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Potentiation of the Toxic Effects of Acetaminophen in Mice by Concurrent Infection with Influenza B Virus: a Possible Mechanism for Human Reye's Syndrome?

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Summary

Using weanling mice of two different genetic strains we demonstrated a potentiation of the toxic effects of acetaminophen by prior infection with influenza B virus. The C57BL/6N (B6) strain of mice is genetically predisposed to increased toxicity from acetaminophen when the hepatic cytochrome P-450 mixed function oxidase system is preinduced. When B6 animals are pretreated with influenza B virus and an mixed function oxidase system inducing agent before administering acetaminophen, we observed a significant incidence of atypical "fatty" liver pathology on light microscopy similar to the microvesicular steatosis seen in human Reye's syndrome. Electron microscopic changes in the liver of these animals resemble those published to date in human Reye's syndrome.

Abbreviations

EID_{50}, egg infectious dose 50%
EM, electron microscopy
ER, endoplasmic reticulum
LM, light microscopy
3-MC, 3-methylcholanthrene
MFO, mixed function oxidase
PAS, periodic acid Shiff's reagent
RS, Reye's syndrome
SER, smooth endoplasmic reticulum
$TCID_{50}$, tissue culture infectious dose, 50%
MID_{50}, mouse infectious dose

This study was designed to test the hypothesis, now supported by several investigators, that RS in humans may be caused by the interaction of viruses, environmental factors, and host genetic factors. There is a body of animal data demonstrating the potentiation of effects of viral infection on the host by chemical agents, especially insecticides (6, 8, 16). Acetaminophen toxicity has been shown to be potentiated by alcohol, and in two patients with mononucleosis (9, 13, 23). Theophylline toxicity is increased by concomitant infection with either influenza A or influenza B virus (12, 20). Viral potentiation of certain chemical toxins, *e.g.*, 4-pentenoic acid and aflatoxin, results in an experimental syndrome very similar to human RS (4, 24, 32). Possible explanations for these effects include the adverse effects of xenobiotics on the integrity and function of the immune system, and effects related to the biotransformation of foreign agents, which may be metabolized to pharmacologically active intermediates by components of the cytochrome P-450 MFO system.

RS, or very similar clinical disorders, are found in many different geographic regions of the world. A review of the literature would suggest that RS can be precipitated by many chemically unrelated compounds, each of which has the capacity to cause subclinical, biochemical, and functional abnormalities of the liver and immune system.

We elected to study the effects of influenza B virus infection (which is commonly implicated in the prodromal illness before onset of RS) on two inbred strains of mice which are known to differ significantly in the way that their hepatic MFO system responds to chemical agents. We chose to study acetaminophen for two reasons. First, because it is a commonly used antipyretic in children which is being promoted in the USA, and can be obtained without prescription. Second, because the metabolic pathways for the drug have been well worked out and linked with the aromatic hydrocarbon responsiveness of the MFO system in mice.

In general, all highly lipophilic xenobiotics are rendered more water soluble and hence, more readily excreted, by the microsomal MFO enzyme system located in the SER of the liver and other tisues. Molecular oxygen and reduced NADPH are required for the reaction. Induction of this enzyme system is associated with an increase in microscopically visible SER, and it is interesting that proliferation of SER has been previously reported in cases of RS (2, 26). Most MFO reactions are mediated through the hemeprotein cytochrome P-450.

Mitchell, *et al.* (14) showed that acetaminophen-induced hepatic necrosis is caused by a toxic metabolite of the drug, which binds covalently to tissue macromolecules, including nucleic acids and proteins. The toxic metabolite represents a small part of the original dose of acetaminophen (approximately 4%) when the drug is taken in therapeutic doses. The majority of the drug is conjugated directly with sulfate of glucuronide and then excreted. The toxic metabolite shown in Figure 1 is thought to be produced by metabolism via the cytochrome P-450 enzyme system. This metabolite is detoxified by preferential conjugation with a nucleophilic sulphydryl tripeptide, glutathione. In animal studies, when glutathione in the liver is either depleted (*e.g.*, due to malnutrition) or overwhelmed by a very large dose of acetaminophen, the activated metabolite remains unconjugated and free to combine covalently with vital nucleophilic macromolecules in the hepatocyte, leading to eventual hepatic cell death in a typical centrolobular distribution (Fig. 2).

Thorgeirseen, *et al.* (30) linked metabolism of acetaminophen by the cytochrom P-450 system to the inducibility of aryl hydrocarbon hydroxylase in inbred mice. The degree of aryl hydrocarbon hydroxylase responsiveness has, in turn, been linked to the presence or absence of the gene locus known as the Ah locus, which is inherited as an autosomal dominant in mice (31). There

is suggestive evidence in the literature that a similar gene locus may exist in man (1, 11). C57BL/6N (B6) mice possess the Ah locus and a hepatic microsomal MFO system, which is responsive to induction by aromatic hydrocarbons such as 3-MC and benzo(a)pyrene. The DBA/2N (D2) mice apparently have a different allele at the Ah locus and are relatively unresponsive to hepatic microsomal induction by aromatic hydrocarbons.

Inducers of cytochrome P-450-associated MFO activity, such as 3-MC and phenobarbital, are known to increase the toxicity of acetaminophen, presumably by increasing the quantity of acetaminophen converted to the toxic metabolite. These inducing agents have also been shown to increase covalent binding of acetaminophen to liver tissue, both *in vivo* and *in vitro* (14). The aims of the study were 3-fold. *1*) To determine whether morbidity and/or mortality is increased in mice given sublethal doses of influenza B and acetaminophen, when compared with mice receiving either agent alone. *2*) To determine whether increased morbidity or mortality resulting from administration of influenza B and acetaminophen is associated with aromatic hydrocarbon responsiveness of the MFO system (*i.e.*, presence or absence of the Ah locus) in inbred mice. *3*) To determine whether the histologic changes produced in the liver by these agents are "similar" or "dissimilar" to those seen in RS in humans, using LM and EM.

MATERIALS AND METHODS

B6 and D2 mice of both sexes were supplied by the National Institutes of Health, Small Animal Division. The mice were kept in plastic cages, on standard soft wood bedding, and fed Wayne lab chow *ad libitum*. An automatic day/night cycle (12 h to 12 h) was maintained and exposure to pharmacologically active compounds such as cigarette smoke, insecticides, and pine or cedar bedding were prevented. Animals infected with influenza B virus were housed separately. Animals were studied at the postconceptual age of 50 d (4 d postweaning). A pilot study was first run to determine dosage regimens for acetaminophen and influenza B virus, and to determine the normal structure of mouse liver under the EM.

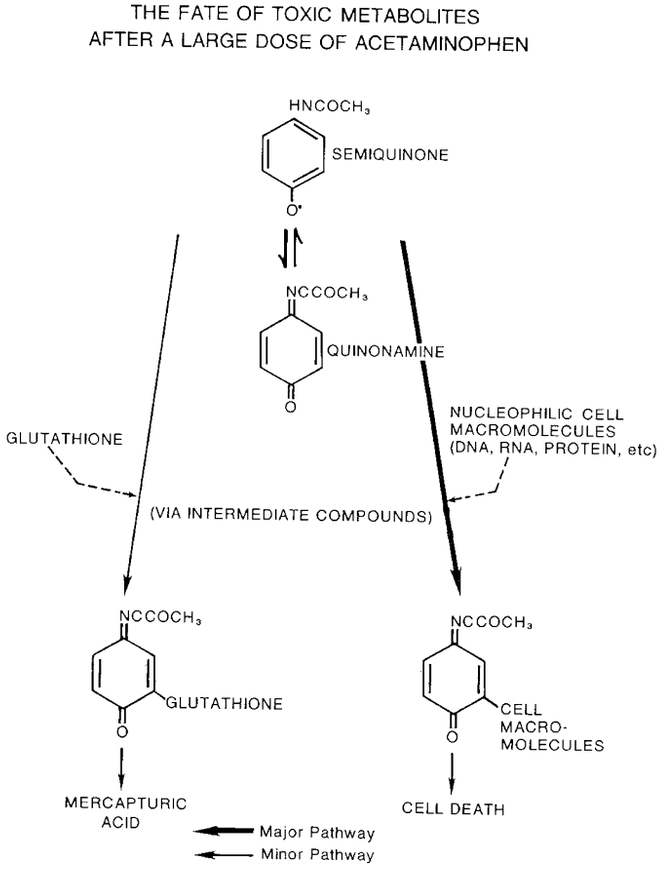

Fig. 1.

Nonmouse-adapted B/Hk/73 seed virus was obtained from the Division of Virology, Bureau of Biologics, U.S. Food and Drug Administration, and initial virus pools were prepared by allantoic inoculation of 9-d-old embryonated eggs. Serial dilutions were then made and injected into 9-d-old embryonated eggs and onto tissue culture plates containing MDCK (canine kidney) cells. The tissue culture plates included liquid overlay, containing trypsin for proteolytic cleavage of the hemagglutinin. Both eggs and tissue cutures were incubated for 72 h. Postinoculation allantoic fluid samples were tested for hemagglutination of chick cells, and culture monolayers were tested for hemabsorbtion, using guinea pig erythrocytes. In this way, the $TCID_{50}$ and EID_{50} were determined.

To determine the MID_{50}, serial 10-fold dilutions of virus were inoculated into mice pernasally, under light ether anesthesia, using 0.05 ml of dilute virus. Forty-eight hours postinoculation the lungs were removed, homogenized in Hank's solution, and inoculated into 9-d-old embryonated eggs. Using a dose of virus 100 times the MID_{50}, we found that virus from lung homogenates from over 90% of study mice would regrow in 9-d-old embryonated eggs after 72 h incubation. Time-course studies gave a period of true infectivity for our mouse population of 7–8 d postinfection. Infection was not associated with readily recognized symptoms. Lung pathology was rarely recognized histologically; minor lung consolidation occurred in approximately 3% of the animals.

Twenty-four hours after virus inoculation, 80 mg/kg, 3-MC was administered intraperitoneally. Twenty-four hours later acetaminophen was administered intraperitoneally (range 150 – 300 mg/kg). Surviving animals were killed by cervical dislocation, 48 h after receiving acetaminophen.

Control groups received: *1*) sterile saline alone or in combination with virus, acetaminophen, or 3-MC; *2*) sterile allantoic fluid alone, or in combination with one or two of the agents in *1*); *2*) virus, acetaminophen, or 3-MC individually or in combination with one of the two other active agents.

Each group consisted of approximately 100 animals. Each experiment was carried out in duplicate, leading to a total *n* of approximately 200/group; however, liver samples from animals dying spontaneously were not examined because personnel were not available to collect samples at the time of death. This was taken into account for purposes of statistical analysis.

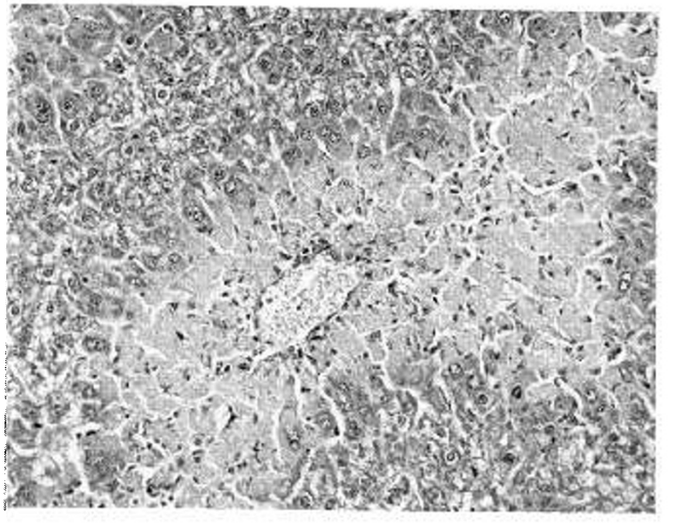

Fig. 2. A section of mouse liver showing the typical centrolobular necrosis caused by acetaminophen.

Liver samples were taken immediately into glutaraldehyde for EM. Other liver samples from the same animals were prepared appropriately for frozen section, and subsequently stained with Oil-red-O for lipid. Further samples were fixed in Bouins for Giemsa and PAS staining, or homogenized for microsomal preparation. Sections for LM were prepared by Baker Histology (Great Falls, VA) and were examined by two of us, M.G.M. and D.N., a veterinary pathologist. D.N. was unaware of which treatment schedule the animals had received. Necrotic and non-necrotic lesions were quantified and graded by the method of Chalkley (3).

Seventy number coded electron microscopic samples, representative of study groups and controls, were examined by P.M. and G.W., also without knowledge of treatment schedule. When macroscopic lesions were recognizable in the liver, representative sections were taken from these areas and, when possible, also from apparently normal areas of the same livers.

Microsomal preparations were made from the livers from 15 animals in each study group (including controls) (33). The animals were killed by cervical dislocation. The livers were immediately removed and, after rapid collection of specimens for LM, weighed and washed with ice cold 0.02 M Tris-HCl/0.1 5 M KCL buffer, pH 7.4. They were then homogenized in 1.5 volumes of the same buffer, using a glass homogenizer with a Teflon pestle. The homogenate was centrifuged at 12,000 *g* for 15 min, and the supernatant was decanted through a double layer of fine meshed gauze. The supernatant was then centrifuged at 105,000 *g* for 45 min at 4°C. The resulting pellet was washed by resus-

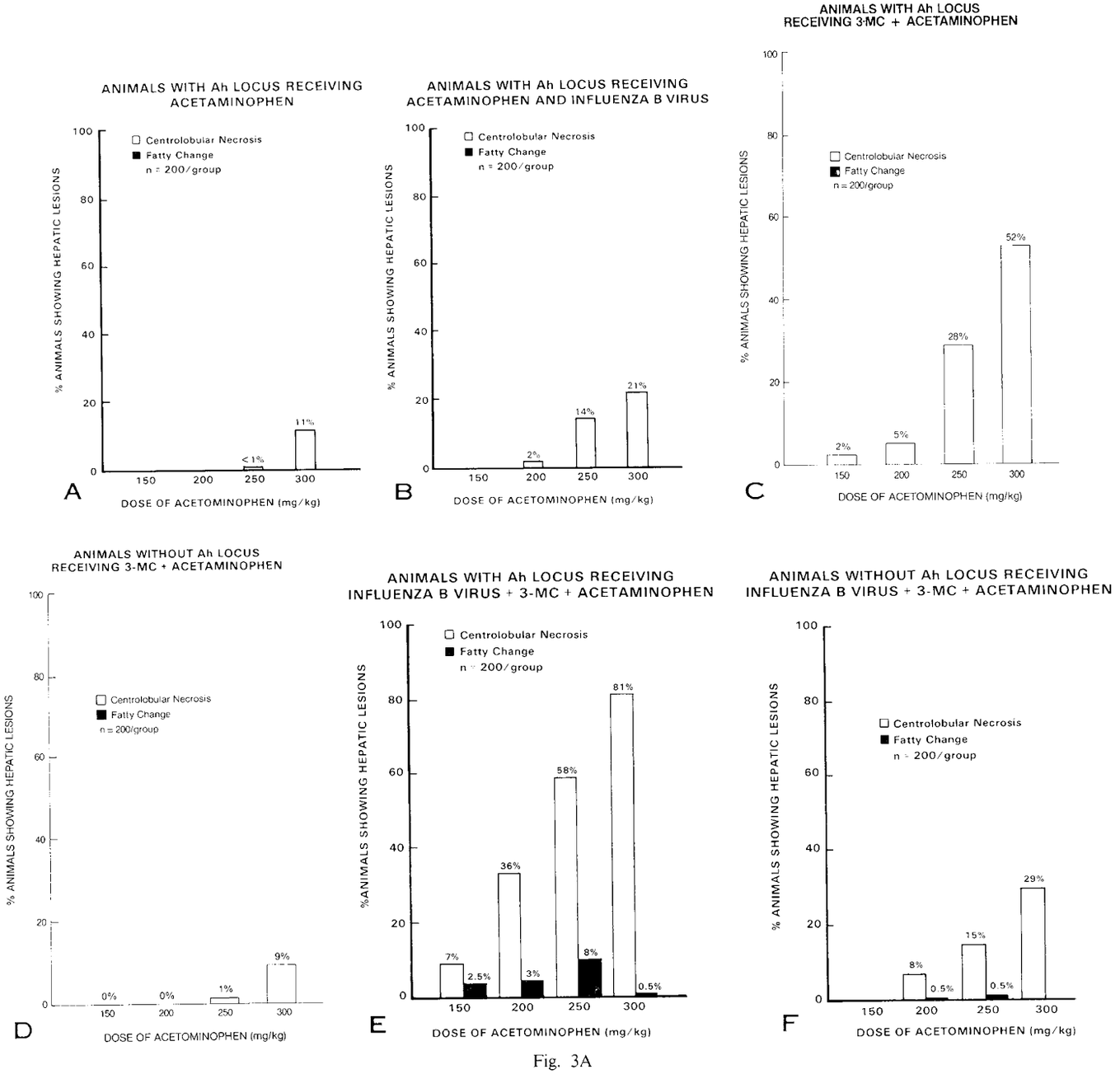

Fig. 3A
Fig. 3B
Fig. 3C
Fig. 3D
Fig. 3E
Fig. 3F

pension in 0.1 M sodium pyrophosphate buffer, pH 7.4, to remove remaining hemoglobin and recentrifuged at 105,000 *g* for 45 min. The pellet was resuspended in 2 ml of 0.02 M Tris-HCl/0.15 M KCL buffer.

Enzyme assay. The protein concentration of the microsomal suspension was determined by the method of Schacterle and Pollack (25) and the cyrochrome P-450 concentration by the method of Omura and Sato (17) from the CO difference spectra of reduced microsomes, using an extinction coefficient of 91 mM-1 cm-1 for Δ 490–450 nm.

RESULTS

LM findings (34).

1) When acetaminophen was given alone, significant centrolubular necrotic lesions (35) typical of acetaminophen toxicity were first recognized on LM at a dose of 300 mg/kg in both B6 and D2 mice (Fig. 3a).
2) When acetaminophen was given to B6 and D2 animals preinfected with influenza B virus, significant centrolobular necrosis first appeared at a dose of 200 mg/kg suggesting some potentiation of acetaminophen toxicity by the virus (Fig. 3b).
3) When the cytochrome P-450 enzyme system was induced with 3-MC, as would be expected, the toxic effects of acetaminophen were markedly potentiated in the B6 mice, and significant centrolobular lesions were first recognized in these animals at a dose of 150 mg/kg (Fig. 3c). A minor potentiation of acetaminophen effect was seen in D2 animals (Fig. 3d).
4) Significant potentiation of the toxic effects of acetaminophen was recognized in both B6 and D2 animals when pretreated with influenza virus before receiving acetaminophen and 3-MC (Fig. 3e and f). In a small number of animals, treated with all three agents, we recognized the appearance of a distinct histologic pathology in the liver: a diffuse microvesicular steatosis. Sixty percent of those with microvesicular steatosis did not have coexisting centrolobular necrosis. The incidence of this finding was significantly greater in the B6 animals, and appeared to be dose related over the range 150–250 mg/kilo.
5) Mortality (within 48 h of acetaminophen administration) was markedly increased in B6 animals, by prior induction of the MFO system with 3-MC when acetaminophen was given at a dose of 250 mg/kilo, in non-induced animals, there was no excess mortality over control animals (approximately 0.5%). With prior induction of the MFO system the rate rose to 6%. When these animals were also pretreated with influenza B virus the mortality rate increased to 19%. [A smaller increase in mortality (~12%) was seen in infected D2 mice].

Inflammatory lesions were seen in less than 2% of the liver samples. Glycogen depletion, recognized on PAS-stained liver sections, was most often associated with severe centrolobular necrosis, but was not specific to any treatment regimen.

EM findings. A total of 70 mouse livers were examined by EM, including livers from B6 and D2 mice, receiving 150 or 200 mg of acetaminophen plus influenza B virus and 3-MC. Also examined were livers from untreated animals, and animal receiving: *1*) sterile saline alone or in combination with virus, acetaminophen, and/or 3-MC; *2*) sterile allantoic fluid alone, or in combination with one of the two other active agents and/or 3-MC.

No remarkable changes were seen in the controls, except in the case of some D2 animals receiving undiluted influenza B virus examined 8 d after infection. These animals showed an increase in number of microbodies in some cells and some disruptions of the mitochondrial ultrastructure (Fig. 4).

The D2 animals receiving 150 mg acetaminophen plus 3-MC and virus showed some loss of glycogen and slight distention of the endoplasmic reticulum. The D2 animals receiving 200 mg of acetaminophen plus 3-MC and virus showed almost complete loss of glycogen, giving the liver parenchymal cells a moth-eaten appearance. Except for a slight sporadic distention of the ER, the other cytoplasmic organelles were spared (Fig. 5).

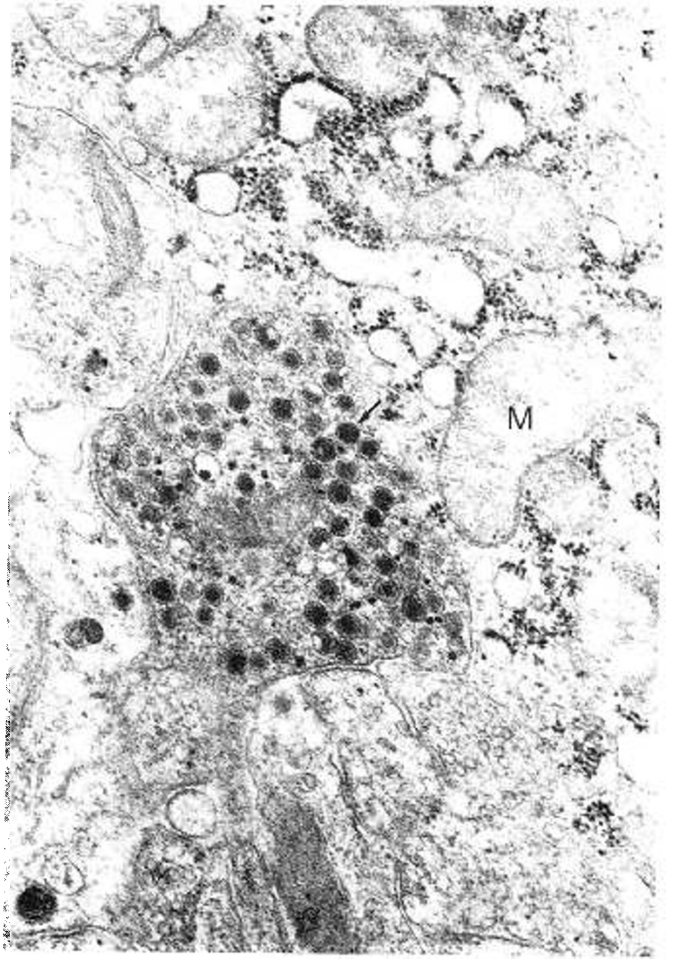

Fig. 4. D2 mouse. Receiving influenza B virus only, showing increase in microbodies (*arrow*) and some mitochondrial changes (*M*).

The B6 mice receiving acetaminophen, 3-MC, and virus showed profound changes. The B6 animals receiving 150 mg of acetaminophen plus 3-MC and virus showed what appeared to be numerous vacuoles in the cell cytoplasm. Closer examination of these vacuoles revealed the fact that these structures were distentions of the endoplasmic reticulum. Other organelles appeared relatively normal (Fig. 6).

The B6 mice receiving 200 mg of acetaminophen plus 3-MC and virus showed progressive distention of the ER, with almost complete disruption of the normal cytoplasmic architecture in many of the liver parenchymal cells (Fig. 7). In some animals the organelles appeared so profoundly disrupted that many of the cells were dead, and the nuclear ultrastructure showed loss of normal organization. Fatty accumulation, corresponding to the microvesicular steatosis seen on LM, appeared to be within the distended ER (Fig. 6).

These changes seen in the B6 mice were mostly confined to the liver parenchymal cells, but an occasional Kupfer cell showed similar changes. The other cells of the liver, such as the cuboidal epithelial cells of the bile ductules and endothelial cells, appeared unaffected.

Enzyme assays. Arithmetic means of the experimental data were compared using Student's *t* test, with $P < 0.05$ for the two-tailed test as the limit of significance. The expected increase in cytochrome P-450 on induction with 3-MC was confirmed. But

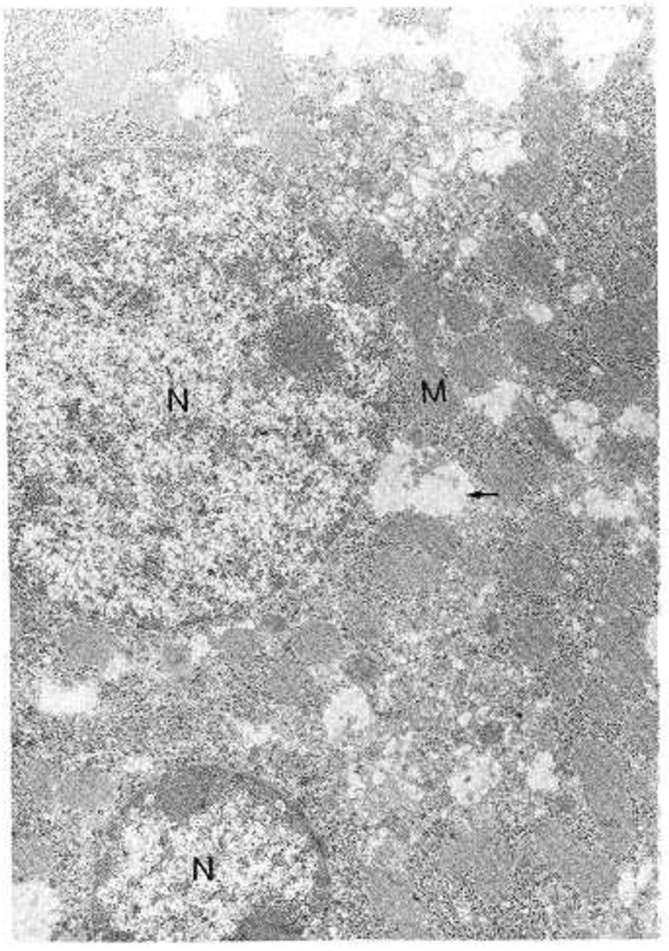

Fig. 5. Hepatocyte from D2 mouse which received 3-MC virus and 200 mg of acetaminophen, showing loss of glycogen (*arrow*). (N) nucleus and (M) mitochondrium.

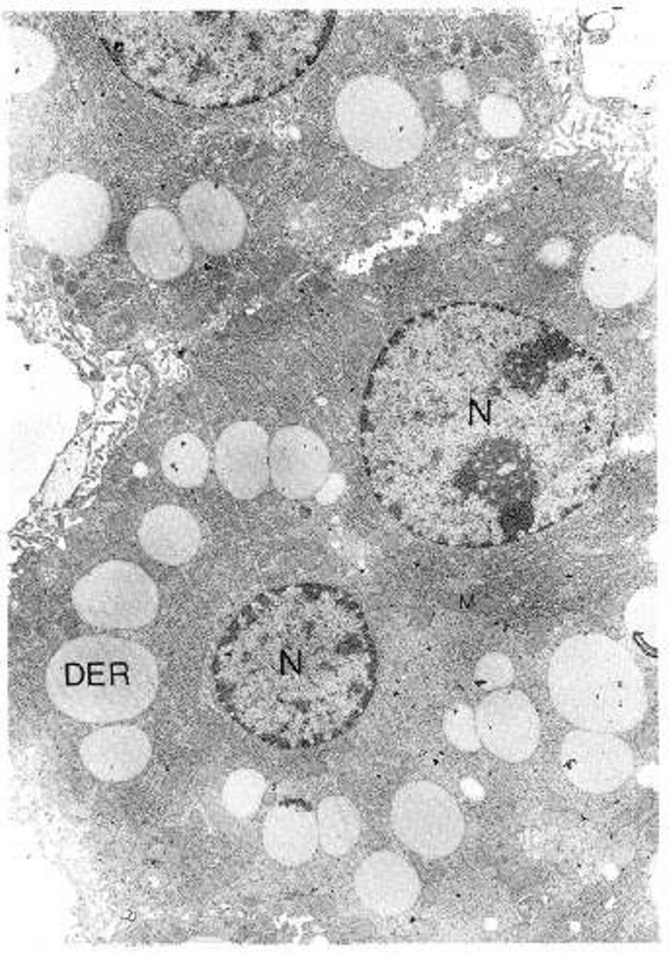

Fig. 6. Hepatocytes from B6 mouse receiving 3-MC, virus, and 150 mg of acetaminophen showing distention of the endoplasmic reticulum (DER). (N) nucleus and (M) mitochondrium.

no significant change in cytochrome P-450 values was detected as an additional response to viral infection.

Clinical signs. No symptomatology or liver pathology was recognized in animals receiving 3-MC alone, or in combination with influenza B virus. Symptoms were recognized in approximately 5% of animals receiving all three agents. These consisted of ruffling of the fur, unsteady gait, and terminal irregular respirations. Death, when it occurred spontaneously, tended to be within 36–72 h after administration of acetaminophen.

DISCUSSION

This study demonstrates that concomitant infection with influenza B virus has a significant enhancing effect on the hepatic toxicity of acetaminophen in mice. In addition, an atypical fatty liver pathology, resembling that seen in human RS under the light microscope, appears to result from an unknown interaction between influenza B virus and acetaminophen, in animals whose cytochrome P-450 MFO system is genetically predisposed to induction by aromatic hydrocarbons.

It has been suggested that an individual might be rendered more likely to develop RS as a complication of minor viral infection, by prior contact with chemical environmental pollutants (6). We chose to use 3-MC as representative of aromatic hydrocarbons which are commonly encountered in the environment (*e.g.*, in cigarette smoke and charcoal-broiled meat).

Nonmouse-adapted virus was used because preliminary studies using mouse-adapted virus produced a disease in the mice which was far more severe than typical human disease, *i.e.*, the virus produced severe pneumonia with lung consolidation, associated with a significant mortality.

The finding that dose-related hepatic centrolubular necrosis was potentiated by prior induction of the MFO system in the B6 mice is compatible with previously reported experience (14). Remarkable, however, was finding that a significant number of animals receiving all three study agents showed changes in the liver on light microscopy which were indistinguishable from the microvesicular steatosis described in human RS. This type of fatty infiltration is not typical of the hepatic pathology produced by the toxic metabolite of acetaminophen. Further, in approximately ⅔ of the animals demonstrating the fatty changes, the latter were present without the typical necrotic changes seen in classic acetaminophen hepatotoxicity. Of major interest also, in light of the suggestion that there may be a component of genetic susceptibility rendering an individual more likely to develop RS, is the finding of a significantly greater susceptibility to fatty change in the B6 as compared with the D2 mice.

The EM examination of the liver from the two strains of mice infected with influenza B virus and treated with the two xenobiotic agents, 3-MC and acetaminophen, showed remarkable differences in susceptibility to the effects of these agents. The changes seen in D_2 mice without the Ah locus were serious, but not as profound as those seen in the Ah suceptible B6 mice, where initial distention of the ER, led to eventual complete disruption of the cells. The disruption of the ER, the site of the MFO cytochrome P-450 enzyme system activity, was only recognized in B6 animals who had received influenza B virus, in

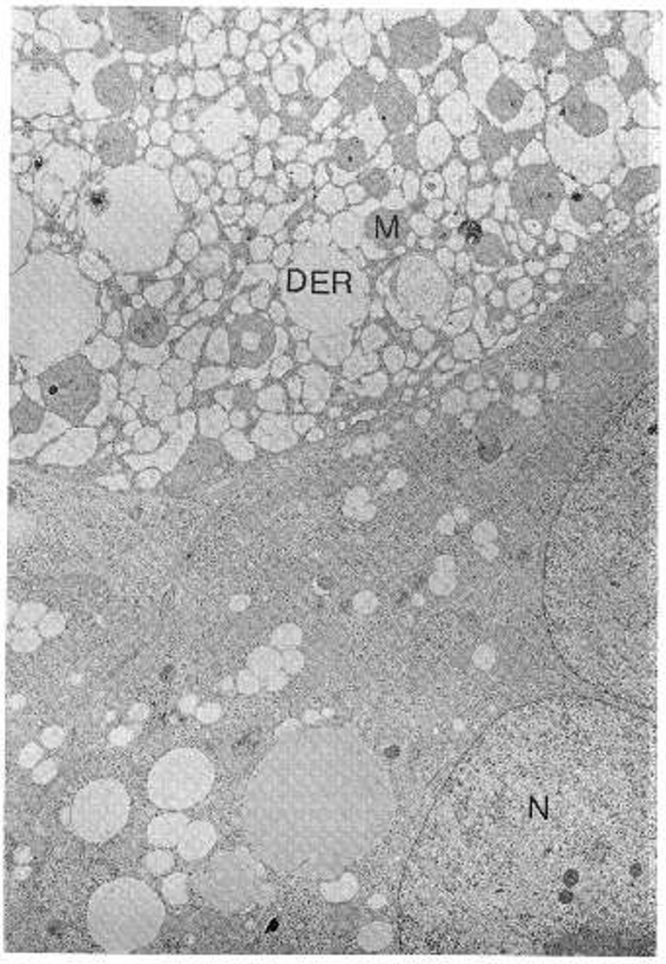

Fig. 7. Hepatocyte from B6 mouse receiving 3-MC, virus, and 200 mg of acetaminophen, with distended endoplasmic reticulum (DER) and ultrastructural alteration of mitochondria (M).

addition to acetaminophen and 3-MC. Similar changes in the ER have been reported on EM examination of the liver of an adult male suffering from acetaminophen overdose, and by Thaler *et al.* (29) Svoboda *et al.* (28) in human RS (18). Fat deposits, corresponding to those seen on LM, were within distended areas of the ER.

Svoboda *et al.* (28) studied the livers of six patients with RS by LM and EM, and attempted to correlate the changes seen with the clinical course of the disease. They found that on LM, inflammation and necrosis were especially prominent in fatal cases and that "Aside from loss of matrix dense granules, alterations in mitochondrial structure were minimal or absent. No ultrastructural features served to distinguish patients who died from those who survived." They concluded that "Although derangement of mitochondrial function may be important in the pathogenesis of RS, such derangement is not necessarily reflected in the ultrastructure of the mitochondria."

Thaler *et al.* (29) noted the presence of numerous microbodies in liver cells on EM. They were not impressed by any significant mitochondrial change. Several incomplete reports have been published, such as the one by Morales *et al.* (15) which merely refers to "mitochondrial changes and dilation of the endoplasmic reticulum."

Structural changes in the hepatic cell mitochondria in RS have been emphasized by Partin *et al.* (26). These authors believe that there is a parallel between recovery of mitochondrial ultrastructure and clinical improvement of the patient, and suggest that mitochondrial damage is important in the evolution of RS.

Although mitochondrial changes did become apparent with progression of the toxic process in our study mice, they were invariably preceded by swelling of the ER. Our findings resemble those described by Svoboda *et al.* (28) and to a lesser extent by Thaler *et al.* (29). Direct comparison of our mouse samples with liver samples from humans was handicapped by the lack of availability of human biopsy specimens, because liver biopsy is no longer regarded as a routine procedure in diagnosing RS (22). Our review of published EMs led us to conclude that dilatation of the ER preceeds significant hepatic mitochondrial damage in the progression of human RS, but that the ER changes are often overlooked by the authors when describing EM findings.

Studies to date on the effects of drug-virus interaction on the immune system have indicated that stimulation of interferon production by the virus will depress the MFO system (7, 12, 20). Our histologic data suggest that activation of certain components of the cytochrome P-450 MFO system might be a mechanism for potential acetaminophen toxicity in our study mice. In light of this, the results of cytochrome P-450 enzyme assays are contrary to expectations; however, interpretation is handicapped by methodologic problems. It was our intention to attempt to correlate cytochrome P-450 values, not only with individual treatment regimens, but also with histologic findings in the individual mouse livers. This was the reason for attempting enzyme estimations on the same livers from which samples were obtained for LM and, in some cases, EM. In fact, enzyme-histology correlation was not possible, because animal livers had to be pooled in order to separate sufficient microsomal material for subsequent enzyme assay. Although our results do not indicate that microsomal P-450 induction is a mechanism by which the toxic effects of acetaminophen are potentiated by influenza B virus in these animals, it is certainly possible that studies of larger numbers of animals and use of more sensitive methods for enzyme assay, along with methodology to separate out individual types of P-450, might produce differing results.

The pitfalls of direct extrapolation from animal data to the human are well recognized. But, the finding that the typical liver toxicity of acetaminophen is significantly enhanced in mice by concomitant viral infection is of major potential significance to the human. Animal studies by McLain *et al.* (13) and Goldfinger *et al.* (9), on the combined effects of alcohol and acetaminophen have been confirmed in humans. Alcoholic subjects may suffer serious toxic effects from usually innocuous quantities of acetaminophen, particularly if they are abusing other chemical substances. Acetaminophen toxiciy also appears to be potentiated by concomitant mononucleosis (23).

Acetaminophen is a commoly used antipyretic in children and is being promoted in the United States. This promotion is being facilitated by the apparent indictment of salicylates as a causal agent in RS (21). The evidence that other commonly used drugs are not etiologically associated with RS is extremely weak.

Taking into consideration the numerous agents which have been causally related to an RS-like syndrome in man, we hypothesize that viral-xenobiotic interaction may be an underlying mechanism by which many foreign chemicals might produce RS in a susceptible individual (27). Those affected may have rendered susceptible by prior contact with some noxious environmental substance, and/or by genetic factors, perhaps manifested through the immune system.

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33. In order to obtain a microsomal pellet of sufficient size for enzyme assay, it was found necessary to pool the livers from a minimum of three animals.
34. Using Stepwise Progressive Logistic Regression, all comparisons quoted in b–e were significant to $P < 0.001$.
35. Centrolobular necrosis was considered significant when it was clearly recognized in 10% or more of the hepatic lobules examined.
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