Characterization of Acute 4,4'-Methylene Dianiline Hepatotoxicity in the Rat
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Methylene dianiline (DDM; 4,4'-(diaminodiphenylmethane) is a primary aromatic amine that is used extensively in a variety of industrial synthetic processes. DDM is an important intermediate in the production of isocyanates and rigid polyurethane foams and is a component of epoxy hardening agents. Because of its widespread use and large-scale production, there is potential for human exposure and intoxication. A mass intoxication occurred in Epping, England, in the late 1960s. The pathologic changes in exposed individuals were limited primarily to the liver. Liver biopsies revealed portal edema and inflammation with an abundant cellular infiltrate composed of neutrophils, eosinophils, and mononuclear cells (1).

Few investigations of acute DDM-induced hepatotoxicity in animals have been reported. Schoental (2) described the lesions associated with long-term exposure of rats and mice to DDM. These included liver tumors and hepatic lesions consisting of focal midzonal and portal necrosis with marked leukocytic infiltrates. These findings were consistent with the lesions described by Kopelman in humans intoxicated with DDM (1). Pludo et al. (3) carried out a rudimentary evaluation of the acute and subacute toxicity of DDM in rats. Kanz and co-workers (4) characterized the histologic lesions associated with a high dose of DDM. This study revealed early injury to bile duct epithelium, necrosis of hepatic parenchymal cells, and altered secretion of biliary constituents in rats.

In this study, we further characterized the hepatotoxic effects of DDM. Our aim was to delineate the biochemical and histologic changes associated with acute DDM intoxication and to understand the dose relatedness and development of DDM hepatotoxicity in rats. In addition, we investigated the possibility that the cytochrome P450 monooxygenase system (MO) is involved in DDM-induced liver injury in vivo.

Male Sprague Dawley rats [CF: CD (SD)BR; Charles River, Fortage, MI] weighing 175–300 g were fasted for 24 hr

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talnecrotizingvasculitisoftheportalvein

Figure 2. Photomicrographs of the livers of rats 24 hr after oral administration of 100 mg/kg methylene dianiline (DDM) or vehicle. (A) Photomicrograph of the liver from a rat receiving corn oil. Note the normal portal tracts and periporal parenchyma. H&E, 129x. (B–D) Photomicrographs of livers from DDM-treated animals. (B) Focus of hepatocellular necrosis extending segmentally from the portal tract and involving the midzonal parenchyma, 128x. (C) Neutrophil infiltration (white arrow) and fibrin exudation (black arrow) within the necrotic hepatic parenchyma, 257x. (D) Perportal edema (arrow), neutrophil infiltration, and segmental necrotizing vasculitis of the portal vein (extending from A to B), 125x.

before and after receiving DDM. We treated rats with one of several doses (25–225 mg/kg) of DDM (Aldrich Chemical Co., Milwaukee, WI) or an equivalent volume of corn oil per os. All doses were administered in a 2 ml/kg volume. We anesthetized the rats with sodium pentobarbital (50 mg/kg, IP) 24 hr after treatment and placed the rats on a heating pad to maintain body temperature. We cannulated the common bile duct as previously described (5) and collected bile flow for 30 min. The rats were then exsanguinated, and blood was collected for determination of indices of liver injury. We quantified total serum bilirubin concentration spectrophotometrically (Sigma kit 605-D, Sigma Chemical Co., St. Louis, MO). Gamma-glutamyl transferase (GGT) activity was determined spectrophotometrically by monitoring the GGT-catalyzed formation of p-nitroanilide from the substrate, L-γ-glutamyl-p-nitroanilide (Sigma kit 419-20). As a marker of hepatic parenchymal injury, we determined serum alanine aminotransferase (ALT) activity via a kinetic spectrophotometric assay (Sigma kit 59-20).

Oral administration of DDM caused a dose-dependent change in all markers of liver injury (Fig. 1). The threshold for toxicity was between 25 and 75 mg/kg DDM. DDM administration caused concomitant changes in all markers of liver injury measured, including serum ALT, bile flow, serum bilirubin concentration, GGT activity, and liver weight (Fig. 1). Pludro et al. (3) reported that the oral LD50 of DDM in rats was 830 mg/kg. They found histologic changes in liver and changes in plasma electrophoretic patterns after subacute administration of DDM at a dose equal to one-tenth of the LD50. Our findings are consistent with theirs.

Liver sections from animals that received DDM (100 mg/kg, per os) had multifocal lesions consisting of hepatocellular necrosis with hemorrhage and moderate

| Time after DDM (hr) | Bile duct necrosis | Edema | Fibrin | Bile ductular neutrophil infiltrate | Portal venous vasculitis | Hepatocellular necrosis | Hemorrhage | Parenchymal neutrophil infiltrate |
|---------------------|--------------------|-------|--------|------------------------------------|--------------------------|------------------------|-----------|----------------------------------|
| 4                   | 1.5                | 0.0   | 0.3    | 0.0                                | 0.0                      | 0.0                    | 0.0       | 0.0                              |
| 6                   | 2.5                | 2.0   | 2.0    | 1.3                                | 1.3                      | 0.5                    | 0.0       | 0.0                              |
| 12                  | 4.0                | 2.0   | 2.0    | 2.0                                | 1.5                      | 2.3                    | 1.3       | 1.5                              |
| 16                  | 4.0                | 2.0   | 2.0    | 3.0                                | 1.8                      | 4.0                    | 2.3       | 3.5                              |

Animals received 100 mg/kg DDM, per os. At various times thereafter rats were killed and tissues were fixed in 10% buffered formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin and eosin. Histologic changes were assessed from slides that were coded. The pathologist had no knowledge of treatment groups. Liver sections were evaluated for relative severity of lesions present and scored on a 0–4 scale (0 = normal relative to controls, 4 = most severe changes). Control livers demonstrated normal hepatocellular architecture and no pathologic changes. These livers were assigned 0 as an arbitrary score. Three sections of liver from different lobes were evaluated per animal. Each value represents the mean score of 3–5 animals.
Table 2. Effect of P450 monooxygenase modifiers on methylene dianiline (DDM)-induced hepatotoxicity

| Pretreatment   | DDM dose (mg/kg) | ALT (IU/L) | Bile flow (μl/g liver/30 min) | Bilirubin (mg/dl) | GGT (IU/L) | LW/BW |
|----------------|------------------|------------|-------------------------------|------------------|------------|--------|
| Saline         | 100              | 524 ± 65   | 5.5 ± 3.4                     | 4.0 ± 0.5         | 5.4 ± 0.6  | 4.1 ± 0.09 |
| ABT            | 145 ± 26         | 28 ± 0.4   | 2.2 ± 0.3                     | 2.8 ± 0.4         | 4.2 ± 0.13 |
| Saline         | 100              | 540 ± 82   | 0.3 ± 0.1                     | 4.2 ± 0.3         | 6.2 ± 0.8  | 3.8 ± 0.07 |
| SKF-525A       | 507 ± 63         | 5.2 ± 3.1  | 4.1 ± 0.3                     | 4.6 ± 0.8         | 3.8 ± 0.06 |
| Corn oil       | 50               | 279 ± 57   | 27.3 ± 6.1                    | 0.6 ± 0.2         | 5.6 ± 0.9  | 3.7 ± 0.10 |
| BNF            | 136 ± 31         | 30.9 ± 3.4 | 0.4 ± 1.0                     | 2.8 ± 0.3         | 4.5 ± 0.08 |
| Saline         | 50               | 588 ± 122  | ND                            | 3.0 ± 0.7         | 6.5 ± 0.9  | 3.6 ± 0.18 |
| Phenobarbital  | 88 ± 11          | ND         | 0.1 ± 0.0                     | 0.8 ± 0.2         | 3.9 ± 0.07 |
| Saline         | 100              | 823 ± 100  | 3.9 ± 1.5                     | 4.6 ± 0.5         | 10.0 ± 1.8 | 3.9 ± 0.05 |
| Phenobarbital  | 706 ± 138        | 7.0 ± 4.5  | 3.2 ± 0.7                     | 8.2 ± 1.6         | 4.7 ± 0.1  |

Abbreviations: ALT, alanine aminotransferase; GGT, γ-glutamyltransferase; LW/BW, liver weight to body weight ratio; ABT, aminobenzotriazole; BNF, β-naphthoflavone; ND, not detected.

Two hours before administration of DDM (100 mg/kg in corn oil, per os), rats were treated IP with 40 mg/kg SKF-525A (20 ml/kg in saline), Sigma Chemical Co., St. Louis, MO), 100 mg/kg ABT (2 ml/kg in saline), or an equal volume of saline vehicle. Twenty-four hours later rats were anesthetized, bile flow was assessed, serum was obtained for evaluation of biochemical markers of liver injury, and liver sections for histologic evaluation were taken as described above. Phenobarbital (70 mg/kg in saline, IP), or β-naphthoflavone (80 mg/kg, IP as a suspension in corn oil, Sigma), or their respective vehicles (2 ml/kg) were administered daily for 3 days preceding administration of DDM (100 or 50 mg/kg, per os). Twenty-four hours after DDM administration, rats were processed as described above. Values represent means ± SEM, n = 8–11. *Significantly different from pretreatment controls (p ≤ 0.05).

The lesions of DDM intoxication have common histologic features to those neutrophil infiltration (Fig. 2). The necrosis involved segments of periportal hepatocytes but did not surround portal tracts. Frequently, the parenchymal insult extended into the midzonal regions of hepatic lobules. The lesions associated with the portal triad consisted of bile ductular necrosis, portal edema with fibrin exudate, and neutrophil infiltration. A segmental necrotizing vasculitis of the portal vein was also evident. The earliest change identified with the hepatotoxicity of DDM was bile ductular necrosis, and histologic markers of liver injury continued to increase in severity over the course of 16 hr (Table 1). Histologic analysis of livers from animals receiving corn oil vehicle demonstrated normal hepatic histology with no apparent lesions.

Several investigators have described the distribution of histologic changes in liver associated with DDM intoxication (1,2,4). In rats and mice, the loss of parenchyma appeared to be primarily midzonal (1,2) or midzonal and periportal (4). Our observations were generally similar to those reported by these investigators. However, we found that the parenchymal lesions appeared adjacent to portal regions and extended into midzonal regions of the liver. The lesions did not appear to be strictly periportal in that they did not encircle the portal areas. In addition, the lesions were not restricted to the midzonal areas of the liver lobule, and they varied in distribution. Finally, we observed a prominent necrotizing vasculitis, which has not been reported previously. This event occurs early and therefore might be important to the mechanism of action of DDM.

Recently, Kanz and co-workers (4) evaluated some of the early hepatic changes associated with administration of a large dose (250 mg/kg) of DDM. An early (30 min) increase in bile flow and decreases in bile salts and bilirubin content in bile occurred. Elevations in bile flow and bile glutathione content were concurrent with these changes. Changes in serum markers of hepatic parenchymal injury, and cholestasis were apparent by 4–8 hr. Histologic evidence from the present study suggests that the earliest lesion is probably biliary in nature (Table 1), a finding consistent with the results of Kanz and co-workers (4). The onset of liver damage caused by DDM appears to be somewhat delayed in expression. Although subtle changes in the histologic appearance of the portal regions in the liver are evident by 4–8 hr, the first significant changes in biochemical markers of liver injury do not occur until later (Fig. 3).
caused by another cholangiolitic hepatotoxicant, β-naphthylisothiocyanate (ANIT). Portal edema, biliary epithelial injury, and inflammation occur in both models. However, the histologic distribution of lesions caused by ANIT are almost exclusively limited to periportal areas. Moreover, a distinct hepatic vascular injury has not been reported after ANIT administration. DDM seems to cause a more severe hepatic parenchymal lesion and generates a more marked inflammatory response than ANIT.

In a preliminary report, Ansari and co-workers (7) presented evidence suggesting the presence of metabolites of DDM in rat urine. Kajbaf and co-workers (8) have recently identified oxidized metabolites of DDM from microsomal preparations of rabbit liver. To gain insight to the role of metabolism in DDM hepatotoxicity, animals were pretreated with various agents at doses that have been shown to alter MO activity and to influence hepatotoxicity of agents that require bioactivation by this enzyme system (9–12). Aminobenzotria- zole (ABT), an inhibitor of MO, was effective at ameliorating the hepatotoxic effects of DDM, as demonstrated by attenuation of all markers of injury evaluated (Table 2). However, the MO inhibitor SKF-525A did not protect against DDM-induced liver damage. The difference in the results obtained when using ABT versus SKF-525A may reflect a difference in the spectrum of cytochrome P450 isozymes inhibited by the two agents (10, 12,13). It is plausible that SKF-525A did not inhibit the specific isozyme that bioactivates DDM, whereas ABT effectively attenuated metabolic activation.

The effect of phenobarbital pretreatment varied with the dose of DDM. At a dose of DDM (100 mg/kg) that caused maximal hepatotoxic effects (Fig. 1), pretreatment with phenobarbital had no effect on any of the markers of liver injury (Table 2). At a more moderate dose of DDM (50 mg/kg), phenobarbital pretreatment attenuated the hepatotoxicity. This result is generally consistent with the preliminary results of Kanz et al. (14), who found that toxic effects of a large dose of DDM were only modestly influenced by phenobarbital pretreatment, and protection was largely restricted to extrahepatic effects of DDM. We also evaluated the effect of pretreatment with a second inducer of MO activity, β-naphthoflavone (BNF). This agent had a modest effect on DDM hepatotoxicity (Table 2). Like phenobarbital, BNF pretreatment did not affect the toxicity of a higher dose (100 mg/kg) of DDM. These findings suggest that there is a cytochrome P450-dependent detoxification pathway for DDM. However, it should be noted that phenobarbital has myriad effects, not only on phase 1 MO metabolism but also on other enzyme systems. Thus, the attenuation of DDM toxicity may reflect an action on phase 1 metabolism of DDM or on other metabolic systems, such as phase 2 conjugating systems, which might contribute to DDM hepatotoxicity.

Assuming that the various agents we used act by influencing metabolism of DDM by MO, the results are consistent with the following hypothesis: DDM requires metabolic activation for its toxicity. This activation is carried out by an isozyme of cytochrome P450 that is inhibited by ABT but not by SKF-525A. In addition, there is a competing, P450-mediated reaction that is important in the detoxification of DDM. This competing reaction is enhanced by phenobarbital pretreatment and to a lesser extent by BNF pretreatment. At low DDM doses, the two opposing pathways compete for DDM substrate. Accordingly, an increase in the detoxification pathway by phenobarbital or BNF pretreatment leads to decreased formation of toxic metabolites by the competing bioactivation pathway. At higher DDM doses, enough substrate is present to saturate the bioactivation pathway irrespective of the action of the detoxification pathway. There is precedent for the concept of competing, toxifying, and detoxifying pathways for hepatotoxicants in the cases of acetaminophen and bromobenzene (13,15). Clearly, further study, including the identification of DDM metabolites in the rat, is needed to test this hypothesis completely.

In summary, our results indicate that DDM causes dose- and time-related toxicity classified as multifocal, cholangiolitic hepatitis, the lesions of which are distributed in portal and midzonal regions of liver lobules. The results with inducers and inhibitors of cytochrome P450 monoxygenase suggest that DDM requires metabolic activation for its toxicity. As a histologic feature of DDM hepatotoxicity is a marked inflammatory response, it would be of interest to evaluate the role of inflammatory cells and soluble mediators in DDM-induced liver injury.

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