Azoxymethane-induced fulminant hepatic failure in C57BL/6J mice: characterization of a new animal model

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Matkowskyj, Kristina A., Jorge A. Marrero, Robert E. Carroll, Alexey V. Danilkovich, Richard M. Green, and Richard V. Benya. Azoxymethane-induced fulminant hepatic failure in C57BL/6j mice: characterization of a new animal model. Am. J. Physiol. 277 (Gastrointest. Liver Physiol. 40): G455–G462, 1999.—Without transplantation, ∼50–90% of all patients with fulminant hepatic failure (FHF) die. This poor outcome is due in part to the absence of an appropriate animal model, which would allow for a greater understanding of the pathophysiology of this syndrome. Given the reports of liver injury in humans and livestock fed cycad palm nuts on the island of Guam, we hypothesized that the active ingredient azoxymethane (AOM) could cause FHF. We therefore evaluated AOM in C57BL/6j mice. Histologically, we observed microvesicular steatosis 2 h, sinusoidal dilatation 4 h, and centrilobular necrosis 20 h after AOM administration, and transmission electron microscopy showed that this agent induces a dose-dependent FHF in mice that is highly reproducible, causes death from liver failure in a dose-dependent manner due to hepatic mitochondrial injury, has a long therapeutic window, and generates an associated encephalopathy with evidence of cerebral edema in end-stage disease. AOM is thus the first toxin important, not all toxins currently used in the study of FHF in animals have been reported to cause injury in humans.

A report from Guam in the early 1960s noted that cycad palm nuts induced a variety of cancers of the gastrointestinal tract (15). The active ingredient, azoxymethane (AOM), has since been used for the study of colon cancer in laboratory animals (37). Interestingly, this report described in anecdotal fashion that cycad palm nuts also caused liver injury in humans, rats, and livestock fed meal derived from this nut (15). We were therefore interested to know if, in addition to its established role in causing colon cancers, AOM also could be used as an FHF-inducing hepatotoxin.

In this study we investigated the effects of AOM as a hepatotoxin in C57BL/6j mice. We restricted this investigation to this species because whole animal genetic manipulations (i.e., transgenics, knockouts) can only be performed in mice, and this particular strain is the best characterized (27). We herein demonstrate that AOM induces a dose-dependent FHF in mice that is highly reproducible, causes death from liver failure in a dose-dependent manner due to hepatic mitochondrial injury, has a long therapeutically window, and generates an associated encephalopathy with evidence of cerebral edema in end-stage disease. AOM is thus the first toxin to satisfy all the criteria previously identified as essential for an animal model of FHF (28) that also is associated with the development of hepatic encephalopathy.

METHODS

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METHODS

Mouse care and maintenance. Male C57BL/6j mice (25–30 g body wt) were obtained from Jackson Laboratory (Bar Harbor, ME) and used at 20 wk of age. Mice were fed ad libitum with Lab Diet Formulab 5008 rodent chow (PMI Feeds, St. Louis, MO) and were allowed free access to water. The mice were housed in microisolator cages with GreenTru laboratory bedding (Green Products, Conrad, ID) and exposed to a controlled light cycle of 10:14 h (light:dark) at 25°C. To accommodate video recording, 48 h before AOM (Sigma, St. Louis, MO) administration, mice were exposed to continual light. For most experiments, animals received 100 µg/g AOM dissolved in 100 µl sterile saline (Fujisawa USA, Deerfield, IL) administered by intraperitoneal injection. For all experiments, animals were matched with a saline-injected control group.

Clinical and biochemical evaluation. Mice were monitored continuously with an AG-180 video recording system (Panasonic, Secaucus, NJ) from 48 h before AOM administration until they were killed or died, allowing us to quantify individual eating and drinking events. Specifically, no more than three animals, each in separate cages, were subjected to continuous video monitoring after receiving saline or AOM. The number
of times each animal drank or ate per hour was determined when a reviewer, blinded as to whether saline or AOM had been provided, reviewed the videotape. Animals also were assessed each hour by one of three investigators (K. A. Matkowskyj, J. A. Marrero, or R. E. Carroll) for activity level (asleep or moving about in the cage spontaneously) and reflexes (scattering, ataxia, righting, corneal) as previously described (3, 38). Mice were killed by CO2 asphyxiation at the indicated time points. Freshly killed animals were immediately subjected to cardiac puncture to obtain serum for alanine aminotransferase (ALT), ammonia, glucose, bilirubin, alkaline phosphate, and creatinine. Automated chemistries were determined by the Clinical Pathology Laboratory of the University of Illinois at Chicago Medical Center, with the use of a CX7 Synchron automated analyzer (Beckman Instruments, Fullerton, CA). All data are reported as means ± SE for a minimum of three separate experiments.

To determine whether hypoglycemia may have caused alterations in mental status, stage I and III encephalopathic animals were treated with glucose. After the serum glucose level was determined with a glucometer (Bayer, Elkhart, IN) on venous blood obtained from the tail, sufficient glucose to return serum levels to normal was administered intraperitoneally as a 50% solution. Animals were then rinsed in a running water bath. The sections were incubated for 5 min at room temperature in 3% hydrogen peroxide in a light-impermeable chamber to quench endogenous activity. After the sections were rinsed with 1 x PBS, slides were incubated for 20 min in blocking solution (90% water, 5% skim milk, and 5% H2O2). The excess solution was removed, and sections were incubated with 1:250 diluted glial fibrillary acidic protein (GFAP) primary antibody (Sigma) for 1 h in a humidity chamber. The sections were washed in PBS buffer and then incubated with biotinylated anti-rabbit IgG (DAKO, Carpinteria, CA) for 15 min. The slides were rinsed with PBS and incubated with streptavidin-conjugated horseradish peroxidase (Dako) for 20 min. The slides were then counterstained with Gills' hematoxylin for 1 min, dehydrated in graded alcohols, and mounted with a coverslip with the use of Permount.

Microscopy. Images were acquired with a MicroLumina digital scanning camera (Leaf, Westborough, MA) connected to a Nikon E600 microscope (Tokyo, Japan) with PlanApo objectives. Transmission electron microscopy was performed on freshly resected liver tissue immediately placed in 4%
glutaraldehyde in 0.1 M cacodylate buffer (CB) overnight, then postfixed for 2 h in 2% aqueous osmium tetroxide in CB. The tissue was dehydrated in graded alcohols, and the samples were embedded in pure Epon resin (EMS, Fort Washington, PA) and were polymerized at 60°C for 12 h. Ultrathin sections were stained with uranyl acetate and Reynolds’ lead citrate and were examined with a Phillips 410 transmission electron microscope.

RESULTS

Clinical findings. Animals treated with 100 µg/g AOM developed evidence of HE that was preceded by a novel prodromal phase. The prodromal phase was characterized by decreased spontaneous activity and decreased food and water intake (Fig. 1). This decrease in activity and feeding occurred before the loss of the animals’ scatter reflex, which has previously been defined as characteristic of stage I HE (38). This prodromal phase of decreased activity reproducibly occurred 5–7 h after AOM administration and lasted for ~15 h, at which time the animals lost their scatter reflex. Thus lethargic animals in the prodromal period can be differentiated from those in stage I HE by virtue of the presence or absence of the scatter reflex.

The second phase was characterized by the development of frank encephalopathy that progressed via all four previously defined stages, including loss of scatter instinct (stage I), ataxia (stage II), loss of righting reflex (stage III), and progression to coma (stage IV) (38). Stage I HE was observed 20.2 ± 0.1 h (n = 32) after AOM injection. Ataxia (stage II HE) was observed after 24.3 ± 0.3 h (n = 14), whereas loss of righting reflex (stage III HE) occurred 33.2 ± 1.5 h (n = 18) after AOM exposure. Loss of corneal reflexes and coma (stage IV HE) developed 36.0 ± 0.8 h (n = 25) after injection, with death following ~3 h later (n = 20). Indeed, all animals given 100 µg/g AOM progressed to death within 41 h.

Histological findings. AOM caused a progressive liver injury that preceded alteration in clinical behavior. The earliest histopathological alteration observed was the presence of microvesicular steatosis 2 h after AOM administration (Fig. 2B; normal mouse liver is shown in A). Four hours after AOM injection sinusoidal dilatation was apparent, predominantly in the area around the central vein (Fig. 2C). By the time stage I HE had developed ~20 h after AOM exposure, profound centrilobular necrosis was evident (Fig. 2D). In the preterminal stage IV HE animal, this necrosis was primarily hemorrhagic in nature (Fig. 2E). Given the early observation of microvesicular steatosis, we wondered if this was associated with mitochondrial injury. Consistent with AOM acting as a mitochondrial toxin, transmission electron microscopy revealed profound damage to the cristae (Fig. 2F).

To determine whether AOM affected any other organ besides the liver, we histologically evaluated the brain, kidney, heart, intestines, lung, and pancreas at 4 and 8 h after AOM exposure and in stage I, II, III, and IV encephalopathic animals. The only other organ showing evidence of histological change was the brain, important because central nervous system (CNS) alterations consistent with cerebral edema have been reported in patients and animals suffering from FHF. We detected astrocyte swelling (ballooning), the most sensitive measure of CNS edema (30, 31, 35), in mice only after they had developed stage IV HE (Fig. 3B; normal mouse brain and astrocytes are shown in A). Indeed, swelling was not seen in stage III HE animals or at earlier time points. We also found evidence of Alzheimer type II changes in the CNS (Fig. 3B and C). These changes include twinning and decreased GFAP immunoreactivity and have been described as nonspecific findings associated with HE due to a variety of causes, including portal-systemic encephalopathy (21, 25), as well as FHF (4, 30, 31, 35, and Dr. M. D. Norenberg, University of Miami, personal communication). Importantly, our detection of Alzheimer type II changes was limited to brains also showing evidence of astrocyte swelling (i.e., ballooning). Thus it may be that the development of Alzheimer type II changes is induced by whatever processes cause edema. Regardless, no CNS findings (swelling/ballooning, twinning, GFAP) were observed at earlier time points; indeed, the brains from mice in stage III HE could not be distinguished from those isolated from control animals (data not shown). Thus AOM-induced FHF generates CNS edema similar to that occurring late in the development of FHF-associated HE in humans (22).

Biochemical alterations. We evaluated serum ALT, arterial ammonia, glucose, alkaline phosphate, bilirubin, and creatinine in saline-injected control animals and in animals at varying time points after AOM administration. In addition to each encephalopathic stage, we evaluated animals 4 and 8 h after drug delivery, because at the former time there is evidence of liver damage but no encephalopathy, whereas at the latter time subtle alterations in mental status can be detected.

Fig. 1. Alterations in animal activity after intraperitoneal injection with saline (●) or 100 µg/g azoxymethane (AOM, ○) until time of killing or death. Inactivity of the prodromal phase was differentiated from the lethargy of stage I hepatic encephalopathy (HE) during hourly examinations by observing animal response to manual stimulation. Data represent means. Error bars are not shown in order to retain graphical clarity.
As expected, we observed an increase in serum ALT that corresponded to the histological degree of hepatic injury. ALT was similar in control animals (64 ± 14 U/l) and animals 4 h after AOM injection (56 ± 5 U/l) (Table 2), at which point only sinusoidal dilatation could be appreciated (Fig. 2C). However, by the time stage I HE and centrilobular hepatocyte necrosis occurred (Fig. 2D), ALT increased to 5,196 ± 126 U/l and ultimately peaked in the preterminal animal at 12,231 ± 2,068 U/l (Table 2). In contrast, there were no significant alterations in bilirubin (Table 2), alkaline phosphate, or creatinine (data not shown). The failure to detect any significant increase in serum bilirubin is similar to what has been described for liver injury due to acetaminophen in the dog (10) but is in contrast to what has been reported for thioacetamide in the rat (38) or galactosamine in the rabbit (3). The variability in hepatotoxin-associated hyperbilirubinemia suggests the possibility that such alterations may well reflect the differential effects of any particular drug in different species or that these toxins may have subtle yet different effects on altering liver function.

We did detect elevations in arterial ammonia, which have been reported in association with HE (2, 6, 29, 32, 34). Yet significant increases in this parameter were not detected until animals entered stage III HE or ~28 h after altered behavior was first detected. In contrast, we observed a significant 26 ± 1% (P < 0.01) decrease in serum glucose as early as 4 h after drug exposure, before any detectable alteration in mental status (Table 2). This decreased to ~58 ± 1% (P < 0.01) of the control animal values 24 h after AOM delivery in the stage II encephalopathic mouse but did not decrease further (Table 2). After glucose administration to stage I (n = 2) and III (n = 2) encephalopathic animals, serum glucose levels returned to normal levels within 15 min and remained in the euglycemic stage for at least 2 h. However, no reversal in mental status was observed at any point. Thus, as has been shown to be the case in humans, hypoglycemia per se does not appear to be responsible for the alterations in mental status.

Effect of AOM dose and lot. All results reported to this point were obtained with the use of 100 µg/g AOM injected intraperitoneally. To determine if AOM-induced liver injury was dose dependent, we also studied animals treated with 20 µg/g, 50 µg/g, and 200 µg/g ip AOM. At the lowest dose used, a dose commonly employed for the induction of colon cancer in mice (37), AOM was not hepatotoxic. In contrast, 50 µg/g and 200 µg/g caused identical changes in liver histology as reported for 100 µg/g but altered the rate at which FHF occurred and altered the time to death (Table 3).

Because lot-to-lot variability is commonly observed with the use of other hepatotoxins such as galactosamine (3, 26, 33), we also studied whether this could limit the usefulness of AOM. We therefore obtained 30 separate lots of AOM from the distributor (Sigma). From these 30 lots, we randomly selected 10 and evaluated four animals per lot. We administered 100 µg/g AOM to each of these animals, killed one each at...
Table 2. Blood chemistries of C57BL/6J mice with hepatic encephalopathy due to azoxymethane-induced liver failure

<table>
<thead>
<tr>
<th>Mental Status</th>
<th>Time After AOM injection</th>
<th>ALT, U/l</th>
<th>NH₄, µg/dl</th>
<th>Glucose, mg/dl</th>
<th>Bilirubin, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal/control</td>
<td>Previously reported</td>
<td>70</td>
<td>NR</td>
<td>262</td>
<td>0.89</td>
</tr>
<tr>
<td>Normal</td>
<td>This study</td>
<td>64 ± 14</td>
<td>275 ± 2</td>
<td>281 ± 18</td>
<td>0.75 ± 0.1</td>
</tr>
<tr>
<td>Normal</td>
<td>4 h</td>
<td>56 ± 5</td>
<td>257 ± 11</td>
<td>208 ± 27</td>
<td>0.75 ± 0.2</td>
</tr>
<tr>
<td>Prodromal (passive)</td>
<td>8 h</td>
<td>44 ± 6</td>
<td>168 ± 27</td>
<td>148 ± 31</td>
<td>ND</td>
</tr>
<tr>
<td>HE stage I (lethargic)</td>
<td>20.2 ± 0.1 h</td>
<td>5,196 ± 126*</td>
<td>350 ± 36</td>
<td>84 ± 4*</td>
<td>0.9 ± 0.1</td>
</tr>
<tr>
<td>HE stage II (ataxic)</td>
<td>24.3 ± 0.3 h</td>
<td>ND</td>
<td>383 ± 35</td>
<td>93 ± 16*</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>HE stage III (loss of RR)</td>
<td>33.2 ± 1.5 h</td>
<td>ND</td>
<td>646 ± 164*</td>
<td>101 ± 22*</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>HE stage IV (coma)</td>
<td>36.0 ± 0.8 h</td>
<td>12,231 ± 2,068*</td>
<td>1,110 ± 303*</td>
<td>119 ± 12*</td>
<td>1.0 ± 1.0</td>
</tr>
</tbody>
</table>

Values are means ± SE for a minimum of 3 separate experiments. Control values are for animals used in this study and for CD-1 outbred albino mice (Charles River, Wilmington, MA) as previously reported (13). AOM, azoxymethane; ALT, alanine aminotransferase; HE, hepatic encephalopathy; RR, righting reflex; NR, not reported; ND, not done. *P < 0.05 compared with control values determined in this study by the Kruskal-Wallis test, a nonparametric equivalent of a one-way ANOVA.

stage I HE and stage III HE, and allowed the remainder to progress to death. In all instances, the time of HE, progression of liver injury, and time to death were identical.

DISCUSSION

FHF is a life-threatening condition resulting in a 50–90% mortality rate in the absence of liver transplantation (5, 8, 12, 16, 20). In the past 10 years little progress has been made in improving patient outcome, in part because of the lack of a suitable animal model. Other commonly used drugs or toxins do not satisfy all the necessary criteria for an ideal model of FHF (28), in part because they are not reproducible, produce inconsistent toxicity, fail to generate a clinically significant lesion, or require supportive therapy (18, 38). The three most commonly studied toxins include carbon tetrachloride, acetaminophen, and galactosamine. Use of carbon tetrachloride as a model is problematic because of the variability and has no known human correlate as a hepatotoxin (3, 26, 33). Furthermore, most toxins cause a centrilobular pattern of injury, galactosamine produces diffuse rather than zonal injury (3, 26, 33). Thus the three most commonly used animal models of FHF are far from ideal, with all models currently used possessing significant limitations (Table 1).

In this study we demonstrate that AOM is the first toxin to satisfy all the essential criteria for an animal model insofar as it is reproducible, causes death from liver failure, has a long therapeutic window, and poses minimal hazard to personnel when handled properly. In addition, AOM-induced liver injury generates all four previously characterized stages of HE (38), which is especially important because encephalopathy is invariably associated with FHF in humans (6, 12, 16, 20). One of the most important features of the murine AOM model of FHF is the identification of a protracted phase separate and distinct from the first stage of HE (Fig. 1). The encephalopathic phase is ~19 h long and is detectable by the loss of scatter reflex when liver injury is already extensive (Fig. 2D). In contrast, the prodromal phase lasts a similar length of time (Fig. 1) but occurs when liver injury is relatively modest (Fig. 2, B and C). Awareness of this prodromal phase may be critical for future studies attempting to identify factors causing HE.

Although we did not specifically study the pathogenesis of HE caused by AOM-induced FHF, our findings do provide some insight into factors that cannot be implicated in altered mental status. Arterial ammonia has been proposed as a cause of HE (2, 6, 29, 32, 34), yet no increase in arterial ammonia was observed in the prodromal phase, and increases were not statistically significant until the development of stage III HE (Table 2). In contrast, significant decreases in serum glucose were detected as early as 4 h after AOM administration, with the decrease in serum glucose preceding the onset of even the prodromal phase. However, administration of exogenous glucose and maintenance of euglycemia did not improve the mental status of the AOM-treated mice; therefore, hypoglycemia per se does not appear to be responsible for the alterations in mental status. This is similar to what has been previously reported in other species, where hypoglycemia is commonly observed to complicate FHF in humans (5, 8, 23) and other animal models of FHF, including the dog, pig, and rabbit (3, 14, 17, 26). In no instance does correction of the hypoglycemia have any impact on improving the mental status of humans and other species suffering from FHF. This suggests that another factor is involved in the development of mental status changes in this model.

Table 3. Azoxymethane-induced liver injury and time to death is dose dependent

<table>
<thead>
<tr>
<th>Dose of AOM, µg/g</th>
<th>Number Studied</th>
<th>Hepatotoxicity</th>
<th>Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>30</td>
<td>No</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>5</td>
<td>Yes</td>
<td>49.7 ± 1.3</td>
<td>48–52</td>
</tr>
<tr>
<td>100</td>
<td>20*</td>
<td>Yes</td>
<td>37.1 ± 2.6</td>
<td>35–40</td>
</tr>
<tr>
<td>200</td>
<td>3</td>
<td>Yes</td>
<td>28.5 ± 1.4</td>
<td>25–30</td>
</tr>
</tbody>
</table>

The majority of results reported were obtained using 100 µg/g AOM. *Only those animals proceeding to death are included in this number. NA, not applicable because animals did not die from AOM at this dose.
from FHF. Finally, edema is well known to complicate the progression of FHF (20), with up to 25% of humans reported to suffer this as a late complication (7). We specifically used astrocyte swelling as a measure of cerebral edema because the gravimetric method, widely used in rats, has not been validated in mice and because morphological changes consistent with brain edema are the most sensitive marker of this condition (22). Using this technique, we observed astrocyte swelling only in the brains of animals that had progressed to stage IV HE. The inability to observe these findings earlier, however, indicates that, similar to humans, CNS edema is a late finding and may not be responsible for the observed alterations in mental status.

Finally, a major advantage of AOM-induced FHF is its apparent ability to cause liver injury and failure in multiple species, including humans. AOM is the active metabolite of cyasin (36), found in cycad palm nuts only on the island of Guam. In a single anecdotal report from that island in the 1960s (15), pyknotic nuclei and focal necrosis in the centrilobular region were identified in humans, livestock, and rats as early as 48 h after ingesting unwashed cycad nuts. In retrospect, Guamanian FHF in humans, rodents, and livestock all appear to be due to AOM hepatotoxicity.

In conclusion, AOM causes a dose-dependent centrilobular necrosis of the liver, possibly by acting as a mitochondrial toxin, that progresses to liver failure and death. This agent causes both a neurological prodrome as well as frank HE. It is the first agent with a human correlate that may be used across species that is potentially reversible, is reproducible, and causes death from liver failure.

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