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ROLE OF MACROPHAGES IN ACETAMINOPHEN (PARACETAMOL)-INDUCED HEPATOTOXICITY

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SUMMARY

Research into the pathogenesis of acetaminophen (paracetamol)-induced hepatotoxicity has concentrated on the generation of toxic metabolites by the hepatocytes. It has, however, recently been shown that human macrophages cultured with acetaminophen secrete increased quantities of tumour necrosis factor (TNF). This study examines whether macrophages have a direct role in acetaminophen toxicity, using a mouse model in which it is possible to eliminate more that 99 per cent of hepatic macrophages by previously injecting liposomes containing dichloromethylene disphosphonate (DMDP). Acetaminophen-induced liver damage was assessed biochemically and histologically. It was shown that the liver damage occurring 0·5, 1, and 2 h after an intraperitoneal injection of acetaminophen was significantly less in mice previously injected with liposomes containing DMDP than in previously untreated mice, or mice previously injected with empty liposomes. By 4 h there was no difference between the groups. We conclude that macrophages play an early and probably a direct role in mediating the liver damage due to acetaminophen. This is consistent with the role that macrophages have been shown to play in the pathogenesis of alcohol-induced liver damage.

KEY WORDS—**macrophage; acetaminophen; liver; dichloromethylene diphosphonate**

INTRODUCTION

Acetaminophen (paracetamol) has been used as an analgesic and antipyretic since its introduction in the mid-1950s. However, when taken in an overdose it is hepatotoxic and overdoses cause 200 deaths in Britain per year.[1] Until recently, the hepatotoxicity of acetaminophen has been considered to be entirely the consequence of its metabolism by hepatocytes.[2] Acetaminophen is metabolized by hepatocytes mainly by sulphation and glucuronidation, but when the enzyme systems involved in these processes become saturated, metabolism via the cytochrome P450 system becomes increasingly important. Cytochrome P450 converts acetaminophen to *N*-acetyl-*p*-benzoquinone imine (NAPQI) by a pathway utilizing oxygen and NADPH. The potentially toxic effect of NAPQI is initially largely prevented by the binding of NAPQI to intracellular reduced glutathione (GSH), but when the stores of GSH are depleted the free NAPQI binds to liver proteins, especially those with cysteine residues, and to DNA.[2,3] The overall effect is to increase cytosolic Ca^{2+}, which leads to centrilobular hepatocyte damage and, ultimately, hepatocyte necrosis. Acetaminophen can also cause liver damage when taken in therapeutic doses by chronic alcoholics, because ethanol induces the cytochrome P450 system and thereby leads to increased production of the toxic metabolite NAPQI.[4]

Recently it has been shown that acetaminophen, at concentrations seen in the serum of patients taking an overdose, stimulates blood-monocyte-derived human macrophages to secrete tumour necrosis factor.[5] This suggested that macrophages might play a direct role in the pathogenesis of acetaminophen-induced liver damage. To investigate this possibility, we have studied acetaminophen hepatotoxicity in control mice and in mice depleted of liver macrophages by injecting them with liposomes containing dichloromethylene diphosphonate (DMDP).

MATERIALS AND METHODS

Animals

Female C57BL/10 mice aged 8–12 weeks were obtained from BAK Universal Ltd., Hull, U.K. They had free access to CRM pellets and water and were kept at an ambient temperature of 26–30°C.

Preparation of drug-containing liposomes

Dichloromethylene diphosphonate (DMDP) was a gift from Boehringer Mannheim GmBH, Mannheim, Germany. Liposomes containing DMDP were prepared according to the method of van Rooijen *et al.*[6] The concentration of DMDP was measured based on the competitive binding of calcium between DMDP and murexide[7] and was 14–15 μM in a suspension containing $4{\cdot}7 \times 10^8$ liposomes per ml; the drug concentration in the liposomes was 31·9 nmoles per 10^6 liposomes. The modal diameter of the liposomes was 4·4 μm.

Injection of liposomes

Some of the mice studied were injected intravenously with 0·2 ml of DMDP-containing or empty liposomes 24–36 h prior to the administration of acetaminophen.

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Table I—Serum ALT activity in the three groups of animals injected with acetaminophen

Time after injection (h)	ALT (units/l)								
	Previously untreated			Previously injected with empty liposomes			Previously injected with DMDP-containing liposomes		
	n	Median	Range	*n*	Median	Range	*n*	Median	Range
0	15	33	26–41	15	34	28–49	15	35	31–42
0·5	16	192	162–225	15	210	157–289	15	54*	34–98
1	15	405	333–479	16	439	387–487	15	172*	106–299
2	14	816	771–857	—	—	—	15	636*	480–750
4	16	1134	1067–1342	16	1189	998–1286	16	1138	1025–1308

*Significance of difference when compared with the previously untreated group: $P<0·01$.

Injection of acetaminophen (paracetamol)

0·5 ml of acetaminophen (Sigma-Aldrich Company Ltd., Poole, Dorset, U.K.), at a concentration of 500 μg/ml, was injected intraperitoneally.

Experimental design

Five experiments were performed, in each of which 55–65 mice were used. In each experiment there were five groups of mice: (1) previously untreated mice, (2) mice injected 24–36 h previously with DMDP-containing liposomes, (3) previously untreated mice injected with acetaminophen, (4) mice injected 24–36 h previously with empty liposomes and then with acetaminophen, and (5) mice injected 24–36 h previously with liposomes containing DMDP and then with acetaminophen. In the three groups injected with acetaminophen, three animals were killed at each of the following time intervals after the injection: 0, 0·5, 1, 2, and 4 h.

Animals were anaesthetized with fluothane and bled from the femoral veins and/or by cardiac puncture. The blood was taken into plain tubes and the serum separated and stored at −20°C. The liver was removed and tissue from each lobe of the liver fixed in buffered formol saline.

Biochemical studies

In three of the experiments, sera from individual mice were assayed for alanine aminotransferase (ALT) activity using a Beckenham Synchron CX5 analyser.

Histological studies

The fixed livers were processed into paraffin wax and sections were cut and stained with haematoxylin and eosin (H & E), or used in the immunohistochemical studies described below. The degree of centrilobular liver damage was scored independently by two different observers according to the percentage of the lobule showing necrosis. The scoring system used was as follows: no damage=0; less than 10 per cent of the lobule=1; 10–25 per cent of the lobule=2; 25–50 per cent of the lobule=3; and more than 50 per cent of the lobule=4.

Immunohistochemical studies

Sections prepared as described above were stained using the mouse-macrophage-specific rat monoclonal antibody F4/80 (Sigma-Aldrich Company Ltd.). Binding was visualized using a polyclonal rabbit anti-rat antibody, followed by a biotin-labelled goat anti-rabbit IgG antibody and the ExtraAvidin–APAAP immuno-alkaline phosphatase technique (Sigma-Aldrich Company Ltd.).

Statistical analysis

Statistical analysis was carried out using the Arcus Pro Stat package using the Mann–Whitney *U*-test.

RESULTS

Immunohistochemical studies

Animals injected with liposomes containing DMDP showed a greater than 99 per cent decrease in the number of hepatic macrophages (Kupffer cells) as assessed by immunohistochemical staining for F4/80. This degree of macrophage depletion is similar to that reported in previous studies.[8,9]

Biochemical studies

In previously untreated mice not injected with acetaminophen and in mice injected 36 h previously with liposomes containing DMDP but not injected subsequently with acetaminophen, the median serum ALT activity was 35 units/l (range 17–49).

The results for the three groups of animals injected with acetaminophen are summarized in Table I. There was no significant difference in the serum ALT activity between the previously untreated group injected with acetaminophen and the group injected first with empty liposomes and subsequently with acetaminophen. By

Table II—Histological changes in the three groups of animals injected with acetaminophen

Time after injection (h)	Necrosis score								
	Previously untreated			Previously injected with empty liposomes			Previously injected with DMDP-containing liposomes		
	n	Median	Range	*n*	Median	Range	*n*	Median	Range
0	15	0	—	15	0	—	15	0	—
0·5	16	0·9	0–2	15	1·1	1–2	15	0·2*	0–1
1	15	2·2	1–3	16	2·3	1–3	15	1·1*	1–2
2	14	3·4	2–4	10	3·2	3–4	15	1·9*	2–3
4	16	3·9	3–4	16	3·8	3–4	16	3·5	3–4

*Significance of difference when compared with the previously untreated group: $P<0·05$.

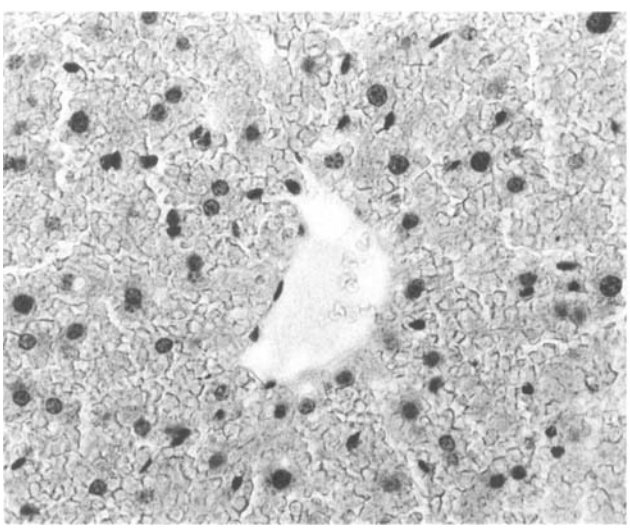

Fig. 1—Liver from a mouse previously injected with empty liposomes showing a pericentral zone of haemorrhagic necrosis 1 h after the injection of acetaminophen (H & E)

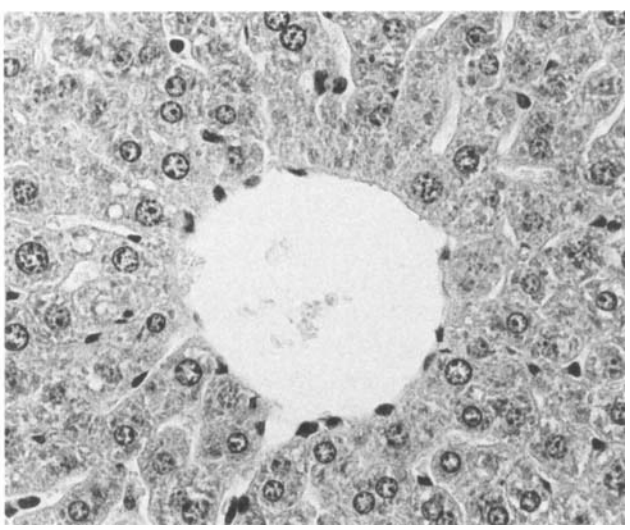

Fig. 2—Liver from a mouse previously injected with liposomes containing DMDP showing no necrosis 1 h after the injection of acetaminophen (H & E)

contrast, between 0·5 and 2 h after the injection of acetaminophen, the ALT levels in the group previously injected with DMDP-containing liposomes were significantly lower than each of the other groups ($P<0·01$ in all three cases). At 4 h, there were no significant differences between the groups.

Histological studies

In animals injected with acetaminophen, there was progressively greater liver damage over the course of the experiment and the extent of damage was similar in previously untreated animals and animals previously injected with empty liposomes (Table II). There were, however, statistically significant differences in the extent of centrilobular necrosis between the previously untreated group and the group injected with DMDP-containing liposomes (Table II; Figs 1 and 2). Between 0·5 and 2 h after the injection of acetaminophen, the extent of liver damage was significantly less in the group injected with liposomes containing DMDP ($P<0·05$ in all three cases). At 4 h, there were no significant difference between the groups.

DISCUSSION

The liver is a common site for drug-induced damage.[10] This is because drugs are transported to it from the gut via the portal blood supply and because hepatocytes are metabolically active cells capable of converting many drugs to toxic metabolites. It has been suggested that macrophages may also have an important role in drug-induced liver damage. The model proposed has been that macrophages are activated secondary to hepatocyte damage and then produce a wide range of inflammatory mediators capable of increasing liver damage, thus escalating the liver damage.[11] Nevertheless, it appears that in ethanol-induced liver disease, macrophages can cause liver damage independently of activation by hepatocytes.[12] Thus, it has been shown not only that macrophages are able to metabolize ethanol, but also that on a

per unit wet weight basis, they do so at a rate greater than hepatocytes and in doing so generate more extracellular acetaldehyde.[13,14] Furthermore, in both a rat and a mouse model of alcoholic liver disease, depleting the Kupffer cells prior to administration of ethanol results in less severe liver disease.[9,15]

In the present study we have used dichloromethylene diphosphonate (DMDP)-containing liposomes to eliminate the macrophages almost completely from the livers of mice; the liposomes are selectively taken up by phagocytic cells and following the subsequent release of the DMDP, these cells are killed. The elimination of macrophages was confirmed using an immunohistochemical marker for murine macrophages, F4/80. Injection of empty liposomes produced no decrease in the number of macrophages and no effect on the liver damage produced by a subsequent injection of acetaminophen. In contrast, mice previously injected with DMDP-containing liposomes (i.e., macrophage-depleted mice) showed less severe liver damage 0·5, 1·0, and 2·0 h after injection of acetaminophen than mice previously injected with empty liposomes. By 4 h, the degree of liver damage was similar in all three groups. Acetaminophen caused less early liver damage in macrophage-depleted animals than in control animals, both when the extent of damage was judged biochemically and when it was judged histologically. These data suggest that macrophages have a potentially important direct role to play in the early stages of acetaminophen-induced liver damage. The nature of this role remains to be determined. In this connection, it is of interest that macrophages contain enzyme systems capable of metabolizing drugs, including the cytochrome P450 system, and therefore of converting drugs such as acetaminophen into toxic metabolites.[16] In addition, we have shown that culturing human monocytes with acetaminophen results in an increased secretion of the inflammatory mediator—tumour necrosis factor.[5] Whether or not the latter phenomenon is dependent on the metabolism of acetaminophen by macrophages is not yet known.

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