Drug-induced liver injury is an unresolved problem, with an impact well beyond the number of actual cases that occur annually. It has emerged as the most frequent cause for aftermarketing withdrawal of medications, despite a rigorous preclinical and clinical review process. This points to serious limitations in current knowledge regarding mechanisms of hepatic toxicity, in methods for identifying susceptible individuals and in preclinical test systems. For compounds that cause predictable dose-related toxicity, such as acetaminophen, mechanisms are generally well understood. However, these constitute a small proportion of the total. For idiosyncratic reactions—recent examples include bromfenac and troglitazone—little is known regarding mechanism; specific kinds of individual susceptibility are involved, possibly related to environmental or genetic factors that remain to be identified. Although this type of reaction is infrequent, it can be fatal. Unfortunately, it cannot be excluded by current approaches to preclinical testing. A workshop sponsored by the National Institutes of Health entitled “New Directions in Drug-Induced Liver Injury: Mechanisms and Test Systems” brought together a group of experts in this area with the goal of reviewing the status of the field, defining areas in need of study, and proposing next steps.

THE BURDEN OF HEPATOTOXICITY
D. E. Kleiner, W. M. Lee, J. R. Senior, and W. F. Balistreri

The pathology of drug-induced liver disease covers a broad spectrum, from acute hepatic necrosis, chronic hepatitis, and vascular injury to bile ductular injury and neoplasms. While not diagnostically helpful, the pattern may provide insight as to the mechanism of injury and prognosis. A role of drugs in liver injury can also be suggested by the characteristic time interval between the administration of the suspected toxin and onset of the injury. Coexisting liver disease also can impact the pattern of injury. The most feared pattern of injury is massive necrosis, presenting as acute liver failure (ALF). About 2,000 cases of ALF occur annually, often in the relatively young and in women (75% of the total). More than 50% of ALF cases in the United States are due to drug toxicity: 36% from acetaminophen and 16% idiosyncratic drug reactions. The latter group tends to involve older patients and carries the worse prognosis, with only 18% recovering without transplantation. The ALF Study Group is prospectively collecting data on patients with ALF admitted to more than 20 referral centers in the United States, in an effort to identify factors associated with this condition and to conduct controlled trials. This is an example of clinical surveillance, which is a necessary first step in the detection and confirmation of any form of drug-induced liver toxicity. A current issue concerns the best method for surveillance. Although alanine transaminase elevation (>3-fold) has been used historically, it lacks specificity; addition of serum bilirubin elevation may improve specificity but reduces sensitivity. Another issue concerns drug reactions in children. Although uncommon, they may indicate a special sensitivity in the pediatric age group: the classic example is aspirin and Reye’s syndrome. Also, drug dosage and frequency for adults cannot be applied directly to children, as absorption, distribution, excretion, and metabolism all may change with development. Often the approval of drugs for adult therapy is not accompanied by adequate data on use in children. The result can be inappropriate dosing, a preventable cause of hepatotoxicity.

DIRECT HEPATOTOXICITY AND CYTOPROTECTION
P. F. Malet, A. Wendel, M. J. Czaja, R. Voellmy, F. P. Guengerich, G. J. Gores, and J. S. Leeder

Troglitazone-associated hepatotoxicity is a recent example of idiosyncratic, direct hepatocyte toxicity. Troglitazone, a peroxisomal proliferator activator receptor γ agonist, was an approved drug on the market for the treatment of diabetes mellitus. Estimates based on post–marketing surveillance suggest that 2% of patients receiving troglitazone developed serum alanine transaminase values greater than 3 times the upper limit of normal, approximately 1:1,250 developed jaundice, and 1:40,000 to 50,000 had irreversible liver failure leading to death or liver transplantation. The drug is predominantly metabolized in the liver by conjugation to sulfate or glucuronic acid. However, metabolism to a quinone intermediate by CYP2C8, CYP3A4, and CYP2C19 also occurs; polymorphisms in the latter enzymes may account for hepatotoxicity. Liver histopathology in the published cases revealed diffuse hepatocyte destruction, fibrosis, and occasionally eosinophils. These findings are consistent with a mechanism of direct hepatotoxicity. Two other proliferator activator receptor γ agonists are now on the market, rosiglitazone and pioglitazone. Although structurally dissimilar, these drugs nonetheless raise concerns of hepatotoxicity. To date, 2 nonfatal
cases of hepatotoxicity from rosiglitazone have been published; none from pioglitazone. This clinical example highlights the challenge of documenting drug-associated hepatotoxicity—the infrequent but occasionally devastating consequences.

Inflammatory mediators may potentiate and perpetuate drug-associated hepatotoxicity. A particular focus is the role of the death ligands, Fas ligand, and tumor necrosis factor α (TNF-α), and their cognate receptors, Fas and tumor necrosis factor receptor-1. They can induce liver injury by triggering hepatocyte apoptosis and necrosis. Hepatic apoptosis may be controlled by the intracellular energy status as well as by the redox status of the hepatocyte. Depletion of intracellular ATP stores (a model relevant to the fasting patient with drug-related impairment of mitochondrial function—see below), blocks TNF-α-associated liver injury but potentiates Fas-mediated damage. In contrast, glutathione depletion protects against apoptosis by both death-receptor pathways, a model relevant to the oxidative stress associated with drug metabolism. The intracellular transcription factor nuclear factor κB, AP-1, and c-myc may play a role in cytoprotection. These signaling pathways block TNF-α-mediated hepatotoxicity but themselves can be blocked by redox changes in the cell. For example, increased expression of CYP2E1, a pro-oxidant enzyme that is up-regulated by chronic alcohol consumption and in patients with nonalcoholic steatohepatitis, sensitizes hepatocytes to TNF-α toxicity. This latter observation may be especially important given the high prevalence of these two conditions in the population. The role of modulators of apoptosis in drug-related liver injury deserves greater attention. High throughput screening systems for assessing liver cell apoptosis, changes in apoptosis-modulating genes, and challenge tests (drug plus an apoptotic stimulus) are all feasible and could be adopted to better screen drugs for hepatotoxicity.

Heat-shock proteins, or molecular chaperones, are proteins induced by various forms of stress including drugs and are important for normal folding of nascent proteins, for protein degradation, and intracellular trafficking. As such, they may exert cytoprotective functions and underlie a tolerance towards potentially damaging toxicants. Although no human polymorphisms in these proteins related to idiosyncratic drug reactions have been described to date, an increase in heat shock proteins may help the liver adapt to and minimize drug cytotoxicity. Many drugs initially cause serum alanine transaminase elevations that may return to normal despite continued drug exposure (e.g., isoniazid). The mechanisms by which the liver adapts to this “reversible” injury deserve further attention.

In addition to potentiating the activity of death receptors, drugs also may activate intracellular stress pathways of apoptosis. Protein adducts generated by intermediary drug metabolism is one such mechanism for activation of this cytotoxic pathway. Cytochrome P450 enzymes are the major oxidative catalysts involved in drug metabolism. Polymorphisms are very common in these genes and lead to significant heterogeneity in drug metabolism. Moreover, these enzymes catalyze oxidation reactions generating reactive intermediates that can cause macromolecular adducts. These adducts can inhibit key cellular enzymes, block protein synthesis, and DNA/RNA replication. The emerging technology in proteomics provides a new approach to evaluate the burden of these adducts on cell physiology and their overall role in drug-mediated hepatotoxicity. Also, transgenic mice that lack the mouse PXR xenobiotic receptor but express the human analog, SXR, under a liver-specific promoter, exhibit a “humanized” response to drugs. This approach is potentially valuable for characterizing compounds in vivo with respect to their ability to induce CYP3A genes.

Pharmacogenomics is a new approach to predicting an individual’s risk for hepatotoxicity after administration of a drug. Given a finite number of drug metabolizing processes and pathways of hepatotoxicity and the prospect of a complete database of the human genome, it should be possible to prepare genetic profiles associated with increased risk for various kinds of drug toxicity. Such profiling supposes rapid and inexpensive genetic typing of drug metabolizing enzymes including all the relevant polymorphisms. Obtaining this information will also require the development of repositories for patients’ samples and accurate drug-related histories. The development of such a national repository was deemed one of the more important goals by the consensus panel discussion at the conclusion of the meeting.

**SINUSOIDAL CELL INJURY**

G. B. McDonald, A. P. Bautista, L. D. DeLeve, D. L. Laskin, and I. G. Sipes

Veno-occlusive disease of the liver (VOD) is a potentially fatal complication of high dose myeloablative therapy, as used in preparation for bone marrow transplantation. It is characterized by a progressive loss of sinusoidal endothelial cells (SECs) leading to impaired hepatic microcirculation and is followed by hepatic necrosis. Alkylating agents such as cyclophosphamide, busulfan, and mephalan are the prototypical inducers of VOD in humans. When cyclophosphamide is administered along with total body irradiation, the development of VOD is directly correlated with the generation of a highly toxic metabolite of cyclophosphamide. Variation in the metabolism of cyclophosphamide may account for the unpredictable occurrence of VOD in patients receiving the identical dosing schedule. Genetic polymorphisms in exporters that remove toxic cyclophosphamide metabolites from cells also may be important. Monocrotaline administration produces a VOD-like lesion in rats. In this experimental model, damage to SECs precedes evidence of parenchymal cell toxicity and appears to relate to SEC glutathione. Depletion of glutathione sensitizes animals to monocrotaline, and constant infusion of glutathione or N-acetylcysteine during monocrotaline administration prevents the injury. Nitric oxide also appears to be important in protecting against VOD by preventing endothelial cell swelling. Beyond VOD per se, SECs may be key targets in a number of liver diseases, including those that develop without obvious circulatory impairment.

Kupffer cells and/or inflammatory neutrophils and macrophages have been implicated as mediators of hepatotoxicity following administration of drugs and chemicals. Model agents include alcohol and endotoxin, acetaminophen and carbon tetrachloride, and 1,2-dichlorobenzene and cadmium. Each of these can elicit production of proinflammatory and cytotoxic soluble factors, which may underlie the development of hepatotoxicity. Of particular importance are chemotactic cytokines (chemokines), TNF-α, reactive nitrogen intermediates (e.g., nitric oxide and peroxynitrite), and reactive oxygen intermediates (e.g., superoxide anion, hydrogen
peroxide, and hydroxyl radical). Macrophage-specific inhibitors such as gadolinium chloride indicate that Kupffer cells contribute to drug-induced hepatotoxicity, while specific blockers of macrophage-derived products such as TNF-α, nitric oxide, and superoxide anion point to these factors as mediators of toxicity. However, it appears that their role in tissue injury depends on the nature of the toxicant and the mechanism by which an individual toxicant acts. Thus, macrophage-derived inflammatory mediators can play both protective and pathologic roles in hepatotoxicity. Moreover, individual differences in hepatocellular oxidative stress and/or the molecular signaling that occurs in hepatocytes following chemical exposure may, in part, explain varying susceptibility to chemically induced hepatotoxicity.

**IMMUNOLOGICALLY MEDIATED LIVER INJURY**

**J. Lewis, A. J. Gandolfi, L. Pohl, and N. Shear**

Idiosyncratic and hypersensitivity responses to drugs are unexpected host-dependent reactions that are not dose related and can be persistent, in contrast to classic hepatotoxic reactions, which are predictable dose dependent and host independent. Prototypical inducers of immune-mediated reactions in the liver include sulfonamide antibiotics, halogenated anesthetics, tienilic acid, and dihydralazine. Injury is manifested by the development of hepatitis and, in some instances, granulomas and can be accompanied by fever, skin rash, lymphadenopathy, atypical lymphocytosis, eosinophilia, and jaundice. Immune-mediated drug reactions appear to involve the generation of neoantigens that are formed by the reaction of liver proteins with reactive drug metabolites. This is inferred from circulating specific antibodies in patient sera, which increase promptly upon rechallenge with the antigen. Additional evidence for an immune-mediated pathology is the finding that the response to the drug is delayed and that multiple exposures are required. With regard to experimental models, the guinea pig has emerged as a model of halothane hepatotoxicity, exhibiting many of the same features as those observed in humans including the presence of circulating antibodies that cross-react with a metabolite of halothane covalently bound to liver proteins. However, there is no direct correlation between antibody levels and the extent of injury. An approach that has been explored for cutaneous drug reactions examines the activation of human peripheral mononuclear cells to a drug or drug metabolite in vitro; if a metabolite is the suspected toxin, the reaction must include an initial exposure of drug to hepatic microsomes and NADPH. These assays, which are host-dependent, are a promising approach to identifying susceptible individuals.

The incidence of immune-mediated hepatotoxicity is relatively low, possibly because the liver is tolerantogenic, a property that may be attributable to the liver’s production of anti-inflammatory cytokines (e.g., interleukin 10 [IL-10], IL-6, IL-4, IL-13) and other inhibitory factors (e.g., prostaglandins). The latter can down-regulate Th1 reactions and thus reduce specific immune responses. Support for this hypothesis is the finding that drug-induced hepatotoxicity is increased in transgenic mice with a targeted disruption of IL-4 or IL-10 and increased in mice lacking COX-2. These mediators may function to prevent allergic hepatitis as well as acute hepatotoxicity through inhibition of gamma delta (CD8+) T cells.

**DRUG-INDUCED CHOLESTASIS**

**E. R. Schiff, J. L. Boyer, F. G. Fitz, and G. Alpini**

Cholestasis, defined as an impairment in bile formation and/or flow, is a common manifestation of drug-induced liver disease. Bile is formed by a secretory unit comprised of hepatocytes and cholangiocytes. These polarized epithelial cells function as transport epithelium to generate bile. As expected, disruption of specific transport proteins and processes by drugs can result in cholestasis. Although over 30 different drugs have been reported to cause cholestatic hepatitis, knowledge regarding drug-mediated disruption of transport has only now become available with the recent cloning of specific hepatocyte transporters and the study of cholangiocyte biology.

Most drugs that cause cholestasis are substrates for several transporting polypeptides, which exist at the canalicular surface of the hepatocytes and are members of the ATP-binding cassette superfamily of transporters. Variant transporters may render individuals uniquely susceptible to drug-mediated transport impairment; this kind of information is central to the emerging field of toxicogenomics. Mechanisms of transporter inhibition are being elucidated. For example, women with heterozygosity for a nonsense mutation of the multidrug resistance-3 transporter are susceptible to cholestasis of pregnancy as a result of the high circulating levels of estrogens. Inhibition of transporters by drugs has the potential to explain many examples of drug-induced cholestasis, although convenient test systems are lacking.

Many severe forms of drug-induced cholestasis persist after the drug has been discontinued. Approximately 1% of patients who develop drug-associated cholestatic hepatitis develop progressive destruction of cholangiocytes resulting in the so-called vanishing bile duct syndrome. The mechanisms are obscure but may involve a prolonged decrease in intracellular ATP levels. Changes in cholangiocyte microvillus architecture are prominent in drug-induced cholestasis. Large intracellular bile ducts express CYP2E1 and may be more sensitive to damage by drugs metabolized by this enzyme. More information is needed regarding the mechanisms triggering cell death in this important liver cell subtype.

**DRUG-INDUCED MITOCHONDRIAL INJURY**

**J. H. Hoofnagle, B. C. Tennant, W. Lewis, and R. J. Sokol**

A relatively uncommon but distinctive form of hepatic injury due to drugs is microvesicular fatty liver, which can be caused by alcohol, aspirin, tetracycline, amiodarone, valproic acid, and several antiviral nucleoside analogues, the most prominent of which is fialuridine. The hallmark of this injury is accumulation of microvesicular fat in hepatocytes and decreased numbers of mitochondria. This early lesion can evolve into macrovesicular fatty liver with focal necrosis, fibrosis, cholestasis, bile ductular proliferation, and Mallory bodies, a picture that resembles alcohol-induced liver disease. The same pattern of injury is seen in acute fatty liver of pregnancy and Reye’s syndrome. Clinically, microvesicular fatty liver is characterized by nonspecific symptoms and insidious onset. Nausea, poor appetite, weight loss, weakness, and fatigue predominant, while jaundice and itching are usually uncommon or late. Laboratory tests show hypoglycemia, hyperammonemia, hypoaalbuminemia, and lactic acidosis. Serum aminotransferase levels are mildly elevated if at all. Pancreatitis, peripheral neu-
ropathy, and myopathy all are associated with mitochondrial failure as seen in “high energy” tissues and can accompany microvesicular fatty liver.

Microvesicular fatty liver can cause acute liver failure, chronic liver injury rapidly evolving into cirrhosis, Reye’s syndrome, or merely a solitary hyperammonemia without other evidence of hepatic injury. Reye’s syndrome is the classic clinical presentation of microvesicular fatty liver. It occurs only in children and is associated with viral infections, particularly chickenpox and influenza B. Epidemiologic data linked Reye’s syndrome with aspirin use during viral infection, and subsequent warnings on the use of aspirin in children led to a marked decrease in the incidence of this disease. This is typical of microvesicular fatty liver disease—misdiagnosis of drug-induced injury.

Fialuridine provides the most striking example of a drug causing microvesicular fatty liver and acute liver failure. This is a fluorinated pyrimidine analogue that had shown marked activity against hepatitis B virus in cell culture systems and in the woodchuck hepatitis virus animal model. When given to patients with chronic hepatitis B for up to 28 days, it had minimal apparent side effects and produced a marked and sustained inhibition of serum hepatitis B virus DNA levels. When administered for more than 2 months, however, fialuridine caused an acute liver failure in the majority of patients. Onset was insidious, with nausea and weight loss followed by jaundice. The condition was irreversible despite discontinuation of drug. The immediate cause of death was usually pancreatitis and lactic acidosis. Analysis of tissues showed depletion of mitochondrial DNA in liver and accumulation of fialuridine in both mitochondrial and chromosomal DNA. This syndrome has also been reported in patients receiving other antiretroviral nucleoside analogue therapy, including retrovir (AZT) and didanosine (ddI) but in a more reversible form.

In vitro and in vivo systems have been devised for assessing mitochondrial injury. These tests include incubation of hepatocyte cultures (primary or transformed) with drug and quantitation of mitochondrial morphology, DNA content, respiratory chain enzymes, and lactate production. The reliability of these assays in predicting toxicity is not established. Similarly animal models do not reliably predict mitochondrial toxicity in humans. In the case of fialuridine, administration of drug for up to 6 months to rats, rabbits, dogs, and rhesus monkeys gave no hint of hepatotoxicity. Woodchucks receiving the drug for up to 4 weeks also exhibited no toxicity. However, after 8 to 12 weeks, the animals developed weight loss and ultimately hepatic failure with lactic acidosis. Liver histology showed microvesicular fatty change and abnormal mitochondrial morphology. Immunoassays showed a marked accumulation of fialuridine in both mitochondrial and chromosomal DNA. Of importance, fialuridine hepatotoxicity occurred equally in woodchuck hepatitis–infected as in noninfected animals. Thus, the underlying liver disease did not predispose to this injury. These factors point to the difficulty of screening for mitochondrial toxicity, which is made all the more complex by the frequent use of combination therapy with nucleosides that individually may have only mild effects on mitochondrial function.

The mechanism of fialuridine and other nucleoside toxicity has been evaluated extensively in vitro and in vivo. Fialuridine inhibits DNA polymerase γ (the enzyme responsible for replication of mitochondrial DNA) but has little effect at physiologic concentrations on DNA polymerase α or β. Similar findings for AZT suggest that differential inhibition of DNA polymerase activities may account for mitochondrial toxicity of nucleoside analogues. Depending on drug pharmacokinetics and distribution, this toxicity may affect different tissues, and the clinical presentation can be neuropathy, myopathy, pancreatitis, or hepatic injury.

Inhibition of mitochondrial DNA is probably not the only mechanism responsible for hepatic mitochondrial injury. Both valproic acid and amiodarone have been associated with severe microvesicular steatosis, with rapid evolution to chronic liver disease and cirrhosis. The cause of the hepatic injury is believed to be inhibition of fatty acid β oxidation and mitochondrial dysfunction. In addition, drugs and toxins can inhibit mitochondrial respiratory chain function thereby reducing oxidative phosphorylation and depleting intracellular ATP levels. Inhibition of normal respiratory chain enzymic activity can also generate excessive reactive oxidative species (ROS), causing further cellular injury. ROS probably play a role in several forms of hepatic injury, including that associated with iron and copper overload.

Mitochondria also play a major role in apoptotic pathways, through induction of the mitochondrial permeability transition pore, which results in a rapid increase in mitochondrial membrane permeability and release of cytochrome c and other proapoptotic factors. Thus, mitochondria participate in many forms of hepatic injury. Drugs and toxins that induce mitochondrial permeability transition include salicylates, valproic acid, ethanol, hydrophobic bile acids, and ROS in general. Importantly, substances that block induction of the mitochondrial permeability transition, including ursodiol and cyclosporine, may be protective against these forms of injury. This characteristic can be tested in model systems, allowing for better approaches to control and management of drug-induced liver injury.

**CONCLUSIONS**

1. Drug-induced hepatotoxicity is a significant clinical problem. It is also a problem with major economic impact as the most frequent cause of post–marketing withdrawal of new medications. Expansion of both clinical and basic research into the mechanisms of drug-induced liver injury is warranted.

2. Current preclinical test systems for hepatotoxicity are inadequate, reflecting our limited understanding of mechanisms of drug toxicity, particularly the “hypersensitivity” or “idiosyncratic” types of reactions. “Humanized” transgenic mice represent a potentially important new tool. The primary target of some drugs may be endothelial or Kupffer cells rather than hepatocytes. The role of specific transporters on hepatocytes or cholangiocytes should be examined.

3. A rigorous, multicenter, active, and prospective database on drug-related hepatotoxicity would help promote basic research on hepatotoxicity and would be particularly useful in the development of pharmacogenomics, i.e., the role of inherited factors in rare types of reactions.

4. Drug hepatotoxicity in the pediatric age group is a neglected area and warrants special attention. Important considerations include age-related changes in drug metabolism and adult-pediatric differences with respect to the types and amounts of medications that can cause toxicity.