chronic motor or predominantly motor neuropathy. 

IgM anti-GM2 antibodies have been found in 25%–80% of cases of MMN. 

GM1 is the ideal target for an IgM antibody to GM1 are found in 25%–80% of cases, as previously stated, crossreacts with GM2. Anti-GD1a could possibly be anti-GalNac-GD1a, motor neuropathy. In the patient we report, IgM (with IgM M-protein) or a demyelinating sensory-motor demyelinating neuropathy with high titers have been associated in rare patients with GM2/GalNAc-GD1a/GalNac-GM1b crossreactive antibodies had a demyelinating motor neuropathy for 8 years.

GM2 antibodies share immunoreactivity with Gal-Nac-GD1a and Gal-Nac-GM1b; these three gangliosides have an identical terminal trisaccharide sequence. GM2 antisera from neuropathy subjects and normal controls bind to the NSC-34 cell line, which expresses high levels of membrane-associated GM2 and are capable of complement-mediated lysis of these cells. However, GM2 is not detectable by standard immunohistological techniques in human or rodent peripheral nerves. This casts doubt concerning the pathophysiological significance of anti-GM2 antibodies in chronic neuropathy, especially in patients with low titers, as the patient we report.

IgM anti-GD1a antibodies are infrequent and high titers have been associated in rare patients with a predominantly motor demyelinating neuropathy (with IgM M-protein) or a demyelinating sensory-motor neuropathy. In the patient we report, IgM anti-GD1a could possibly be anti-Gal-Nac-GD1a, which, as previously stated, crossreacts with GM2. IgM antibodies to GM1 are found in 25%–80% of cases of MMN. GM1 is the ideal target for an immune response causing CB, since it is enriched in the nodal and paranodal region and antibodies to GM1 bind to the Ranvier node.

Intraneural injection or exposure to serum of patients with high anti-GM1 antibodies were capable of inducing focal CB in vivo or in vitro. However, these results were not confirmed in studies using purified anti-GM1 antibodies or in isolated fibers studies even when binding of the antibodies to the node and consequent complement activation was demonstrated. Although the role of anti-GM1 antibodies remains to be fully clarified, we think they are the most likely candidate for inducing and maintaining the multifocal conduction abnormalities in the patient we report. Experimental studies of acute synchronously demyelinating lesions showed that recovery from CB is characterized by dysynchroniztion and increased duration of CMAPs due to TD and slowing of conduction velocity indicative of remyelination. Persistent CB or excessive TD are characteristic of Lewis-Sumner syndrome and MMN. In MMN, pathological changes of nerves at the site of CB have rarely been studied. Taylor et al. found an increased frequency of remyelinated fibers and prominent clusters of regenerating fibers. The authors hypothesized that an antibody-mediated attack, directed against components of axolemma at the nodes of Ranvier, could cause conduction block, transitory paranodal demyelination and remyelination, and axonal degeneration and regeneration.

To explain the persistence of focal conduction abnormalities, it could be hypothesized that a demyelinating lesion, once produced, may persist as a result of impaired remyelination caused by a disrupted blood–nerve barrier or antibody binding.

In conclusion, although we do not have direct evidence, it is likely that in this patient persistent multifocal conduction abnormalities resulted not from chronic nerve ischemia secondary to vasculitis, but from a superimposed immunomediated demyelination and defective remyelination, possibly due to anti-GM1 antibodies.

REFERENCES


