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THE CYTOTOXICITY OF PLASMA FROM PATIENTS WITH ACUTE HEPATIC FAILURE TO ISOLATED RABBIT HEPATOCYTES

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Summary.—The cytotoxicity of plasma from patients with various types of liver disease to rabbit hepatocytes maintained *in vitro* has been investigated using a microcytotoxicity assay system. Plasma from patients with fulminant hepatic failure and uncomplicated viral hepatitis showed significant cytotoxicity compared to controls. The cytotoxic effect of plasma from patients with fulminant hepatic failure was reduced by charcoal haemoperfusion, or heating and dialysis. The bile acids chenodeoxycholic acid and lithocholic acid were cytotoxic when added to control human plasma. Such toxic factors may be responsible for the delay in liver regeneration often seen in patients with fulminant hepatic failure.

LIVER regeneration in man is rapid after resection for hepatic trauma or tumour (Aronsen *et al.*, 1969). After diffuse hepatocellular injury such as that in viral hepatitis, regeneration also occurs, although in the most severe instances—patients with fulminant hepatic failure (FHF)—little evidence of regeneration may be found at autopsy, even when the onset of the illness may have been 10 to 14 days earlier (Gazzard *et al.*, 1975). Since it seemed possible that the blood of these patients might contain substances which prevent or delay liver regeneration, we have studied the toxicity of plasma from such patients using a microcytotoxicity assay system. This is based on the culture of isolated hepatocytes *in vitro*. We have also examined the effect of charcoal haemoperfusion, used as a form of artificial liver support, on the cytotoxicity of plasma from patients with fulminant hepatic failure. For comparison cytotoxicity assays were also carried out in patients with jaundice due to extrahepatic obstruction or chronic liver disease and the possible factors involved in the cytotoxicity reaction were investigated in a series of *in vitro* studies.

PATIENTS AND METHODS

Preparation of liver cells and cytotoxicity assay.—Pieces of liver were removed from adult (3 kg) New Zealand white rabbits immediately after killing with pentobarbitone (Nembutal; Abbott Laboratories). The liver slices (3–5 g) were cut into small 1–2 mm cubes and added to 20 ml of the culture medium RPMI 1640 (Flow Laboratories) in a 100 ml bottle. This contained foetal calf serum (FCS) 10%; hyaluronidase 0·1% (Miles Research Division); collagenase 0·01% (Boehringer, Mannheim) all weight to volume; Hepes 1 mol/l 2·3%; penicillin 200 u/ml; and streptomycin 100 μg/ml. Sometimes DNAase 0·001% was added. The pH was adjusted to 7·35 and the liver was incubated for 12 h at 37° in an atmosphere of 95% O_2/5% CO_2. This procedure, as briefly described previously (Thomson *et al.*, 1974) disrupted the liver into single cells and small fragments. The single cells were collected by repeated centrifugation ($\times$ 3) in the medium TC 199 at 70 g for 5 min. These preparations were kept in tissue culture flasks (Falcon 3012) at 37° in the medium RPMI 1640 containing FCS, Hepes, penicillin and streptomycin. For a cytotoxicity experiment the cells were added to microculture plates (Falcon 3034) to give approximately 100 cells

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per well in 10 μl of RPMI 1640. The plates were then incubated as before for 24 h to allow the cells to adhere to the plastic surface.

Assessment of viability.—A variety of techniques were used to assess metabolic activity.

The uptake of 2 dyes, trypan blue (0·2% w/v aqueous solution) and eosin Y (1% w/v aqueous solution), was observed at various times after the isolation procedure.

Albumin was assayed in the supernatant of liver cells incubated overnight at 37° in RPMI 1640 containing FCS using radial immunodiffusion of dilutions of the supernatant against a specific antibody for rabbit albumin.

The incorporation of amino acid was investigated by the addition of [^{14}C] leucine (0·1 μCi, Radiochemical Centre, Amersham) to the medium RPMI 1640 (10 ml). This was added to approximately 8×10^6 cells, which were then incubated at 37° for 6 h and the amount of radioactivity was determined in the acid precipitable fraction of the cells (Rubenstein *et al.*, 1974). As a control experiment, cells were used after boiling for 5 min.

The incorporation of [^{3}H] thymidine (Radiochemical Centre, Amersham) into acid precipitable material after overnight incubation at 37° was also studied. 8×10^6 cells in RPMI 1640 containing 1 μCi in 10 ml were used. For comparison, the uptake into cells which had been boiled was measured.

8×10^6 cells were labelled with chromium (sodium [^{51}Cr] chromate, Radiochemical Centre, Amersham) by incubating them in 10 ml of RPMI 1640 containing 10 μCi of radioactivity for 2 h at 37°. The cells were then washed twice in medium, and incubated overnight at 37° in 10 ml of medium. The supernatant and a 10 ml saline (0·9%) wash were pooled and counted for radioactivity using a Packard 5023 tandem Auto-Gamma system.

The cells in the microculture test plates were treated with various concentrations (1–1000 μg/ml) of oubain or thimerosal (Sigma) in RPMI culture medium. Ten replicate wells were used for each concentration and the controls on each plate consisted of two rows of 10 wells containing 10% FCS alone. The plates were incubated for 48 h at 37° and then inverted for 2 h before washing with medium, fixing with alcohol, and staining with eosin. The number of cells in each well was counted using a light microscope. The mean number of cells in each well was compared with the mean number of cells in the 10 control wells. This was expressed as the percentage cytotoxicity.

Measurements on patients and in vitro studies.—Peripheral venous blood was taken from 18 normal controls, 20 patients with active chronic hepatitis, 13 patients with jaundice due to extrahepatic biliary obstruction and 10 with uncomplicated viral hepatitis at the height of their illness who had been admitted to hospital. Arterial blood samples were taken from 6 patients without liver disease who were undergoing diagnostic arteriography and from 14 patients with fulminant hepatic failure who were being treated by charcoal haemoperfusion (Gazzard *et al.*, 1974). The aetiology of the liver disease was viral hepatitis in 6 patients (3 were HBsAg positive), paracetamol overdose in 5, drug adverse reaction in 2, and amanita phalloides poisoning in 1 patient. Blood was also taken simultaneously from the "venous" line of the perfusion system after it had passed over the charcoal and was returning to the patient. The plasma was collected by centrifugation and immediately frozen and stored at −20° until use.

Samples of plasma were subsequently made up as a 20% solution in RPMI 1640 containing 10% FCS. In the microculture plate the medium was carefully aspirated and then one drop (approximately 10 μl) of the plasma solution was added to each culture well through a 0·45 μ Millipore filter using a 1 ml syringe and 25 gauge needle. The controls used were 30% FCS. The plates were then incubated at 37° in 95% O_2/5% CO_2 for 48 h and the loss of adherence measured as described above.

In some experiments, the plasma was heated at 56° for 30 min before dilution with medium and testing. This step was omitted for heated samples as it was difficult to millipore-filter the solutions after the procedure.

Samples (2 ml) of the plasma from patients in fulminant hepatic failure were dialysed overnight using a cellophane dialysis membrane at 4° against 0·1 mol/l phosphate buffer pH 7·4 before cytotoxicity testing.

In other experiments, analytically pure chemicals were added to normal control human plasma and the effect on the cytotoxicity was measured. The substances used were bilirubin, chenodeoxycholic acid (Weddel Pharmaceuticals), cholic acid, deoxycholic acid, taurodeoxycholic acid (Sigma) and taurocholic acid (Calbiochem). Bilirubin was used at a concentration of 260 μmol/l and the bile acids 97 to 133 μmol/l.

RESULTS

The various viability studies showed that, after preparation, the liver cells were rounded and possessed an intact plasma membrane. The experiment on dye uptake demonstrated that, after 24 h, the liver cells accumulated dye into their nuclei indicating possible cell damage, but the cytoplasm remained lightly stained. These appearances were found with cells

for up to 2 weeks after isolation when the cells would rapidly accumulate dye to all compartments indicating non-viability. The presence of immunoprecipitable albumin could be detected in the supernatant of overnight cultures of freshly prepared cells and there was a 10-fold greater incorporation of (^{14}C) leucine into acid precipitable material of freshly prepared cells as compared with cells which had been killed by boiling (7260 and 746 cpm/8 $\times$ 10^6 cells: mean of two experiments). When freshly prepared liver cells were incubated in the presence of (^{3}H) thymidine, there was a greater incorporation (39 cpm/8 $\times$ 10^6 cells) than controls which had been killed by boiling (4 cpm/8 $\times$ 10^6 cells), mean of 2 experiments. In both experiments, however, the total incorporation was low.

Using liver cells 1 day after their preparation labelled with ^{51}Cr there was an average loss of radioactivity of 16% from the cells into the supernatant during overnight incubation. In the microculture test system an increase in the concentration of the metabolic poisons thimerosal (an organic mercury compound) and oubain gave greater cytotoxicity values as measured by loss of adhesion to the microculture plate (Table I).

TABLE I.—*The Effect of Increasing Concentration of the Metabolic Inhibitors Oubain and Thimerosal on the Survival of Rabbit Hepatocytes* in Vitro *Expressed as % of Controls*

	Concentration μg/ml	Cytotoxicity %
Oubain	0·001	5
	0·01	3
	0·1	43
	1	64
Thimerosal	5	8
	10	43
	50	62
	100	72

Cytotoxicity of human plasma

The measurements of cytotoxicity using plasma from controls showed that there was no significant difference in the mean values for arterial samples taken from 6 patients undergoing diagnostic radiography (−11 $\pm$ s.e. 13·51%) and peripheral venous samples from 12 normal controls (0 $\pm$ 5·39%). The negative values obtained in some of the measurements with human plasma were indicative of a better survival of the liver cells in the presence of human plasma than foetal calf serum.

The highest cytotoxicity values (Fig. 1) were found in plasma from patients

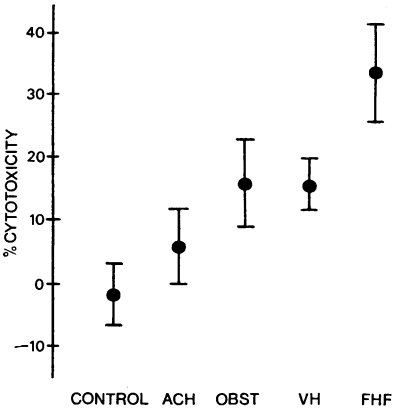

FIG. 1.—Cytotoxicity in the hepatocyte culture system of plasma from 18 control patients, 20 with active chronic hepatitis (ACH), 13 with obstructive jaundice (OBST), 10 with acute viral hepatitis (VH), and 14 with fulminant hepatic failure (FHF). Mean value and standard error are shown.

with FHF (mean values 34 $\pm$ s.e. 7·56%) followed by uncomplicated viral hepatitis (16 $\pm$ s.e. 4·25%). Both these values were significantly greater than the cytotoxicity of plasma from control subjects ($P < 0·005$ and $P < 0·05$ respectively; Mann-Whitney u-test). Although the values in FHF were higher than in uncomplicated viral hepatitis, this did not reach statistical significance. Mean cytotoxicity found with samples from patients with obstructive jaundice and active chronic hepatitis did not differ significantly from the controls. There was no correlation between the biochemical abnormalities as shown by the results of standard liver function tests and the cytotoxicity

values from the microculture test system (Table II).

TABLE II.—*Plasma Bilirubin and Aspartate Aminotransferase in the Different Groups of Patients. The Mean ± SD Values are Given*

Disease	Plasma bilirubin μmol/l	Aspartate amino-transferase i.u./l.
Active chronic hepatitis	27±17	114±112
Obstructive jaundice	342±335	169±96
Viral hepatitis	108±162	276±169
Fulminant hepatic failure	287±217	856±568
Normal range	0–12	10–50

The addition of a variety of substances, including bile acids and bilirubin, to plasma appeared to enhance cytotoxicity (Table III) but only the value for lithocholic acid and chenodeoxycholic acid reached statistical significance.

TABLE III.—*The Cytotoxicity of Normal Plasma Containing Added Bilirubin and Bile Acids*

	Concentration μmol/l	Cytotoxicity %
Bilirubin	26	−14
Cholic acid	122	−5
Taurocholic acid	97	4
Deoxycholic acid	127	33
Taurodeoxycholic acid	100	37
Chenodeoxycholic acid	127	54**
Lithocholic acid	133	44*

* $P < 0{\cdot}005$ (Student's t test).
** $P < 0{\cdot}001$

In the patients with FHF being treated by charcoal haemoperfusion the cytotoxicity of arterial plasma samples was significantly higher ($P < 0{\cdot}02$; Wilcoxon's matched pairs signed rank test) than the samples taken simultaneously from the venous output lines (Fig. 2). Heating the plasma samples (Table IV) produced a significant reduction in mean cytotoxicity in those patients with fulminant hepatic failure. This was also observed in uncomplicated viral hepatitis. To examine the possibility that this reduction in cytotoxicity with heating might be related to the omission of the millipore filtration step for the heated samples, the cytotoxicity of 6 plasma

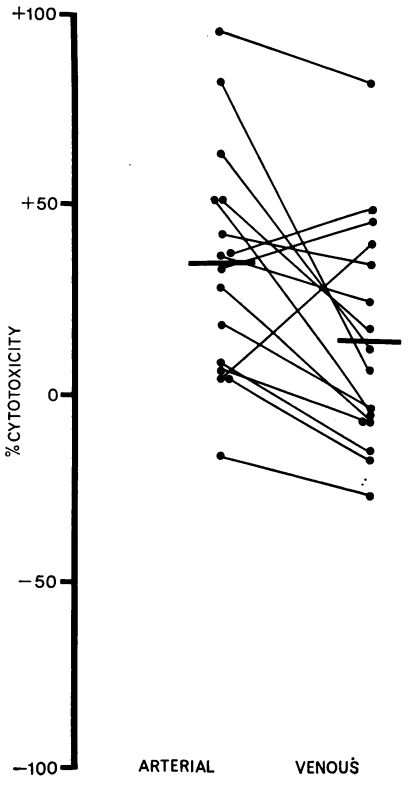

FIG. 2.—Percent cytotoxicity of plasma from the inflow (arterial) and outflow (venous) lines of the charcoal column during the first haemoperfusion in 14 patients with fulminant hepatic failure. Results are also shown for samples taken during the second perfusion in two of these patients. The horizontal line indicates mean value in each group.

TABLE IV.—*Mean Reduction in Cytotoxicity Following Heating Plasma Samples*

Disease group	Number of subjects	Reduction in cytotoxicity %	P*
Control subjects	18	19·5	NS
Obstructive jaundice	13	15·1	NS
Viral hepatitis	10	28·9	0·05 to 0·01
Fulminant hepatic failure	14	39·3	0·01

* Wilcoxon's matched pairs signed rank test.

samples from patients with FHF were examined before and after millipore filtration. There was no significant difference (mean values 20 ± s.e. 11·05% and 25 ± s.e. 7·63% respectively). Reduction in cytotoxicity was observed when plasma from FHF was dialysed against buffer, resulting in a decrease in cytotoxicity of 51 to 4% and 65 to 21% in the 2 patients tested. The cytotoxic factor could not be detected in concentrates of the dialysis fluid after dialysis of the FHF plasma.

DISCUSSION

The principle of the cytotoxicity assay system used in this study is dependent on obtaining viable hepatocytes. This appeared to be so, for although there was some intracellular accumulation of dye which suggested that the cells were damaged to some extent (Howard, Lee and Pesch, 1973), they were still capable of secreting albumin and incorporating amino acids into acid precipitable material. The visual appearance of intact plasma membranes was confirmed by the small loss of ^{51}Cr from radio-labelled hepatocytes. The low incorporation of [^{3}H] thymidine together with the absence of mitoses and cell division was possibly due to the liver cells being obtained from adult rabbits without prior partial hepatectomy. The isolated liver cells were used in the microculture tests for up to 14 days after isolation, providing they retained their well-rounded shape and adherence to the plastic surface. The dose-related response to the metabolic inhibitors oubain and thimerosal suggests that cell detachment, which we have attributed to cytotoxicity, is indeed associated with a depression of cell metabolism. The demonstration of factors in the plasma toxic to these cells could be of relevance to the problem of hepatic regeneration.

Samples of plasma from all patients with liver disease showed an increased cytotoxic effect compared to normal controls. It was most striking in those with fulminant hepatic failure and to a lesser extent in those with uncomplicated viral hepatitis. As the cytotoxicity of plasma from patients with obstructive jaundice was not significantly different from the control subjects, it suggests that the observed cytotoxicity in other patients may be related to substances found in the plasma during hepatic necrosis other than bilirubin, for the mean bilirubin levels in the patients with obstructive jaundice was greater than in both viral hepatitis and acute hepatic failure. Furthermore, the addition of bilirubin to plasma *in vitro* did not enhance its cytotoxic effect. Some of the patients with acute hepatic failure had a raised blood urea, but there was no correlation between the cytotoxicity and uraemia, or the aetiology of the fulminant hepatic failure.

Attempts were made to try and define more closely the substances responsible for the cytotoxic effect. Heating the plasma reduced the cytotoxicity in all cases and this was most marked in the patients with viral hepatitis and fulminant hepatic failure, although the results for heated plasma did not fall to control levels. However, this may indicate that some of the toxic effect is attributable to the accumulation of volatile compounds or proteins such as complement which are inactivated by heat. Heat inactivatable factors which interfere with liver regeneration have been found in uraemia (Chen and Leevy, 1973) and in normal rat plasma (Onda and Yoshikawa, 1973).

The bile acids chenodeoxycholic acid and lithocholic acid which we found to be toxic to isolated hepatocytes, have also been shown to be toxic in other investigations. For instance chenodeoxycholic acid suppresses the metabolism of rat brain and liver slices (Williams and Taylor, 1973), and lithocholic acid is hepatotoxic when administered to rabbits (Holsti, 1960). Their importance in liver disease is uncertain for they accumulate in obstructive jaundice with little evidence of liver cell injury. It is possible, however, that they are more harmful when

liver cell destruction has occurred and new hepatocyte formation is needed to replenish the liver cell mass.

The reduction in cytotoxicity of blood from patients in fulminant hepatic failure taken from the venous line during charcoal haemoperfusion compared with simultaneous arterial samples suggests that the charcoal was adsorbing substances toxic to isolated rabbit hepatocytes. This effect may be related to the apparent increase in survival we have found with this treatment (Gazzard *et al.*, 1974). Although substances up to about 2000 daltons molecular weight are adsorbed by charcoal, the removal of the cytopathic effect by dialysis *in vitro* suggests that at least some of the molecules responsible are of lower molecular weight.

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