HEPATOCYTES AND NONREGENERATIVE HEPATOCYTE CYTOSOL IN THE TREATMENT OF ACUTE HEPATIC FAILURE

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Degree of M.D.

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Research conducted in the ward and laboratories of the Liver Unit, Queen Elizabeth Hospital, Birmingham.

January 1990

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ACKNOWLEDGEMENTS

It is with grateful thanks that I acknowledge the help I received in the undertaking of the experimental work and the encouragement given in the preparation of this thesis. In particular I wish to acknowledge the contributions of the following people.

Mr David Anderson who prepared the rat hepatocyte cell cultures and attended to the maintenance of the cells under the culture conditions. He performed the ^{3}H uptake measurements and the diphenylamine assay of DNA concentration on the rat hepatocytes.

Mr Tariq Ismail who prepared and maintained the human hepatocyte cell cultures and also undertook the ^{3}H uptake study and diphenylamine assay on these cells.

Mrs Jean Fitzmorris who prepared by sectioning and staining all the histological sections from the rat livers.

Mr Keith Garvey and the staff of the Department of Clinical Chemistry, Queen Elizabeth Hospital who undertook all the biochemical measurements of liver function on the plasma obtained from the rats.

Dr Alastair Strain who gave considerable encouragement throughout the undertaking of the experiments and provided much appreciated advice.

I wish to express particular thanks to Mr Paul McMaster who provided the initial inspiration and the encouragement to continue. I appreciate his allowing me to study the patients under his care and those of his consultant colleagues and for putting all the facilities of the Liver Unit Laboratories at my disposal. For his support, encouragement and advice I shall be forever grateful.

SUMMARY

Fulminant hepatic failure is a condition associated with a high mortality. The experience of its treatment over a 26 month period in a referral unit for acute liver failure is reviewed. The overall mortality rate was 45% but for those who progressed to grade IV hepatic coma the mortality rate was 73%. These figures represent the results of a combination of medical management and liver transplantation. The largest group of patients referred had developed liver failure due to ingestion of paracetamol (55%), the next largest group had viral hepatitis, mainly non A non B hepatitis (25%) and the others had an assortment of aetiologies including acute Budd-Chiari syndrome and other drug reactions. These different aetiological groups have been considered separately and predictors of outcome determined from their admission and early clinical and laboratory results. In the paracetamol group degree of coma, bilirubin and creatinine levels, hydrogen ion concentration and prothrombin ratio proved to be useful prognostic indicators but in the other two groups these measurements bore no relationship to the ultimate outcome.

Past and present treatments of fulminant hepatic failure are then reviewed with discussion of the timing and long term consequences of orthotopic liver transplatation. The history of hepatocyte transplantation is presented with particular reference to its use in acute hepatic failure in experimental animals. Reference is made to studies

suggesting that intact hepatocytes are not required to improve survival of the animals but that subcellular fractions from regenerating livers are as effective at improving survival. Cytosol from nonregenerating liver has in the past been shown to be ineffective at improving the animals' survival.

A model of acute liver failure using a single intraperitoneal injection of dimethylnitrosamine was developed in the Wistar rat and produced a reproducible degree of liver necrosis. This model was then used in all subsequent in vivo experiments.

Hepatocyte transplantation was investigated using the intrasplenic route or by intraperitoneal injection. Neither of these treatment modalities significantly improved the survival or any of the other measured indices of severity (blood coagulation, biochemical liver function tests or extent of hepatocyte necrosis) when compared with the control groups. Two of the rats treated with intraperitoneal hepatocytes did survive to show full recovery from liver failure and clumps of intact hepatocytes were found in the peritoneal cavity at post mortem. Some of these cells appeared on light microscopy to be viable but the deposits also contained some dead cells. This finding is contrary to the work of other researchers who have failed to detect any evidence of cells, either dead or alive, when they have been transplanted by this route.

The hepatocytes which had been prepared by in situ collagenase perfusion of the liver were then disrupted by ultrasound bombardment and ultracentrifuged to obtain hepatocyte cytosol. This was injected intraperitoneally in repeated doses to the rats with liver failure and there was a significant improvement in animal survival compared with the control group. This effect may have been even greater but some of the rats developed fatal intraperitoneal haemorrhage because of the repeated injections during a time of defective blood coagulation.

Cytosol was then given by repeated intravenous injection and this produced a significant improvement in survival with 75% of the rats recovering. Different doses of cytosol were tried and the improvement was evident over the three doses tested. When plasma was given the result for this was midway between the cytosol and saline (control) groups and not significantly different from either. Further experiments showed the cytosol to be effective if administered up to 24 hours after the induction of liver failure. In a paired experiment where the cytosol treated rat was sacrificed at the time of death of its control rat no significant difference was seen in the amount of liver necrosis but there was significantly more cellular regeneration in the surviving hepatocytes in the cytosol treated rats.

Human hepatocyte cytosol was also used in the rat model and produced a similar improvement in survival showing that the

substance responsible for this improvement is also present in human liver cytosol.

Rat cytosol was heated at over 56°C and another sample dialysed across a small pore membrane and the fractions tested in the liver failure model. The results of this suggested that the active substance is a molecule of molecular weight below 8,000 daltons and is heat labile.

Both rat and human cytosols were added to hepatocytes in cell culture and changes in the amount of DNA synthesis measured. Neither of the cytosols produced a demonstrable change, suggesting no increase in cell regeneration.

A possible explanation for these findings is that by using pure hepatocyte cytosol this preparation is not contaminated by cytosol from nonparenchymal cells as the liver cytosol produced by homogenization of liver slices used by other workers must be. This could be the reason that my cytosol from nonregenerative liver is effective treatment of liver failure and that the contribution from the Kupffer and endothelial cells is what is inhibitory to cell growth. The factor producing the beneficial effect is obviously not acting directly on the hepatocytes to stimulate increased regeneration but must require an interaction with other tissues (possibly the reticuloendothelial system) in the intact animal to produce its effect.

Liver transplantation is at present the best therapy for clinical fulminant hepatic failure but is not appropriate for all patients and is not without long term morbidity. Alternative treatments to support the failed liver or increase regeneration of the surviving hepatocytes require to be found and developed. In this thesis a method of producing hepatocyte cytosol is described and its administration to rats with acute liver failure is reported. When given intravenously in repeated doses it improves the survival of the animals and appears to do so by stimulating regeneration. If this technique could be applied to humans it might improve the prognosis for some patients with fulminant hepatic failure.

INTRODUCTION

Fulminant hepatic failure has been defined by Trey and Davidson (1970) as the development of hepatic encephalopathy within 8 weeks of the onset of liver failure. It poses a major problem for the clinician because of its considerable mortality when treated medically (Vickers 1988).

Within the last 5 years there has been the treatment option of liver transplantation which can give better patient survival rates. Unfortunately a donor liver is not always available at the most appropriate time. Even if an organ can be found, it may not be the most suitable for the intended recipient, in terms of size, age, ABO blood group, or be from a suboptimal donor. Not all patients with acute liver failure are suitable candidates for liver transplantation and decisions require to be made about which patients should be offered this treatment. A further weakness of orthotopic transplantation as treatment is that it involves immunosuppressive therapy for the rest of the patient's life. This carries an increased susceptibility to infection and malignancy. Episodes of graft rejection and possible loss of the liver from this make transplantation less than the ideal solution to the management of hepatic failure.

Numerous liver support systems have been tried but the initially reported improved survivals have not stood up to

critical study, neither would many achieve public acceptability.

Hepatocellular transplantation showed promise as a possible solution to the problem but despite initial enthusiasm these attempts have not been adapted to the clinical situation.

In this study clinical experience in the Liver Unit of the Queen Elizabeth Hospital was recorded over a 2 year period. Then experiments are undertaken to develop an animal model of acute liver failure and to use this to explore different treatments of liver failure. Isolated hepatocytes were transplanted and then subcellular components of the hepatocytes from normal (nonregenerating) liver were injected. Different routes of administration of both cells and cytosol were also explored.

By these methods it is hoped to develop a readily available, easily administered, effective, temporary therapy for acute liver failure which has the potential for adaptation to the human situation.

AIM

i) to assess the number of, and result of therapy in, patients presenting with and developing acute hepatic failure and to investigate the potential help of prognostic factors in their management;

ii) to set up and validate a model of acute hepatic failure in a small animal;

iii) by means of hepatocyte transplantation or injection of hepatocyte-derived products by various routes to alter the outcome of the hepatic failure in relation to survival, the rate of recovery of clotting factor synthesis, biochemical liver function tests and histopathological features;

iv) to attempt partial physical and chemical purification of any substances proving effective;

v) to test cell-derived substances in vitro by their effect on DNA synthesis in hepatocyte culture.

vi) to determine if any substance found in rats is present in human hepatocytes.

ACUTE HEPATIC FAILURE - A CLINICAL PERSPECTIVE

Fulminant hepatic failure as defined by Trey and Davidson (1970) is a syndrome of failure of liver function with the development of hepatic encephalopathy within 8 weeks of the onset of the illness in a person with no previous liver disease. It is associated with a considerable mortality and even when treated aggressively survival rates only reach 20-30% by conventional medical management (Vickers 1988). The advent of hepatic transplantation has given a lifeline to some of these patients but the supply of donor organs is not sufficient to provide adequate numbers of livers for these emergency transplantations as well as supporting the ever increasing demand from patients with chronic hepatic diseases. The extent of the need for emergency liver replacement must be discerned, with identification of the patients most likely to benefit from the limited resources, and other methods of supporting the temporarily failing liver found.

Attempts to help this problem have been made by Christensen (1984), Tygstrup (1986) and O'Grady (1989) by defining those subgroups of patients with acute liver failure who have the worst prognosis, and identifying patients who cannot be expected to recover adequate hepatic function. Those factors which principally affect the outcome are the age of the patient (with older and younger people faring worse) and the underlying aetiology (with patients who have taken a paracetamol overdose usually recovering better than those who have viral hepatitis). Even within the types of

viral hepatitis those with non A non B hepatitis have a greater mortality than those with hepatitis A or hepatitis B.

In an attempt to investigate the numbers involved, all patients with acute liver failure referred to a supraregional liver unit were studied.

Patients and methods

From the beginning of January 1987 till the end of February 1989, 89 patients with acute hepatic failure were admitted to the liver unit at the Queen Elizabeth Hospital in Birmingham. All were treated by conventional medical means with attention to the fluid balance, controlled by central venous pressure monitoring; correction of hypoglycaemia, by the infusion of 50% dextrose solution; supplementation of blood clotting factors with fresh frozen plasma to avoid spontaneous haemorrhage; renal dialysis, either peritoneal or haemodialysis if needed to compensate for inadequate renal function and mechanical ventilation if the respiratory function of the patient was unable to ensure satisfactory oxygenation. They received enteral lactulose, intravenous ranitidine and vitamin K. Signs of raised intracranial pressure detected clinically or by intracranial pressure monitoring were treated by intermittent boluses of intravenous mannitol and/or by controlled hyperventilation.

Paracetamol levels were measured, often in the referring hospital, in all patients in whom drug overdose was considered likely and a full drug history was obtained from the patient or family. Other causes of acute liver failure were sought including diagnostic investigations for Wilson's disease, acute viral infections; hepatitis A and B serology, and also for cytomegalovirus and Epstein Barr virus. Radiological investigations such as ultrasound and angiograghy were used to diagnose such conditions as Budd-Chiari syndrome. The clinical and laboratory measurements previously shown by other groups (O'Grady 1989) to indicate disease outcome; age, aetiology, extent of hepatic coma, clotting derangement as measured by the prothrombin ratio, transaminase and bilirubin levels, white cell count, hydrogen ion concentration and serum creatinine were measured on admission and daily thereafter. These measurements were analysed, both by admission values and the most abnormal value in the first 48 hours in the unit. The time until admission from onset of symptoms or taking of a drug overdose was recorded. Whether the patients who had taken paracetamol were treated with acetylcysteine was also noted..

Some of the patients were considered for liver replacement. Whether they received a hepatic transplant, died while awaiting a donor organ or recovered without transplantation was recorded, as was the ultimate outcome of the episode of liver failure.

Results

During the 26 months, 89 patients with acute hepatic failure were admitted. The median age was 31 years with a range of 18 months to 66 years and there were 25 males and 64 females. 48 patients had taken paracetamol overdoses, 23 had viral hepatitis of which 3 were due to hepatitis B infection and the others, by exclusion, diagnosed as non A non B hepatitis. 18 patients had other causes for their hepatic failure including 6 with other drug causes, usually idiosyncratic reactions, 4 with acute Budd-Chiari syndrome, 2 women who developed liver failure post partum, 2 patients whose hepatic failure followed surgery and/or anaesthesia, and one patient each with Hodgkin's lymphoma, alcoholic hepatitis, a haematological problem and one lady who developed acute veno-occlusive disease of the liver following a bone marrow transplant for leukaemia.

In the total group, 49 patients survived (55%). Of the 37 patients who developed grade IV hepatic coma 10 recovered giving a mortality rate in this most severe group of 73%.

The different aetiological groups will now be considered separately.

Paracetamol overdose

48 patients were admitted with hepatic failure due to paracetamol. One claimed to have taken large doses over a period of time to treat severe headaches but the rest took the tablets at one or occasionally two episodes because of psychological or emotional stress. 36 (75%) of this group were female. 20 of these patients developed fulminant hepatic failure with progression to grade IV coma. The overall mortality rate for this group was 35% with 17 of the patients dying, but in the fulminant subgroup the mortality rate was 75% (15 of 20). The overall survival of the females was 61% and 75% for the males. 47% of the females developed fulminant failure compared with 25% of the males.

Whether or not the patient received acetylcysteine was not known in 8 of the cases but of the other 40, 13 were given this treatment. Most of the patients not treated with this drug attended hospital too late for it to be considered effective. The median time from overdose to admission to the liver unit was 3 days with a range of 1 to 5 days. Most of the patients were referred from other hospitals having presented there at least 2 days after taking the tablets.

3 of these patients were considered for liver transplantation. One died before a donor liver could be found, one underwent a liver transplant but despite postoperative evidence of function in the graft she failed to recover neurologically and was found to have sustained

cerebral coning. The third patient recovered without a transplant.

The survivors and non survivors were compared with reference to the prognostic factors discussed and the results shown in table 1. (median and range given for each variable)

Table 1	survivors		non survivors		signif.
age	25	(14-46)	31	(14-56)	N.S.
sex	F/M	22/9	F/M	14/3	
admission coma grade	1	(1-4)	3	(1-4)	p<0.005
AST	4470	(619-12000)	5126	(2826-14000)	N.S.
bilirubin	64	(13-128)	96	(71-129)	p<0.05
H+ conc.	33	(30-37)	42	(37-81)	p<0.05
PT ratio	4	(1.5-9)	5	(3-15)	p<0.02
WBC	13	(6-20)	16	(7-26)	N.S.
creatinine	110	(54-533)	274	(95-415)	p<0.05
first 48 hrs coma grade	1	(1-4)	4	(2-4)	p<0.005
AST	3339	(435-12000)	6133	(3000-14000)	p<0.05
bilirubin	108	(18-290)	154	(103-235)	p<0.05
H+ conc	31	(28-37)	41	(30-81)	p<0.05
PT ratio	4	(1.5-12)	6	(3-15)	N.S.
WBC	15	(6-20)	17	(5-28)	N.S.
creatinine	108	(67-756)	516	(95-852)	p<0.05

Viral hepatitis

Of the 23 patients who presented with viral hepatitis 3 were infected with hepatitis B virus, none with hepatitis A and by exclusion the remainder were considered to by suffering from non A non B hepatitis. When these patients presented and this study was undertaken serological testing for hepatitis C was not routinely available and so this cannot be considered as a separate diagnosis.

11 of the patients (48%) developed grade IV coma and this subgroup had a mortality of 64% compared with a mortality rate of 52% for the whole group. 17 people received liver transplants, 10 of them once they had deteriorated to grade IV coma and the other 7 before this stage but with indications of a deteriorating course. Of the patients transplanted in grade IV coma 4 survived (40%) compared with 4 of 7 surviving if transplanted before they reached this stage. Three other patients were considered for transplantation but died before a donor organ could be found. There were 8 males and 15 females in this group with ages from 18 months to 60 years (median 35 years). The prognostic factors for this aetiological group between the survivors and non survivors are compared in table 2.

Table 2	survivors		non survivors		signif.
age	41	(1.5-60)	35	(4-57)	N.S.
sex	F/M	8/2	F/M	6/6	
admission coma grade	4	(3-4)	4	(1-4)	N.S.
AST	1176	(925-1241)	296	(107-5000)	N.S.
bilirubin	594	(340-607)	625	(532-900)	N.S.
H+conc.	35	-	28	-	N.S.
PT ratio	2.5	(2-4)	3	(1-9)	N.S.
WBC	10	(5-16)	11	(6-20)	N.S.
creatinine	86	(77-356)	80	(68-144)	N.S.
first 48 hrs coma grade	4	(3-4)	3.5	(1-4)	N.S.
AST	1050	(796-1241)	305	(107-5000)	N.S.
bilirubin	633	(377-796)	672	(365-900)	N.S.
H+conc.	31.5	(29-34)	27.5	-	N.S.
PT ratio	3.5	(2-6.5)	3.4	(1-11)	N.S.
WBC	9	(5-16)	11	(7-20)	N.S.
creatinine	180	(80-356)	124	(90-309)	N.S.

Other causes

This group of 18 patients had a variety of aetiologies for their hepatic failure. Their ages ranged from 15 to 60 years with a median of 37 years and there were 5 males and 13 females. 8 survived the episode of acute liver failure (44%). 9 developed fulminant hepatic failure of whom 1 survived (11%). 2 of these patients has a sub-acute form of hepatic failure with a prolonged period of over 8 weeks before they deteriorated neurologically and even then they did not reach grade IV coma. Three of the patients were

treated with a liver transplant 2 of whom survived. One further patient died on the list awaiting transplantation. The comparison of prognostic factors between the survivors and non survivors is shown in table 3.

Table 3	survivors		non survivors		signif.
age	40	(31-66)	35	(15-60)	N.S.
sex	F/M	7/1	F/M	6/4	
admission coma grade	2	(1-4)	2	(1-4)	N.S.
AST	502	(225-1384)	2580	(61-18000)	N.S.
bilirubin	233	(96-302)	312	(108-607)	N.S.
H+conc.			58	-	
PT ratio	2	(1-2.5)	3.25	(1.5-5)	N.S.
WBC	24	(5-25)	11	(1.6-49)	N.S.
creatinine	100	(59-296)	161	(53-792)	N.S.
first 48 hrs coma grade	2	(1-4)	4	(1-4)	N.S.
AST	363	(127-1384)	802	(62-18000)	N.S.
bilirubin	134	(31-302)	34	(96-675)	N.S.
H+conc.			38	(28-50)	N.S.
PT ratio	2.5	(1-5)	3.5	(1.8-5)	N.S.
WBC	24	(5-25)	13	(2-52)	N.S.
creatinine	213	(59-450)	186	(66-819)	N.S.

The different aetiological groups are compared in table 4

Table 4	paracetamol	viral	others
age	27 (14-56)	35 (1.5-60)	37 (15-66)
sex M/F	12/36	8/15	5/13
% developing FHF	42%	47%	50%
overall survival	65%	43%	44%
survival in FHF	25%	36%	11%
% transplanted	2%	74%	17%
% transplant survival	0%	47%	67%
time from onset of symptoms till admission (days)	3 (1-5)	14 (7-90)	14 (2-100)
prognostic factors effective at predicting outcome	YES	NO	NO

Discussion

From these results it is seen that acute hepatic failure, particularly in its fulminant form, is a disease associated with a considerable mortality. Despite the improvements in this situation predicted a few years ago (Buckels 1987, Bismuth 1987) with the introduction of liver transplantation as a treatment for this condition the longer term results have not been as good as anticipated. Reasons for this include donor shortage and the short time interval during which liver transplantation can be undertaken.

A better understanding of the natural history of fulminant liver failure must be achieved so that those patients who

will benefit from transplantation can be identified early and selected for this mode of therapy and the others who can be managed by conventional medical therapy do not use up the restricted number of donor organs or have to undergo unnecessarily the future disadvantages of a life of immunosuppressive therapy.

Several hepatologists have assessed prognostic factors in order to identify the group of patients likely to do worst with conservative management and so indicate those who should be offered transplantation. However, our results show that these parameters of degree of acidosis, prothrombin ratio, white cell count, transaminase or bilirubin levels, coma grade or creatinine level are only applicable to the patients who took paracetamol overdoses and that they do not predict the patients who will do badly with viral hepatitis or from the other assorted hepatitis aetiologies. In understanding other series assessing prognostic factors the relative proportions of the various aetiologies must be known since the size of the paracetamol group will distort the findings.

The viral group contained a higher proportion of patients who underwent liver transplantation and the direct consequences of this therapy may have altered the predictive accuracy of the prognostic factors; to the extent that survival in this group might reflect the ability to overcome the transplantation procedure rather than the natural history of the disease.

The results from this study indicate that for patients who have liver damage due to the ingestion of paracetamol the admission severity of hepatic coma, prothrombin ratio, bilirubin, hydrogen ion and creatinine levels are adequate to identify those who are unlikely to survive with conservative therapy alone.

Whole or partial organ transplantation cannot be the best answer to the treatment of liver failure in all patients. It is a considerable trauma to an already seriously ill patient and commits him or her to a life time of immunosuppression and all of its associated problems. Even if the liver is successfully transplanted and functions satisfactorily there is sometimes no return of neurological activity as happened in 3 of our patients, one in the paracetamol group and 2 in the viral group. Organ transplantation is not applicable to the patient with psychological or emotional imbalance and should only be considered in a small number of the instances of paracetamol overdose.

Comment

These findings prompted an investigation into providing a temporary method of liver support using cellular material and subcellular substances in an experimental model of acute hepatic failure to try to determine a short term, readily available, hopefully easily administered treatment which would improve survival and be adaptable to fit the clinical needs in fulminant hepatic failure.

TREATMENTS OF ACUTE HEPATIC FAILURE - PAST AND PRESENT

Diseases attributable in origin to the liver were recognized early in medical history (5th and 4th centuries B.C.) but since no diagnostic investigations were possible treatments were to say the least empirical, entailing various ways of draining abscesses and at about 300-250B.C. Erasistratos is reported as opening the abdomen and applying medication directly to a diseased liver. At this time paracentesis through the umbilicus was employed to remove ascites.

Major changes in therapy did not develop until the 19th century when, with the aid of the microscope, it was possible to differentiate different types of liver disease. A diagnosis of hepatitis could be made but the clinicians of that day considered it to be stage in the development of a liver abscess. Liver congestion was similarly thought to be a forerunner of hepatitis. Treatment for this sequence of diseases was based upon blood-letting particularly by English physicians such as Annesley, Twining and Morehead working in tropical countries. In 1887 however the German pathologist Thierfelder stated that he was unable to show any direct effect of blood-letting on the inflammatory process. A more direct approach was instituted by MacLean and Harley who plunged a trocar 15cm into the liver to drain pus and/or blood. Despite the exponents' enthusiastic reporting of the healing produced, this therapy was not generally accepted. Other treatments used at this time

included emetics and purgatives, calomel, nitric acid, mercury, blistering plasters, mustard fermentations and iodine compounds. This "hepatitis" is unlikely to be the pathological entity that is now recognized by that name.

Outbreaks of endemic jaundice were encountered in the 17th, 18th and 19th centuries and these may have been similar to some of the infective types of hepatitis diagnosed today. The treatment of this disease was mainly by purgatives and dietary measures and often no therapy was given since it was observed that "for the most part this endemic hepatitis cured itself spontaneously".(reported by Franken, 1974)

Fulminant hepatic failure was first described by Lucke and Mallory in 1946 as severe, acute impairment of hepatic function culminating in hepatic encephalopathy. This was modified in 1970 by Trey and Davidson who stated that the encephalopathy must develop within 8 weeks of the onset of the illness in a person with no evidence of previous liver disease. This definition excludes a group of patients in whom the long term sequel and survival are in many ways similar to the group with acute fulminant hepatic failure but in whom the development of encephalopathy takes longer to manifest itself. This more protracted form has received a variety of names including late onset hepatic failure, subacute or subchronic atrophy of the liver, subacute hepatitis, subacute (fatal) hepatitis and subacute hepatic necrosis. Most of these cases are thought to be viral in aetiology, have similar symptoms and signs to the more

familiar acute form but to have a higher proportion of older patients in the series.

General Supportive Measures

The mainstay of treatment of acute fulminant hepatic failure is by general supportive measures, paying attention to fluid balance usually with central venous pressure monitoring and closely observing urine volumes with recourse to renal stimulation with drugs such as frusemide or dopamine if necessary and early resort to dialysis either peritoneal or haemodialysis if indicated. Hypoglycaemia must be detected and treated by continuous dextrose infusions, often in increasing concentration as the patient's condition dictates. Biochemical monitoring of electrolyte concentrations and acid base balance is essential with correction of parameters to physiological levels. Reduction in liver produced coagulation factors leads to a prolonged prothrombin time making the patient susceptible to haemorrhage and fresh frozen plasma is administered when there is a danger of bleeding. As the level of hepatic coma increases the patient is unable to protect his own airway and endotracheal intubation often with mechanical ventilation is needed.

The onset of seizures is a serious complication associated with raised intracranial pressure and the presence of increased pressure can be detected and treated immediately by monitoring the intracranial pressure by placing a probe into one of the lateral ventricles. Treatment involves the administration of mannitol or other diuretics, ensuring a

high ventilatory pressure and nursing the patient in a head elevated position.

With increasing intensive care facilities the application of these measures has improved the survival prospects of patients with acute fulminant hepatic failure in the last 20 years. While in the 1960's the reported mortality was 95% (Gazzard 1974) now in the late 1980's the survival rate of patients reaching grade 4 coma has only reached 20% (Christensen 1984). Mortality rates vary depending on the underlying aetiology of the hepatic failure; patients with hepatitis A, hepatitis B and paracetamol overdose doing significantly better than people with non A non B hepatitis or drug reactions. Survival rates quoted in different studies are dependant on the relative proportions of the principle aetiologies and the age distribution of the patients studied.

Corticosteroids

Steroid therapy has gone through phases of popularity since it was first advocated by Ducci and Katz in 1952, being considered beneficial in viral hepatitis and in reducing cerebral oedema. This was countered by the increased incidence of gastric erosions and susceptibility to infections. The first randomised trials of steroid therapy (Ware et al 1974, Redeker et al 1976, Gregory et al 1976) failed to show any benefit but were criticised because of the small numbers of patients studied. In the multicentre trial instituted by the European Association for the Study of the Liver there was no difference in survival or side

effects of therapy between the steroid group and the controls although the number of patients included in the study was still only 40 and the aetiologies were not similar in each group.

Charcoal haemoperfusion

Gazzard et al in 1974 reported spectacular success using charcoal haemoperfusion in patients with grade 4 coma; a 50% recovery rate and 45% ultimate survival rate. They compared these results to their previously published series of patients who received the same supportive treatment but no charcoal haemoperfusion with a survival rate of 10%. The distribution of aetiologies was said to be similar but the number of patients studied was small. The same group then extended this therapy to those patients in grade 3 coma (Gimson, 1982) in a more strict regime with prostacycline infusion as platelet protection. Daily haemoperfusion, started when the patient was in grade 3 coma, achieved a survival rate of 65% compared to a 20% survival rate if the haemoperfusion was started in grade 4 coma. All the grade 3 patients did deteriorate into grade 4 so the severity of liver failure was comparable in both groups. There was less cerebral oedema in the group treated earlier; 49% compared with 78% in the group treated later. Despite the authors' concluding comment that a randomised control trial of this therapy against intensive supportive therapy would be difficult to justify ethically, they did proceed to this.

In 1988 O'Grady et al published the results of two trials of charcoal haemoperfusion therapy. Different durations of

haemoperfusion were compared in grade 3 patients and no difference was seen in the survival or cerebral oedema results between the two groups. In grade 4 coma patients charcoal haemoperfusion was compared in a randomised study with conventional therapy and no difference was found. There were slightly more patients from the aetiological groups with poorer prognoses in the control group but not a large enough difference to be significant. Even in this trial numbers were still insufficient to demonstrate small changes in survival rates; 29 patients in the treatment group and 33 in the control group.

As an alternative to charcoal the same group from King's College Hospital employed a mesh of XAD-2 resin in the haemoperfusion columns as a way of removing protein bound toxins from the circulation of experimental animals with ischemia-induced acute hepatic failure. (Weston et al 1974) This was technically unsuccessful because of haemorrhagic problems due to platelet adherence to the resin producing thrombocytopenia in the animals.

Large pore membrane haemodialysis

Haemodialysis can not only be used to substitute deficient renal function in patients with acute hepatic failure but also to filter out of the circulation some of the substances normally metabolised by the liver. To do this the usual haemodialysis membrane must be substituted for one with a larger pore size. Silk et al (1977) used a polyacrylonitrile membrane in a conventional haemodiaysis set-up as part of the treatment of 24 patients with

fulminant hepatic failure who had reached grade 4 coma. They concluded that their survival figure of 33% was similar to that they obtained during their phase of enthusiasm for charcoal haemoperfusion and better than that for conservative therapy. Their results were not supported by the study performed by Denis et al (1978) who failed to show any difference in survival over conservative management, although they achieved an increased occurrence of the return to consciousness.

In 1983 de Groot again employed this large pore membrane in haemodialysis in pigs with ischemically induced acute hepatic failure and found a significant improvement in survival compared to a control group which did not receive haemodialysis. In this study one period of 6 hours of dialysis appeared to be sufficient to improve the survival but all animals eventually died because the dialysis was unable to support the animal indefinitely in this model of irreversible acute liver failure.

Exchange transfusion

Other methods have been used to try to remove toxic substances from the circulation. In 1967 Jones et al employed exchange transfusion of blood to try to wash out harmful particles in seven patients with hepatic failure; six in grade 4 coma at the start of the treatment and one in grade 3. Despite the transfusion of between 5 and 58 litres of fresh blood per patient all died of liver failure with other complications directly attributable to the therapy. This study was in contrast to previously published

work giving encouraging results for this treatment. (Trey 1966, Berger 1966). All these reports had a small number of patients (one in the paper from Berger), the ages of patients were different between the groups and so were the predominant aetiologies and severities of the liver failure.

Cross circulation

In the early 1970's when mechanical means of liver support were unsuccessful the procedure of cross circulation was tried allowing the blood from the patient with hepatic failure to circulate through the body of another human in the hope that the "donor's" liver would perform the functions that of the patient was unable to do. (Burnell 1973) Donors used included patients with widespread malignancy, chronic renal failure and in one case the healthy son of the hepatic failure patient. Survival of the patients was in the region of 20% so it contributed nothing more than conservative treatment and not only did most of the donors develop some morbidity from the procedure but 2 died of causes directly attributable to the technique. Cross circulation was also used connecting the human patient to animals such as baboons (Bosman 1968).

Extracorporeal liver perfusion

This approach of perfusing through whole people or animals was modified by the removal of a donor liver from the body and setting up an extracorporeal liver perfusion with the blood of the patient circulating through the isolated liver. Various animals have been used in this way for the

treatment of humans and Abouna (1970) reported a case in which one patient with hepatic failure underwent a series of 16 extracorporeal liver perfusions employing the livers of 10 pigs, 3 baboons, 1 calf, 1 monkey and 1 human as well as 20 exchange blood transfusions. Although there were temporary improvements in the patient's condition there was no return of liver function and the patient died after 76 days of this treatment. Human livers obtained from cadavers were found to be better than livers from other species since they functioned for longer (Abouna 1970, Sen 1966).

Liver transplantation

A logical progression from this treatment has been the placement of the donor liver inside the patient with fulminant hepatic failure. Since 1984 increasing numbers of liver transplant centres have reported their experience of orthotopic liver transplantation in the treatment of acute hepatic failure.(Williams 1984, Adams 1986, Ringe 1986, Buckels 1987, Bismuth 1987, Brems 1987, Peleman 1987, Vickers 1988). This mode of therapy has run parallel to the improved results of liver transplantation for chronic liver diseases. This form of treatment for selected patients in grade 4 hepatic coma produces survival rates of 55%-74%. The differences in the results of all the quoted studies are dependant upon the ages of the recipients and the neurological status of the patients before transplantation. Early reports dealt only with a few patients but now results are available for larger series.

Liver transplantation is unfortunately not an option available to all patients developing fulminant liver failure. A small number will recover spontaneously with conservative medical management and for them transplantation is not needed and would be detrimental. A few patients will have developed irreversible neurological damage by the time of referral to a transplant centre and for them liver replacement would be a waste of resources. Moral decisions are required to justify transplantation of selected patients with paracetamol overdose liver failure, since some of these people will proceed to take further overdoses (Burglass 1974, Goldacre 1985). Viral hepatitis (hepatitis B, delta hepatitis and some forms of non A non B hepatitis) can recur in the new liver (Rizetto 1987, Ferla 1988) and most patients who are positive for hepatitis B antigen retain this antigenicity after transplantation.

In an attempt to reduce the long term morbidity associated with orthotopic transplantation, heterotopic (auxiliary) liver transplantation appears a sensible therapeutic option in acute liver failure. The insertion of a second liver below the native one to supplement its function while it recovers from the temporary failure, and then removal of the donor liver once the original organ is again functioning satisfactorily would have obvious advantages. This would involve only a limited period of immunosuppressive therapy. To date results of heterotopic liver transplantation in acute hepatic failure have not been very successful (Diaz 1977) and with 1 out of 3 surviving in the series reported by Terpstra (1989).

Comment

Despite the recent advances in the treatment of acute hepatic failure particularly in relation to liver transplantation survival figures, though improved, are not universally attainable for all sufferers with this disease. Death still occurs due to cerebral oedema in a substantial number of patients and although therapies such as charcoal haemoperfusion reduce the duration of hepatic coma they do not prevent a fatal outcome. The therapy of liver transplantation has produced major advances for selected patients but it means major surgery in a critically ill individual and commits the patient to life long immunosuppression. Some of the initial mortality and subsequent morbidity might be reduced if isolated hepatocytes or hepatocyte derived substances could be administered as temporary support.

EXPERIMENTAL HEPATOCELLULAR TRANSPLANTATION

Hepatocyte transplantation was first reported by Rugstad in 1970 when he implanted cells from a rat hepatoma into the subcutaneous tissues of a Gunn rat. The Gunn strain is deficient in the enzyme bilirubin uridyldiphosphate glucuronyl transferase and therefore has an unconjugated hyperbilirubinaemia (Gunn 1938). Following transplantation the rats were capable of conjugating bilirubin and the serum levels fell. Obviously the transplantation of neoplastic tissue did not hold much clinical application and so in 1973 Mukherjee placed fragments of normal liver from a Wistar rat into the liver of a Gunn rat and also achieved a reduction in serum bilirubin.

Isolated hepatocytes grown in cell culture had been shown to be metabolically active and so Matas and Sutherland (1976) infused a hepatocyte suspension from a Wistar rat into the portal vein of a Gunn rat and demonstrated that the bilirubin fell. However when the cells were placed intraperitoneally a drop in bilirubin level was not observed, neither was a positive result obtained when cells from other organs e.g. spleen were injected into the portal circulation. These findings were confirmed by other workers (Groth 1977). The intramuscular route also proved unsuccessful for hepatocyte function. The transplanted cells were identifiable in the portal vein branches within the liver in the early post transplant period but they became less easy to locate later in the study. Various explanations were suggested for these results; including

bilirubin conjugation having occurred within the donor hepatocytes, the possibility that the infused cellular material blocked up the reticuloendothelial system and delayed the breakdown of old red blood cells with reduced bilirubin synthesis being responsible for the lower serum levels (Woods 1981). He supported this latter theory by demonstrating that splenectomy reduced the serum bilirubin level in the Gunn rat. Vroemen in 1985 showed by using an allogeneic rat model that it was in fact the transplanted cells that produced the missing enzyme because the bilirubin level fell initially but after rejection and loss of the hepatocytes and bilirubin returned to pretransplant levels. Hepatocyte transplantation was then tried for different enzyme deficiencies in the experimental rat (Demetriou 1986a, Kebukuro 1987).

Since allogeneic hepatocytes are immunologically rejected (Ebata 1985) when non syngeneic cells are transplanted immunosuppression needs to be given to ensure donor cell survival (Makowka 1986, Darby 1986, Cobourn 1987).

In 1977 Sutherland showed that the mortality of rats with hepatotoxin induced hepatic failure could be reduced from 80% to 30% by hepatocellular transplantation. This was initially thought to be due to the transplanted cells taking over the hepatic function until the native liver regenerated, but on histological examination it was difficult to see new cells in the liver when they had been injected intraportally and no evidence of viable cells was found in the peritoneal cavity in the rats which received

the cells intraperitoneally. Sommer (1979) confirmed these observations in a model using a different hepatotoxin. He also transplanted hepatocytes into the spleen where they appeared to survive with an increase in animal survival from 20% to 70%. When this therapy was applied to rats with a 70% hepatectomy there was also improved survival (Minato 1984). Different groups of workers have found this technique effective if the cells are given at certain restricted time periods after the induction of acute hepatic failure (Makowka 1980a, Cuervas-Mons 1984) but the differences are probably related to the different hepatotoxins involved, doses of hepatotoxin or volume and concentration of hepatocyte suspensions and to the different strains of rats used. All of these variables make comparison of the results difficult.

Makowka (1980a,b,c) showed that allogeneic hepatocytes were effective at reducing the mortality as were xenogeneic cells (pig or rabbit hepatocytes transplanted into rats). If the cells were given intraperitoneally there was also an improvement in survival but no functioning hepatocytes could be detected histologically in the peritoneal cavity. Irradiated hepatocytes which could not divide (Kawai 1987) and cryopreserved hepatic cells with a limited life (Kusano 1981) were also able to reduce the mortality rates of rats with acute hepatic failure. Radiolabelling of the donor hepatocytes showed that the transplanted cells were not repopulating the native liver.

These experiments made it difficult to believe that intact, metabolically active hepatocytes were responsible

for the improvements in survival. It was postulated that the cells could be producing or releasing a substance that was increasing the rate of regeneration of the surviving native hepatocytes. Makowka (1981) demonstrated that cytosol prepared from the liver of a rat that had undergone a substantial hepatectomy was capable of producing the same results as isolated hepatocytes when injected intraperitoneally. Similar results were obtained when cytosol from fetal or weanling liver was used but cytosol from normal adult rats liver was not effective in reducing the mortality from acute hepatic failure. Cell-free supernatant from hepatocyte cell culture was administered in a similar way and also proved effective therapy in the rat (LaPlante O'Neill 1982) as did implantation of cells from other tissues e.g. bone marrow cells (Makowka 1980c).

Therefore although intact functioning hepatocytes appeared to be needed to produce a substitute liver in the enzyme deficient rat, the mode of action of the transplanted hepatocytes in the acute liver failure experiments did not appear to be dependant on cellular function.

Various sites of implantation have been tried in each experimental group; ranging from liver (Sutherland 1977), which makes identification of the donor cells very difficult, spleen which appears to be the most suitable environment (Mito 1979), pancreas (Vroemen 1988b, Jaffe 1988), under the capsule of the kidney (Ricordi 1988), the peritoneal cavity (Sutherland 1977),, the dorsal

subcutaneous fat pad (Jirtle 1982), muscle (Groth 1977) and the lung (Selden 1984).

Long term experiments have shown healthy looking hepatocytes present in the spleen more than a year after transplantation. By this time they have settled into an orientation which looks identical to liver parenchyma with the establishment of columns of hepatocytes with sinusoidal spaces detected between (Maganto 1988). These hepatocytes can be seen to account for up to 40% of the spleen, suggesting cellular replication. On electron microscopy bile canalicula, desmosomes between the cells and the appearance of endothelial cells lining the sinusoids can be detected. The intracellular organelles appear to be the same as those in non transplanted hepatocytes.

Vroemen(1988a) showed that the transplanted hepatocytes in the spleen showed mitotic activity and cellular proliferation throughout the first 20 weeks after transplantation not as a late phenomenon as was initially believed following the work of Kasano and Mito (1982). Most workers have failed to show definite evidence of cell proliferation in these hepatisized spleens (Cuervas-Mons 1985) and to date the mechanism of hepatic establishment is purely speculative but may be controlled by "trophic" factors.

Cryopreservation studies have enabled isolated hepatocytes to be stored in a frozen state at -196^{o}C and then function after thawing and transplantation into the spleen of syngeneic rats (Maganto 1986).

Demetriou (1986a,b) has developed a method of attaching isolated hepatocytes to collagen coated microcarriers which can then be injected intraperitoneally into experimental rats. These microcarrier-attached hepatocytes were also functional when transplanted after 2 weeks storage at -80^{o}C.

Hepatocytes have also been encapsulated in alginate microspheres prior to transplantation to reduce the antigenic stimulus when injected into allogeneic animals and these encapsulated groups of cells appear to function similarly to isolated hepatocytes (Wong 1986, Sun 1986).

Comments

Hepatocellular transplantation has now developed to the stage when groups of isolated hepatocytes can successfully be injected into experimental animals with long term survival of the cells and good cellular function even to the extent of the development of ectopic hepatic tissue within the recipient spleen. The cells can be adapted for storage and their immunogenicity modified to allow timing and interspecies flexibility of use.

MATERIALS AND METHODS

RATS

All the rats used except one in the cell culture experiment, which was female, were adult male Wistar rats obtained from the breeding colony in the Department of Biochemistry, University of Birmingham. They had free access to standard laboratory rat chow and water throughout the experiments and were maintained in the Department of Surgery Animal House in a room at a constant temperature and exposed to normal diurnal variation in light.

RAT HEPATOCYTE ISOLATION

Apparatus

A perfusion platform was mounted on the edge of a water bath which was heated to maintain the water at a constant temperature of 37°C. In the water bath were placed the following which were connected in order by means of plastic tubes; (i) a glass reservoir for the perfusion solution which could be filled via a funnel inserted in its neck and which emptied by gravity via a spout in its base. It also had 2 entry spouts near the neck to facilitate recirculation. Tubing from the reservoir exit was connected to the input spout of (ii) a bubble trap, to prevent air bubbles entering the system. The tubing from here passed out of the water bath and through a rotating pump head, the speed of which could be varied to produce different flow rates and then back into the water bath where it was connected to (iii) a heat exchange coil, the outflow from which could be attached to a cannula for tissue perfusion. The perfusion platform drainage channel could be connected by tubing to one of the input spouts of the reservoir.

Solutions

(a) Hank's balanced salt solution (HBSS) without calcium or magnesium with phenol red (Sigma) reconstituted from powder with addition of sodium bicarbonate as directed by the suppliers and adjustment of the pH to 7.44 immediately prior to use.

(b) Collagenase solution. 50 mg of collagenase H (Boehringer Mannheim) was added to 100 ml of HBSS (sol. a) to which was also added 1.5 ml 500mM calcium chloride.

(c) Hank's balanced salt solution with calcium and magnesium (HBSS+) (Sigma) which was reconstituted with the addition of sodium bicarbonate according to the suppliers instructions and the pH adjusted to 7.44 immediately prior to use.

Procedure

The method used was a modification of that described by Seglen (1976) which was based on the initial work by Berry and Friend (1969). Perfusion was performed without oxygenation or the addition of a biological buffer, but with intermittent addition of sodium hydroxide to the outflow from the liver before return to the reservoir during the collagenase perfusion phase.

The rat was anaesthetised with an intraperitoneal injection of pentabarbitone at a dose of 60 mg/kg and placed supine on the perfusion platform. A bilateral subcostal incision with sternal extension was made and the gastrointestinal tract reflected to the animal's left. The infrahepatic inferior vena cava (IVC) was encircled with silk ties and the IVC cannulated with an 18G cannula which was secured in position with the ties. 200 units of heparin was immediately injected via the cannula. The portal vein was divided and the intrathoracic IVC was ligated. The perfusion tubing was then connected to the cannula and perfusion commenced with 500 ml of HBSS at a flow rate of 28 ml/min. This flow rate was maintained throughout. Following this the liver was perfused with collagenase solution which was recirculated with correction of the pH

as necessary with sodium hydroxide. The collagenase perfusion was continued for about 10 minutes, the duration assessed by the appearance of the liver. When the liver looked digested the pump was stopped and the liver removed from the animal and placed in a tray containing HBSS+. The capsule was incised and the liver raked with forceps to release the hepatocytes from the vascular network. This crude cell suspension was passed through a double layer of gauze and then through a nylon mesh. The suspension was divided into centrifuge tubes and centrifuged at 300rpm for 3 minutes. The supernatant was removed and discarded and the loose pellet of cells resuspended in HBSS+. The centrifugation and resuspension was repeated twice and the volume of the resultant suspension adjusted to a manageable amount. Cell viability was assessed by Trypan blue exclusion and a total cell count made after suitable dilution of a sample of the suspension. (Figs. 1-12)

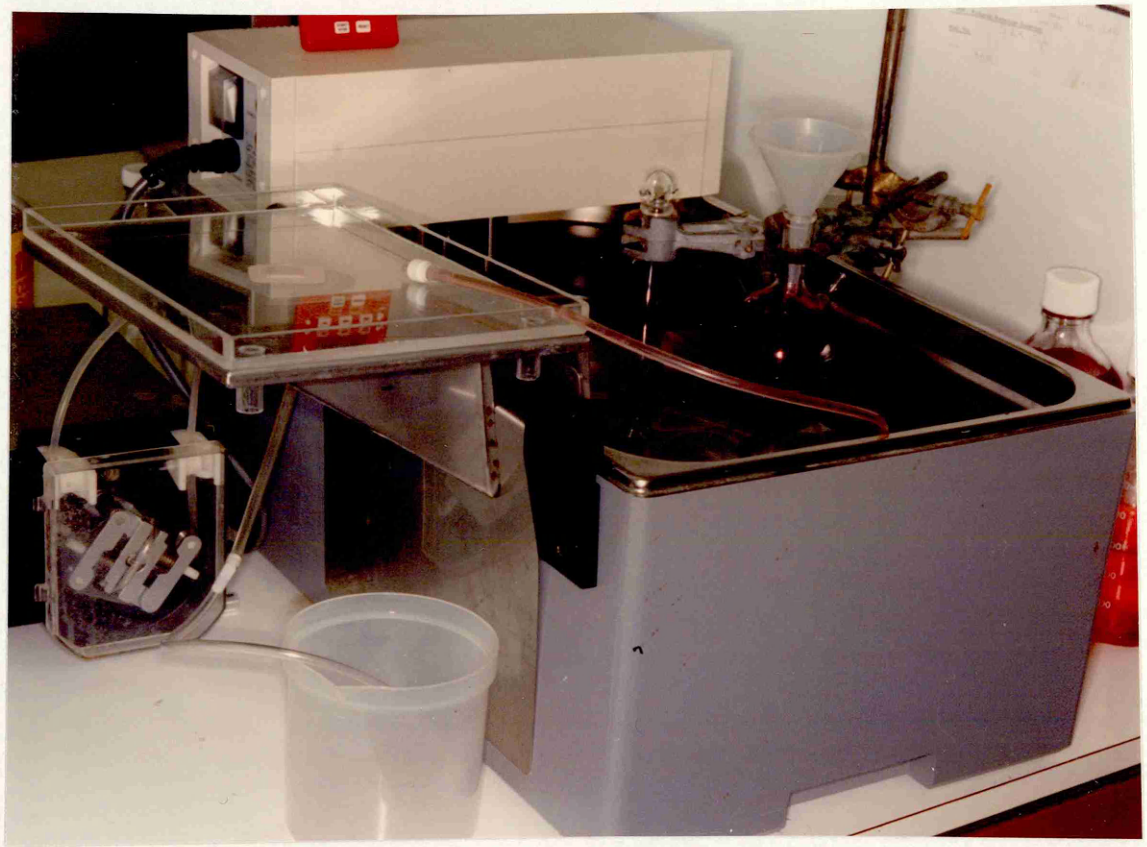

Fig. 1. Apparatus.

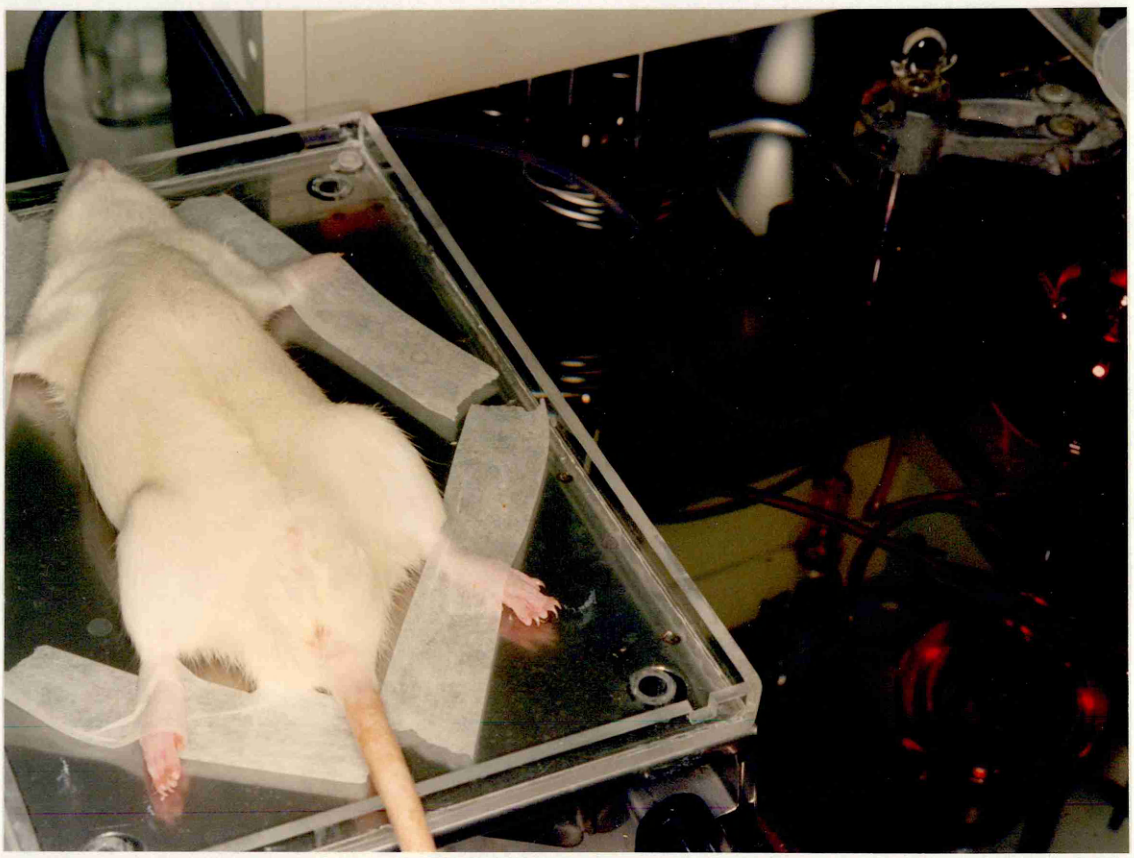

Fig. 2. Rat on perfusion platform.

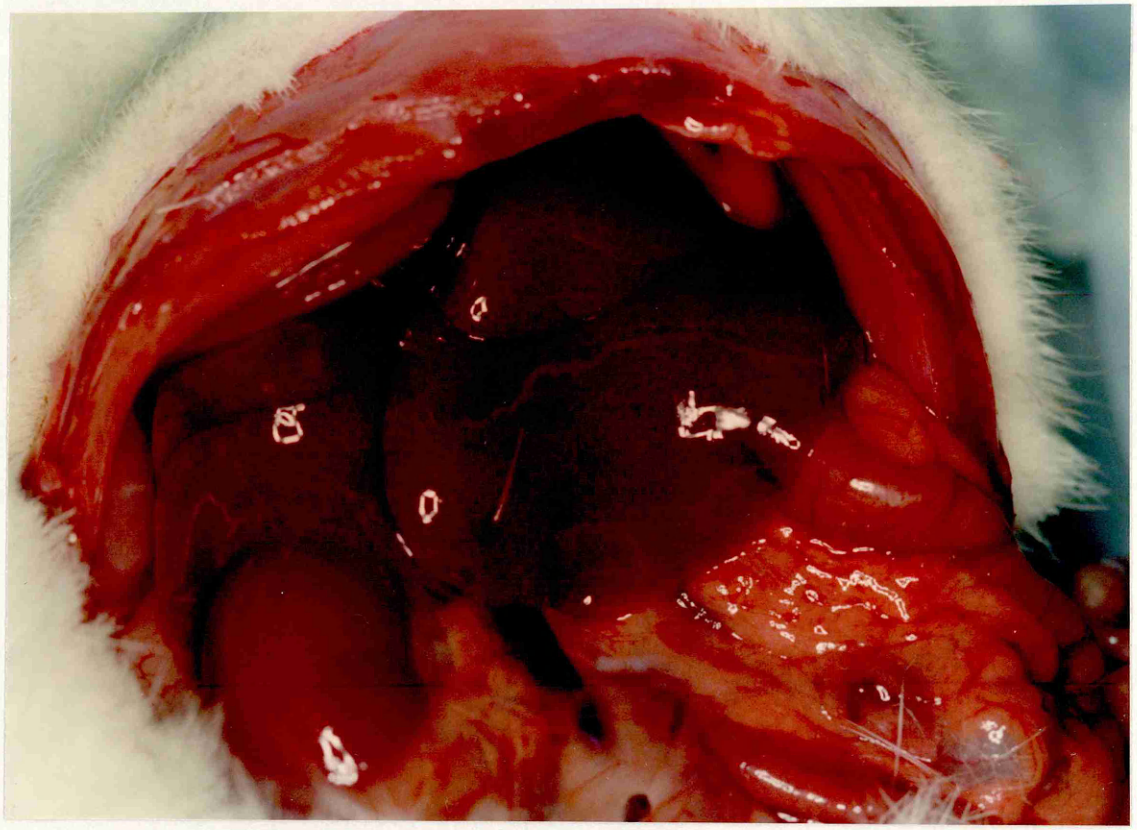

Fig. 3. Normal liver exposed.

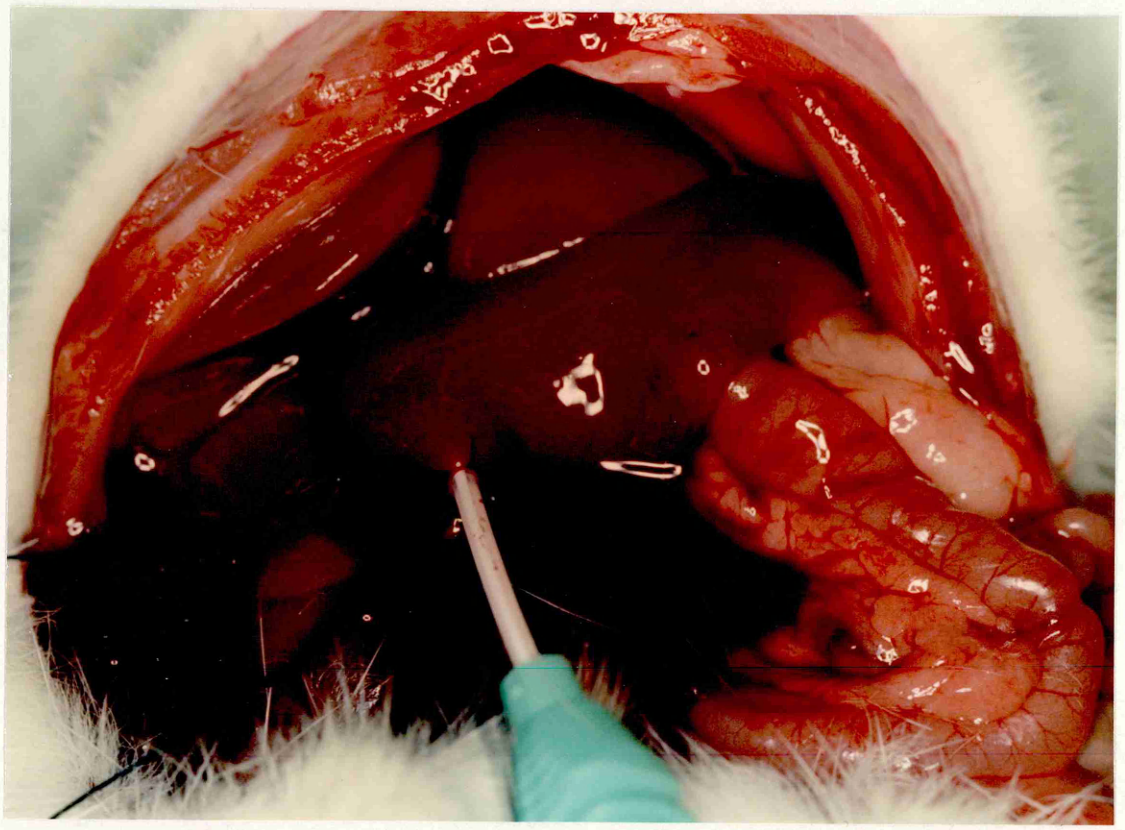

Fig. 4. Cannula inserted into inferior vena cava.

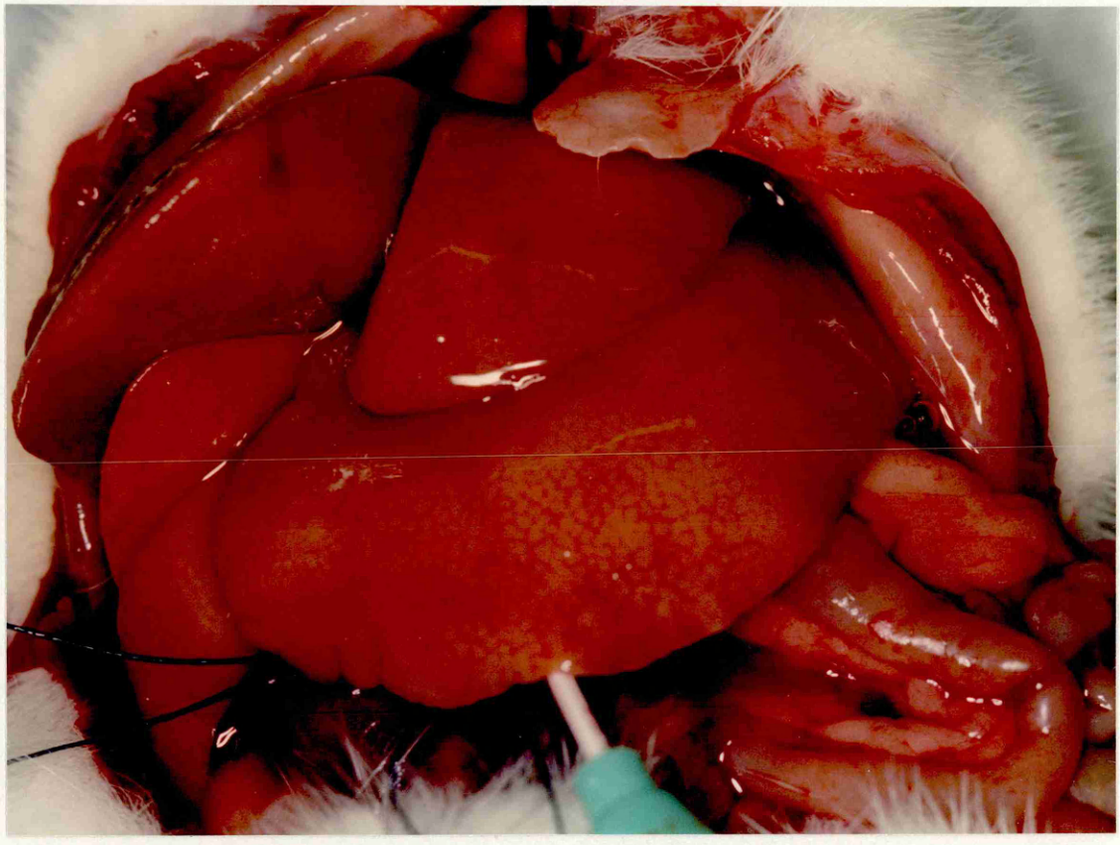

Fig. 5. Liver perfused with HBSS.

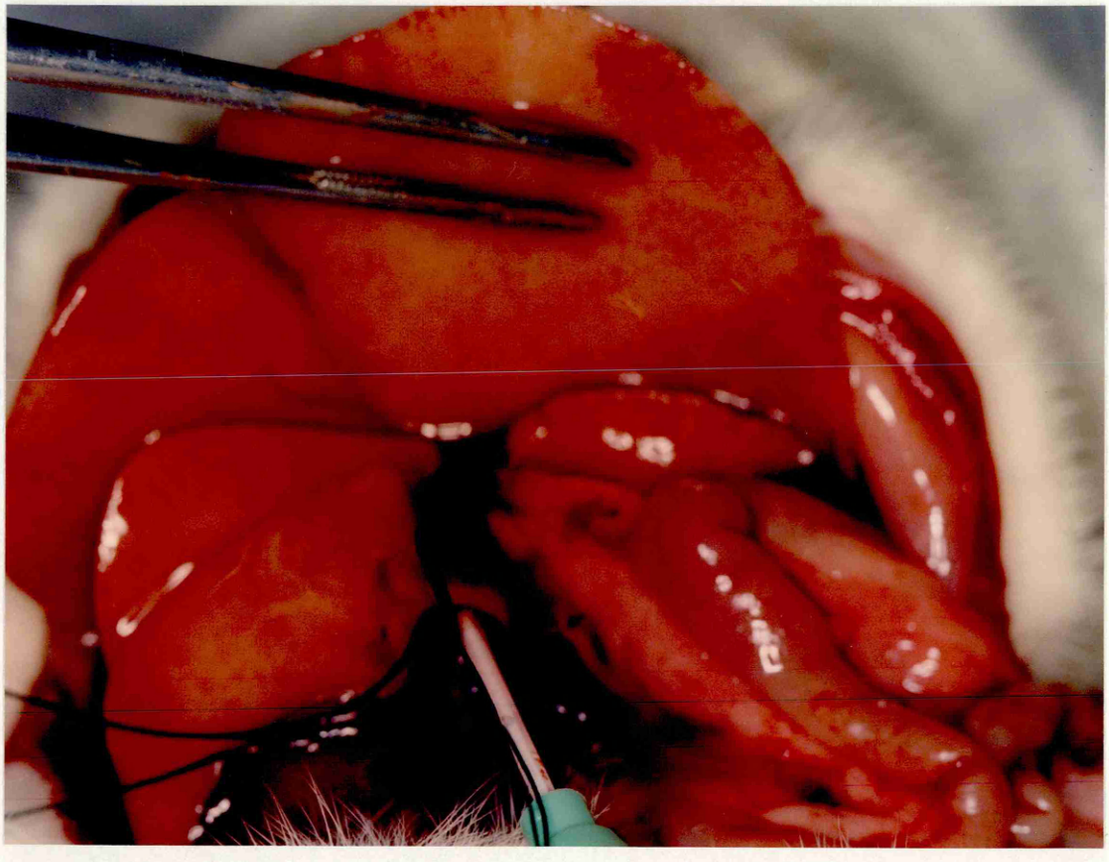

Fig. 6. Liver perfused with HBSS, view of porta hepatis.

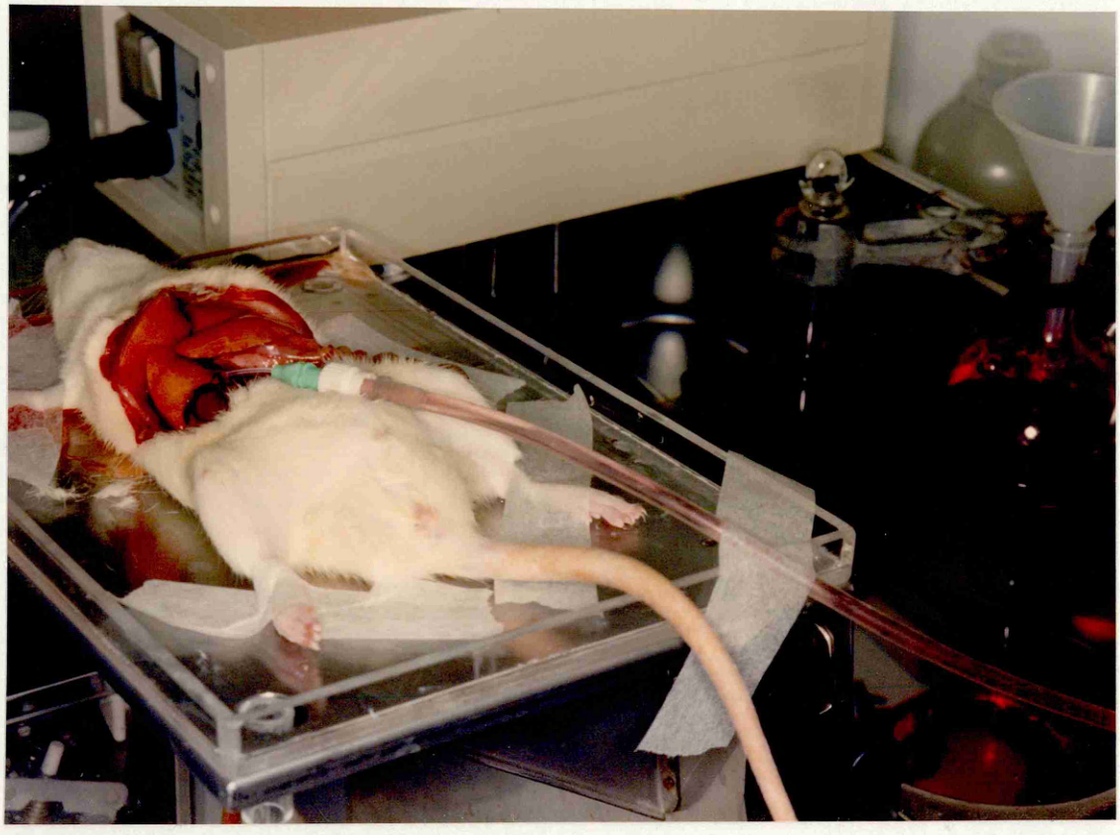

Fig. 7. Perfusion with HBSS.

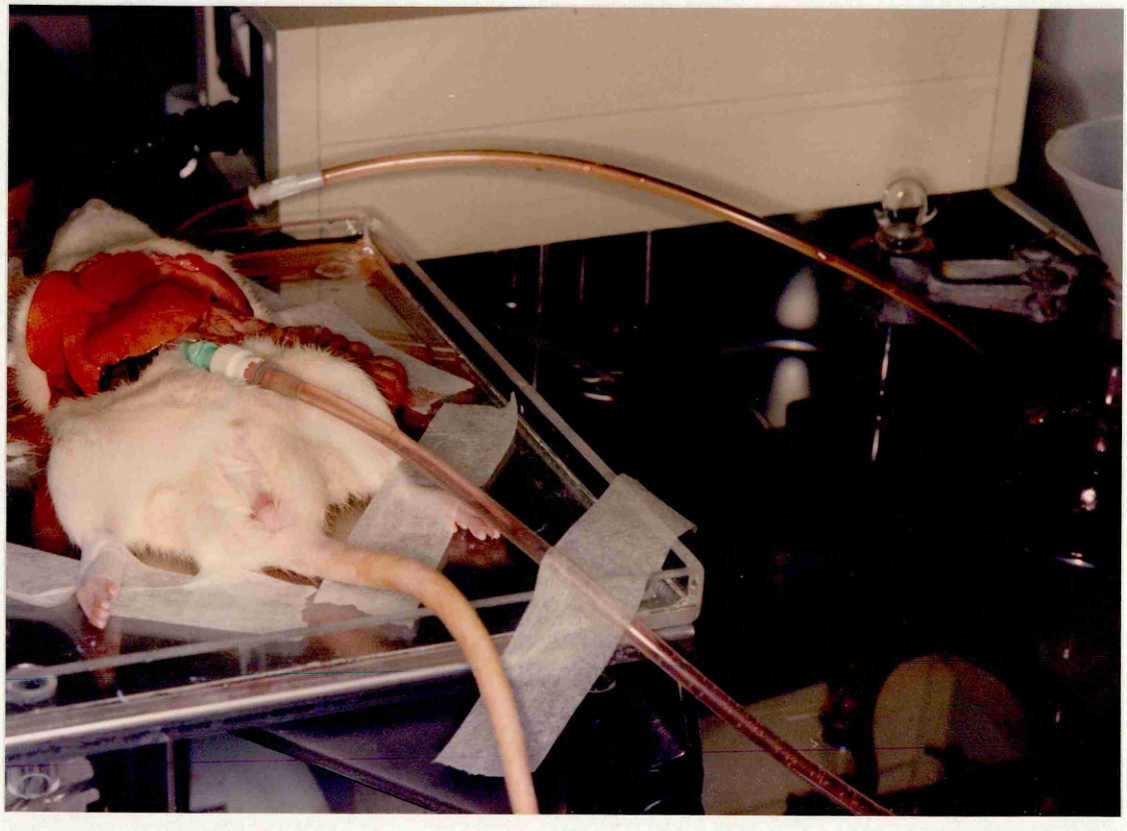

Fig. 8. Recirculating perfusion with collagenase soln.

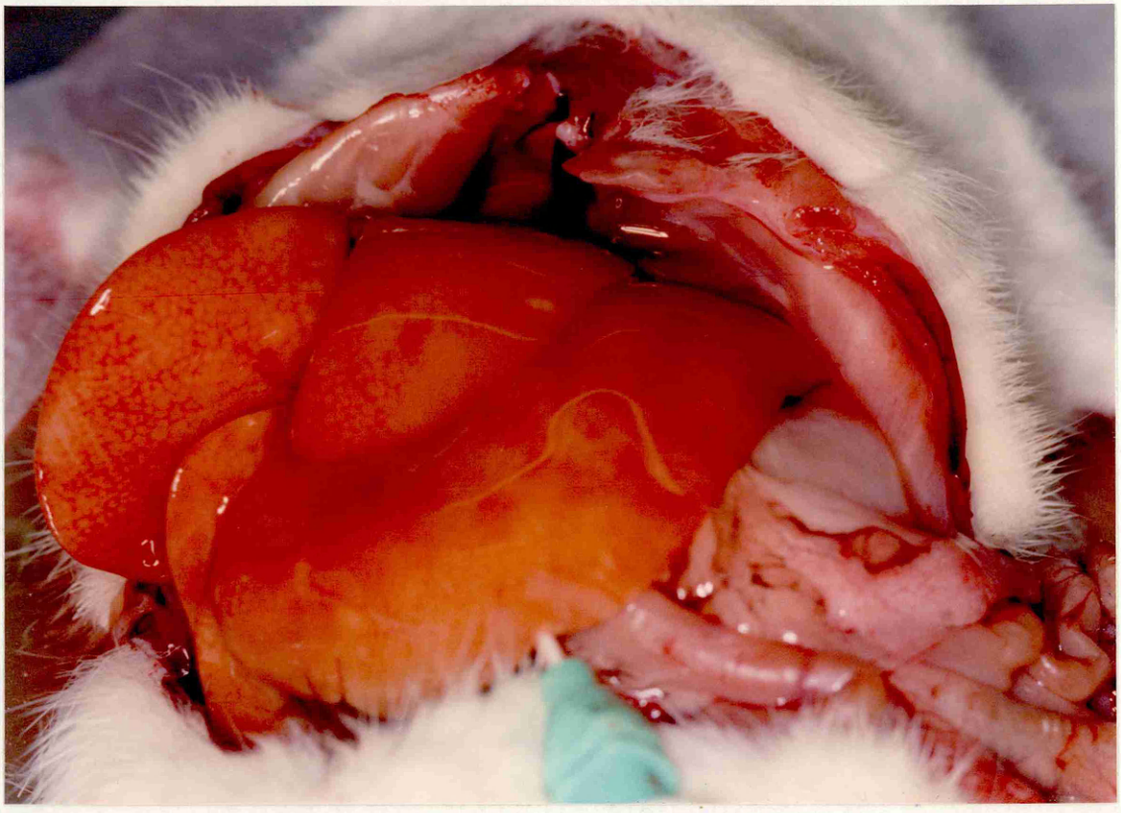

Fig. 9. Collagenase perfusion.

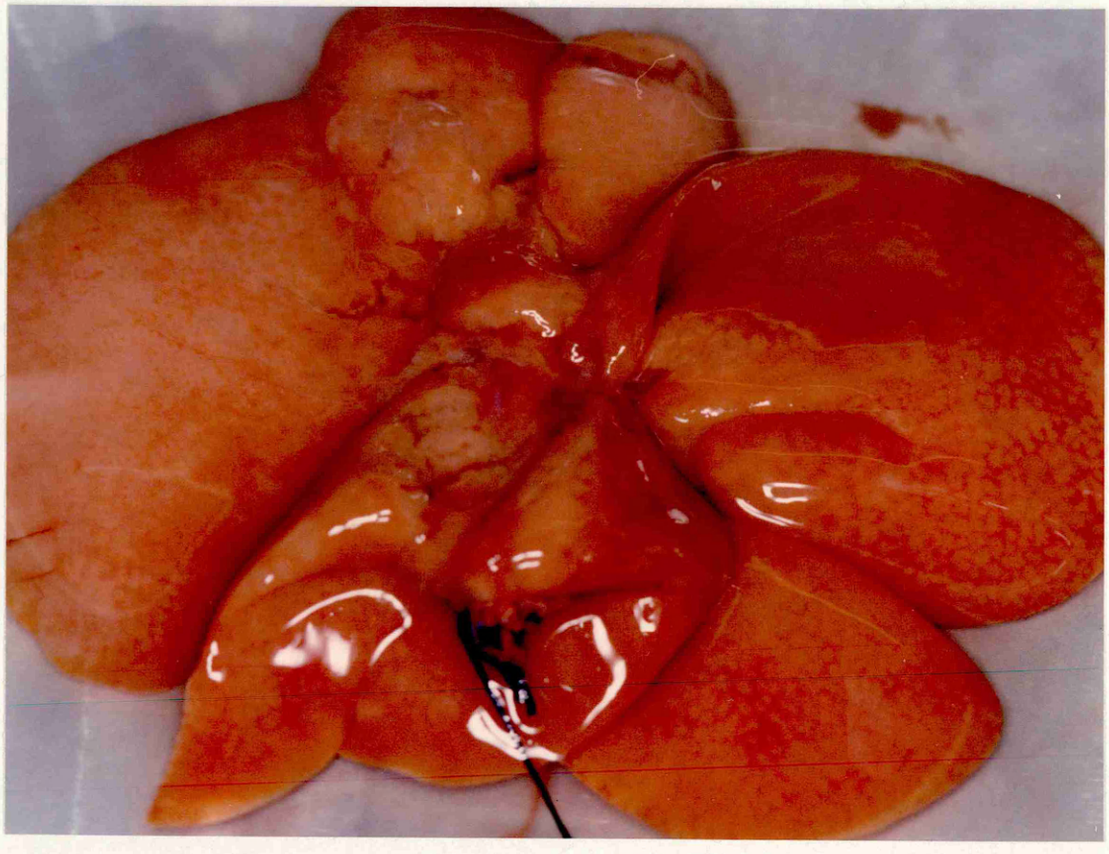

Fig. 10. Liver after removal.

Fig. 11. Hepatocyte suspension prior to centrifugation.

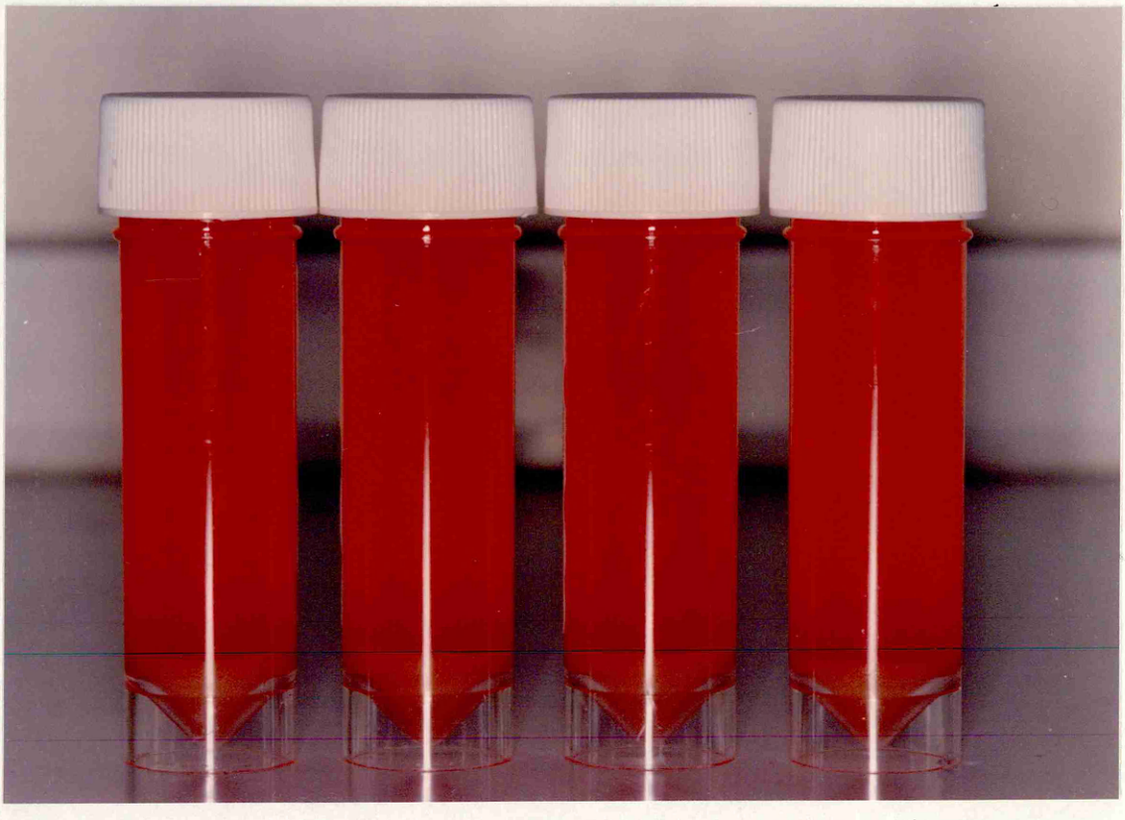

Fig. 12. Hepatocytes during centifugation and resuspension.

Trypan blue exclusion

0.1 ml 0.4% Trypan blue (Sigma) was mixed with diluted cell suspension (usually 0.1ml cells with 0.8 ml HBSS+) to give a final cell dilution of 1 in 10. This was instilled into a cell counting chamber and viewed under a microscope. The number of cells taking up the dye was counted, as was the total number of cells within the same area. The viability was calculated from the total cells minus the blue ones, divided by the total number and the result expressed as a percentage.

Total cell count

The total number of cells in the prescribed area of the counting chamber was adjusted for the dilution and the volume of the chamber to give the number of cells per ml of the suspension. This could be multiplied by the total volume to determine the yield from the liver.

PREPARATION OF CYTOSOL

Cytosol was prepared from a suspension of isolated hepatocytes which was produced as described before. After centrifugation and washing of the cells the suspension was subjected to ultrasound bombardment in the Soniprep at power setting of 14 for 5 minutes to break down the cell structure. (Fig. 13) The resultant mulch was ultracentrifuged at 40,000 rpm for 90 minutes and the supernatant removed. This solution of intracellular substances was filter sterilised, divided into individual doses and frozen at -20^{o}C till required. Immediately prior to use it was thawed at 37^{o}C. Dosages were calculated according to the number of hepatocytes used in the preparation.

Fig. 13. Cells disrupted in Soniprep, prior to spinning in ultracentrifuge.

COAGULATION AND BIOCHEMICAL MONITORING

Monitoring of the animals' progress to recovery or death was by the measurement of "Normotest" (Nycomed Diagnostics) to determine the activity of coagulation factors II, VII and X. The test reagent was reconstituted according to the supplier's instructions by adding 11ml sterile distilled water to the contents of the ampoule. This solution was divided amongst small tubes each containing 0.25ml of reconstituted reagent solution. These were stored at -20^{o}C and heated in a water bath at 37^{o}C for 10 minutes prior to use. Blood was obtained from the rat and 0.1ml of blood was placed in a tube containing 0.01ml of 0.109M trisodium citrate. 0.025ml of this citrated blood was pipeted into the reagent and the time taken for coagulation to occur in a water bath at 37^{o}C was noted. This Normotest time in seconds was then plotted on the correlation curve supplied by the company to calculate the Normotest percentage.

Biochemical liver function tests were measured on plasma obtained by centrifugation of fresh blood from the rat and the plasma stored at -20^{o}C till biochemical analysis was undertaken. Alanine aminotransferase was measured on a Cobas Bio centifugal analyser using Boehringer Mannheim reagent (product No.124524). Aspartate aminotransferase was measured on a Hitachi 737 fully selective analyzer with Boehringer Mannheim reagent using the "optimised standard method". Alkaline phosphatase was measured by the "optimised standard method" using Boehringer Mannheim

reagents on the Hitachi 737 analyzer. Bilirubin was measured on the same analyser.

Biochemical analyses were performed by the staff of the Department of Clinical Chemistry, Queen Elizabeth Hospital.

STATISTICAL METHODS

In the clinical study the various results obtained did not fit the normal distribution and so these measurements were analysed nonparametrically with the medians and ranges being given.

Within each test group of rats the results for each modality tested appeared to conform to a normal pattern and so within the group the analysis was by parametric methods with means reported. However when comparing one group with another the analysis was by nonparametric methods.

Statistical analyses were performed by computer using the "Statgraphics" software package (STSC, Inc. USA.)

ACUTE HEPATIC FAILURE MODEL

For the purposes of the study it was necessary to develop a reproducible model of acute hepatic failure in a small animal so that different treatment options could be tried. The administration of a known hepatotoxic agent was the most convenient available method and so dimethylnitrosamine was chosen as the hepatotoxin.

Dimethylnitrosamine (DMNA) is a liver specific toxic agent having its effect on the hepatocyte by alkylation of the molecules of the nucleus and the cytoplasm and hence by means of a metabolic injury producing tissue necrosis. Histologically DMNA produces a zonal necrosis of the centrilobular type (zone 3) similar to the pattern encountered in clinical practice following ingestion of hepatotoxic agents such as paracetamol. (Zimmerman 1987)

DMNA was implicated as a hepatotoxin in 1954 by Barnes and Magee who in a series of experiments in different species produced both acute and chronic liver injury by using different doses of the substance over different time schedules. They showed that acute hepatic failure could be produced in albino rats by a single intraperitoneal injection or by one oral dose. The results of oral administration were less dependable and less reproducible than if the substance was given intraperitoneally. In the rats they used the L.D. 50 by intraperitoneal injection was 26.5mg/kg. and the average oral dose to produce the same effect was 40 to 50mg/kg. The rats died between 2 and 5

days after administration of DMNA. Magee (1956) demonstrated that DMNA is rapidly metabolised and that 4 hours after dosage to the rat none was recoverable from the animal's body.

Adult male Wistar rats were chosen as the experimental animals and it was decided to administer the hepatotoxin in a single intraperitoneal injection.

The doses of DMNA originally chosen for testing in this strain of rat were 40mg/kg and 50mg/kg and following the results of these doses 45mg/kg was also given.
There were 3 rats in each test group all weighing between 300 and 390g.

All rats were anaesthetised with ether and a blood sample was taken from the tail vein of each for coagulation testing with "normotest" and biochemical liver assessment measuring alanine transaminase (ALT), aspartate transaminase (AST), alkaline phosphatase (AP) and bilirubin (Bil). The volume removed (0.2ml) was replaced with saline intravenously. While still anaesthetised each rat was injected intraperitoneally with DMNA, 1g of which had been diluted with 50ml of normal saline to produce a concentration of 20mg/ml. Dosages of 40mg/kg, 45mg/kg and 50mg/kg were administered. The rats were allowed to recover, their physical states were observed, and blood samples, with volume replacement were taken regularly under ether anaesthesia for the same investigations as before. All rats surviving the episode of acute liver failure were

sacrificed when they appeared well. Postmortem examination was performed on all animals at death or sacrifice with removal of the liver for histological assessment of the degree of liver necrosis

Results.

All animals appeared unwell, becoming inactive, slow to respond and puffed out their coats.

Of the rats receiving 40mg/kg 1 died at 48 hour after the administration of DMNA while under anaesthesia and the other 2 survived with signs of full recovery to be sacrificed at 192 and 316 hours. All the rats given 50mg/kg died at 48 hours having been in a very poor physical state for the preceding few hours. One rat given 45mg/kg died at 122 hours and the other 2 showed signs of recovery and were sacrificed at 151 hours.

Normotest (mean)

hours	0	6	16	24	48	72	96	120	144	sac.
dose										
40mg/kg	45%	38%		21%	12%	31%	41%	33%	34%	45%
50mg/kg	50%		20%	4%						
45mg/kg	44%			12%	4%	4%	39%	47%	52%	

ALT (mean)

hours	0	6	16	24	48
dose					
40mg/kg	101	61		94	1463
50mg/kg	54		199	1064	
45mg/kg	57			1189	252

hours	72	96	120	144	sac.
dose					
40mg/kg	934	392	184	97	65
50mg/kg					
45mg/kg		197	77	50	

AST (mean)

hours	0	6	16	24	48
dose					
40mg/kg	225	128		300	5402
50mg/kg	144		605	2628	
45mg/kg	106			2047	293

hours	72	96	120	144	sac.
dose					
40mg/kg	1710	676	323	267	100
50mg/kg					
45mg/kg		283	134	127	

AP (mean)

hours	0	6	16	24	48
dose					
40mg/kg	396	432		442	508
50mg/kg	406		326	448	
45mg/kg	393			381	884

hours	72	96	120	144	sac.
dose					
40mg/kg	696	803	723	667	536
50mg/kg					
45mg/kg		664	587	519	

Bil (mean)

hours	0	6	16	24	48	72	96	120	144	sac.
dose										
40mg/kg	10	10		10	10	10	10	10	10	10
50mg/kg	10		10	40						
45mg/kg	10			10	10		10	10	10	

Histology of the livers removed at postmortem showed 81% liver necrosis in the centre of a lobe in the rat which died at 48 hours in the 40mg/kg group and no liver necrosis was evident in the rats which survived in this group. In the 50mg/kg group there was a mean of 93% liver necrosis (at 48 hours) and 49% liver necrosis in the rat which died in the 45mg/kg group, with the remaining rats in this group showing 0% necrosis at sacrifice.

From these results it was clear that a dose of 40mg/kg of DMNA was insufficient to produce reproducibly fatal acute hepatic failure in the Wistar rat. A dose of 50mg/kg

produced the necessary biochemical derangement to indicate significant liver damage but the rapidity of fatal outcome might preclude any demonstration of effectiveness for treatments of the liver failure. 45mg/kg was given in an attempt to find a suitable compromise dose but this too proved insufficient.

50mg/kg appeared to be the only workable dose to produce the necessary degree of hepatic failure so this dose of DMNA was given to a further 5 rats to validate the model, demonstrating its consistency.

Validation of model

5 adult male Wistar rats weighing 400 to 450 g were anaesthetised with ether and blood samples were taken from their tail veins as before with intravenous volume replacement with saline. Each rat received an intraperitoneal injection of DMNA at a dose of 50mg/kg and was allowed to recover. Its physical state was observed and further blood samples were taken at intervals with volume replacement. Postmortem examinations were performed and the liver histology was assessed.

Results

All 5 rats became physically unwell and died, 1 at 41 hours after DMNA injection, 2 at 42 hours and 2 at 43 hours

Normotest (mean)

hours	0	18	24	42
dose 50mg/kg	44%	11%	5%	4%

ALT (mean)

hours	0	18	24	42
dose 50mg/kg	56	1216	2679	828

AST (mean)

hours	0	18	24	42
dose 50mg/kg	97	1083	6985	9488

AP (mean)

hours	0	18	24	42
dose 50mg/kg	246	480	184	529

Bil (mean) hours	0	18	24	42
dose 50mg/kg	10	10	12	27

The livers removed at postmortem all showed hepatic injury with a mean of 89% liver necrosis. (Figs. 14,15)

During this and subsequent experiments the histological appearances of the livers at different times during the recovery from acute hepatic failure were observed. After the initial injury with hepatocyte necrosis the central parts of the lobule surrounding the central vein appeared to involute and "cave in" leaving a hole which then developed an endothelial lining becoming a dilated central vein. As the recovery phase progressed the vein returned to normal size. Regeneration of hepatocytes from the periportal areas extended to reduce the size of the central vein. In some case the recovered liver showed evidence of bridging fibrosis and in one instance a granuloma. (Figs. 16-18)

DMNA 50mg/kg administered in a single intraperitoneal injection to the adult Wistar rat produces severe liver injury which was fatal in all the animals. Despite the short survival time it was decided to use this model of acute hepatic failure in the subsequent experiments.

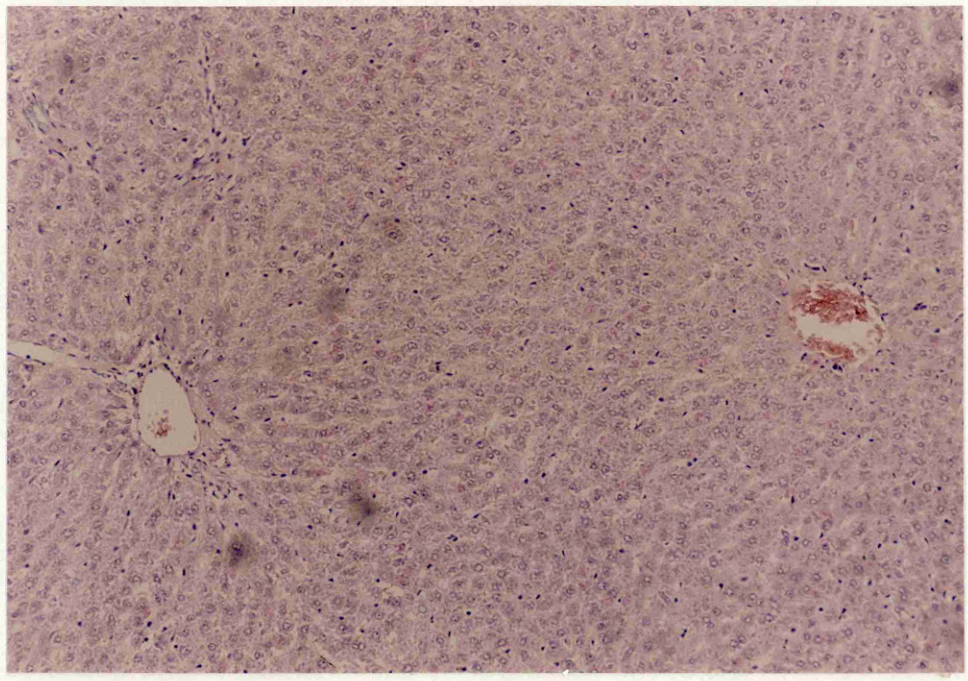

Fig. 14. Normal liver. (x 10)

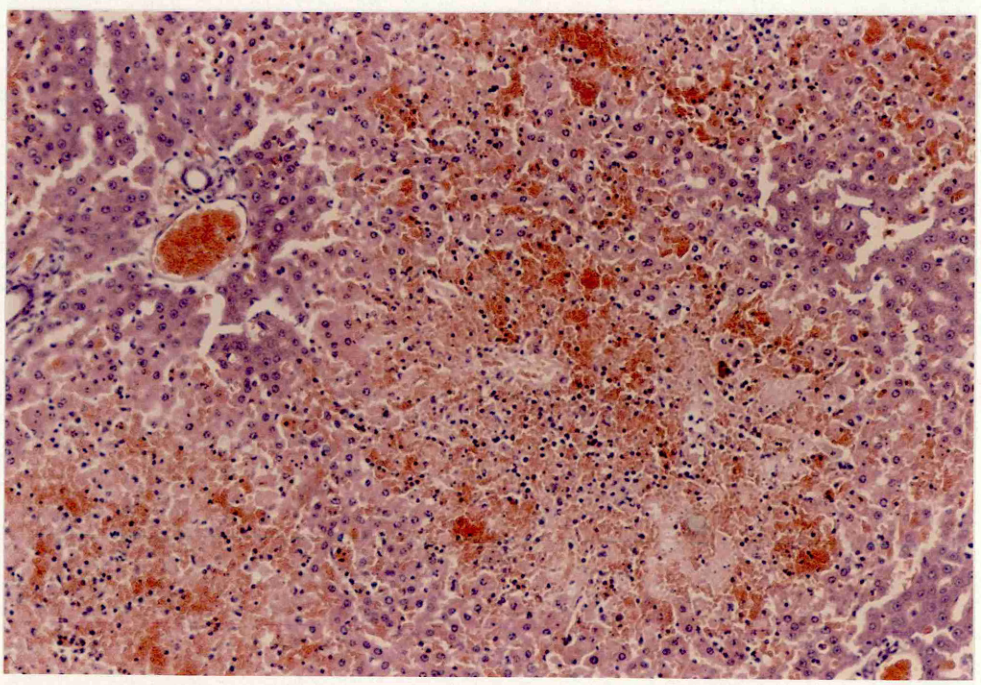

Fig. 15. Acute hepatic failure due to 50mg/kg DMNA. (x10)

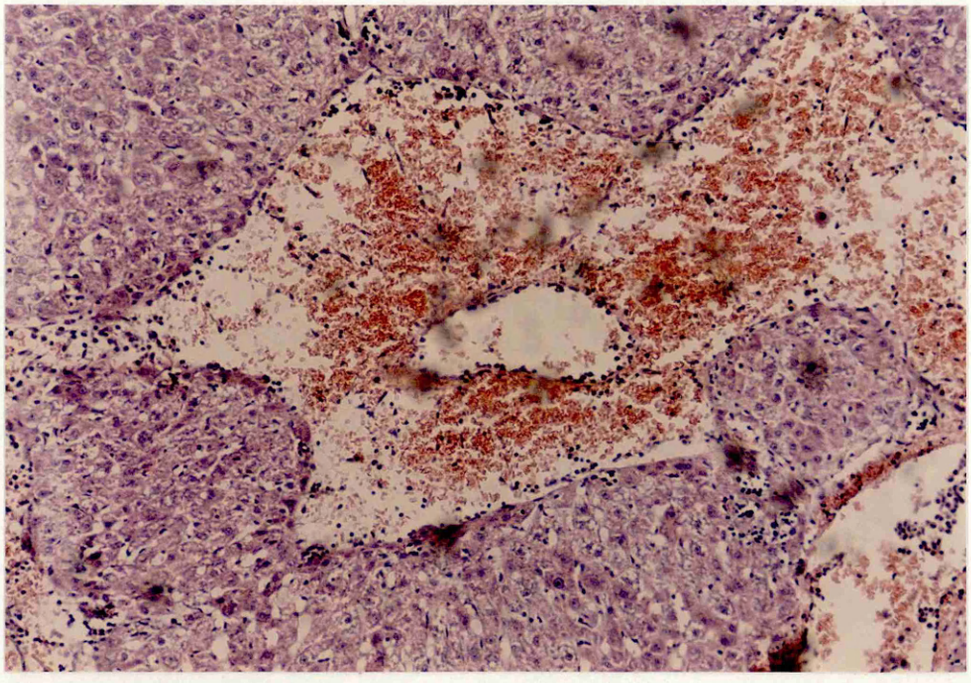

Fig. 16. Resolving acute hepatic failure with central vein involution. (x10)

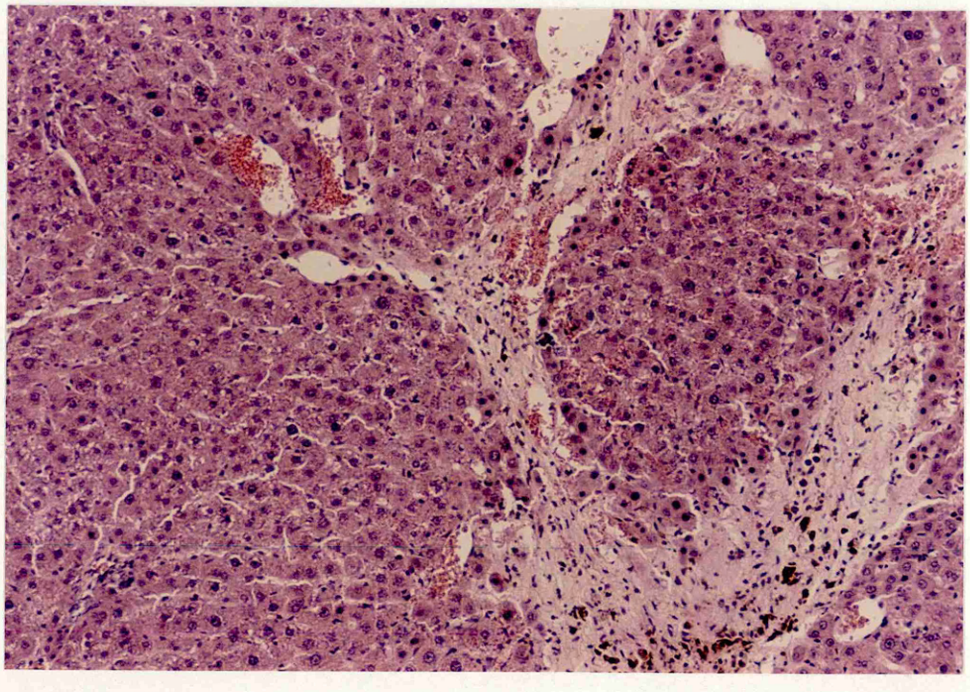

Fig. 17. Resolved acute hepatic failure with bridging fibrosis. (x10)

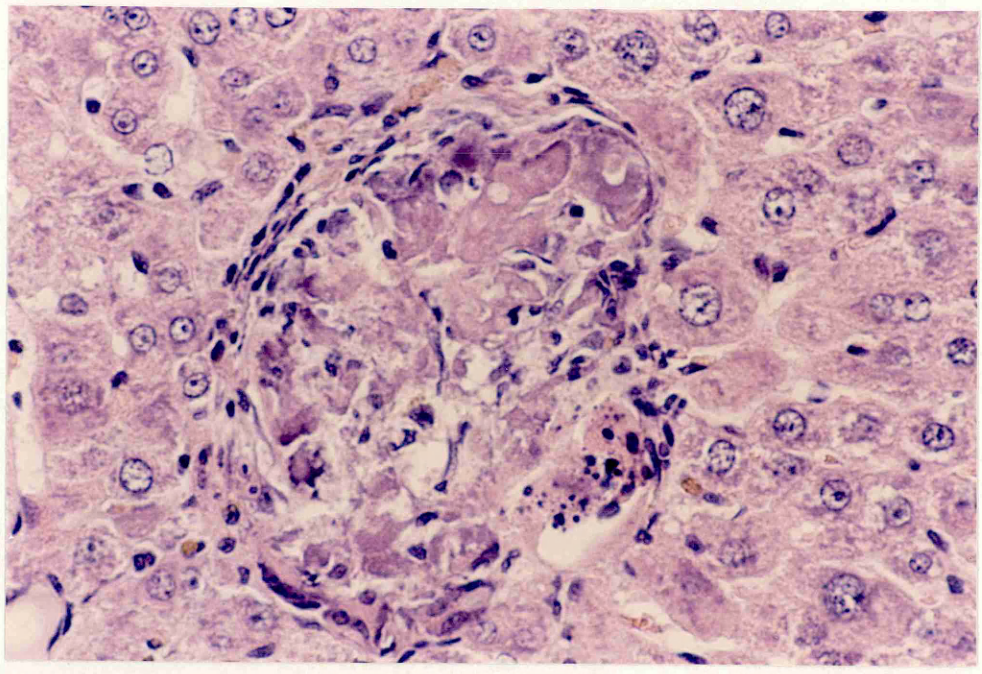

Fig. 18. Resolved acute hepatic failure with granuloma. (x40)

HEPATOCYTE TRANSPLANTATION

Hepatocyte transplantation has been shown to be effective as a means of therapy in the rat with experimentally induced acute hepatic failure (Sutherland 1977), and so it was decided to assess its efficacy in the DMNA model in the Wistar rat as a modality with which to compare other treatments. Hepatocytes were prepared as described previously and administered into both the intrasplenic and the intraperitoneal sites.

INTRASPLENIC HEPATOCYTE TRANSPLANTATION

Method

19 male adult Wistar rats were divided into 3 groups. Group I - induction of acute hepatic failure followed 6 hours later by intrasplenic injection of hepatocyte suspension; group II - induction of acute hepatic failure followed 6 hours later by intrasplenic injection of Hank's solution (HBSS+); group III - intrasplenic injection of hepatocyte suspension without induction of acute hepatic failure.

Acute hepatic failure was induced by a single intraperitoneal injection of DMNA at a dose of 50mg/kg. In group I two rats received $2.5x10^7$ hepatocytes with viability of 90%, two received $2.0x10^7$ hepatocytes with viability of 80% and five received $3.15x10^7$ hepatocytes with viability of 74%. In group III five rats each received $2.86x10^7$ hepatocytes with viability of 88%. All the hepatocyte suspensions were given in a volume of 1ml.

The rats weighed 320 - 460g and were divided into 3 groups; group I - 9 rats, group II - 5 rats, group III - 5 rats. Acute hepatic failure was induced in groups I and II but not in group III. Six hours later in groups I and II and at time 0 in group III the rats were anaesthetised with an intraperitoneal injection of pentabarbitone and a small left subcostal incision was made. The spleen was delivered into the wound and the lower pole was injected via a 25 gauge needle with hepatocyte suspension in groups I and III, and with HBSS+ in group II. Bleeding was stopped by pressing on the injection site with a bacteriology swab. The splenic vein was not tied or clamped. The spleen was returned to the peritoneal cavity and the wound closed with an absorbable suture and the skin closed with a prolene suture. Blood samples were taken before induction of acute hepatic failure, before intrasplenic injection and at daily intervals afterwards. At death or sacrifice all animals underwent postmortem examination with removal of the liver and spleen for histological examination.

Results

The mean survival was 50 hours in group I, 39 hours in group II and indefinite in group III with all animals sacrificed between 164 and 356 hours. The difference in survival between groups I and II was not statistically significant when analysed by the Mann-Whitney test.

Normotest (mean)

hours group	0	6	24	48	72	96	120	144	192	300
I	48%	29%	6%	4%	8%					
II	60%	63%	12%							
III	41%		32%	57%	40%		36%	59%	44%	50%

ALT (mean)

hours	0	6	24	48	72
group					
I	90	84	4062	1172	3002
II	44	53	1415	6335	
III	60		526	165	94

hours	120	144	192	300
group III	51	58	49	41

AST (mean)

hours	0	6	24	48	72
group					
I	95	153	4098	7785	7143
II	169	208	1387	14150	
III	104		874	324	267

hours	120	144	192	300
group III	111	151	132	94

AP (mean)

hours	0	6	24	48	72
group					
I	405	196	650	616	894
II	391	212	306	584	
III	386		476	377	229

hours	120	144	192	300
group III	315	410	358	398

Bil (mean)

hours	0	6	24	48	72	96	120	144	192	300
group										
I	10	10	10	24	30					
II	10	10	10	48						
III	10		10	11	11		10	10	11	10

Histology

In group I the one rat which died 7 hours after induction of acute hepatic failure showed no histological evidence of liver necrosis but the other 8 all had liver destruction with a mean of 73% necrosis. In 8 of the spleens it was possible to identify groups of hepatocytes but these were small in number although the cells appeared viable. (Figs

19,20) In all the livers there were deposits of hepatocytes within some branches of the portal vein and in most cases these contained viable cells. (Fig. 21)

In group II the livers had a mean of 80% necrosis and no cellular deposits were seen in the portal vein branches. The spleens all showed some haemorrhage from the injections but were otherwise normal.

In group III there was no evidence of liver necrosis and only 2 of the livers had any hepatocyte deposits within the portal vein branches but this was at a mean of 259 hours after transplantation. All the spleens in this group contained collections of hepatocytes but these were neither large nor numerous.

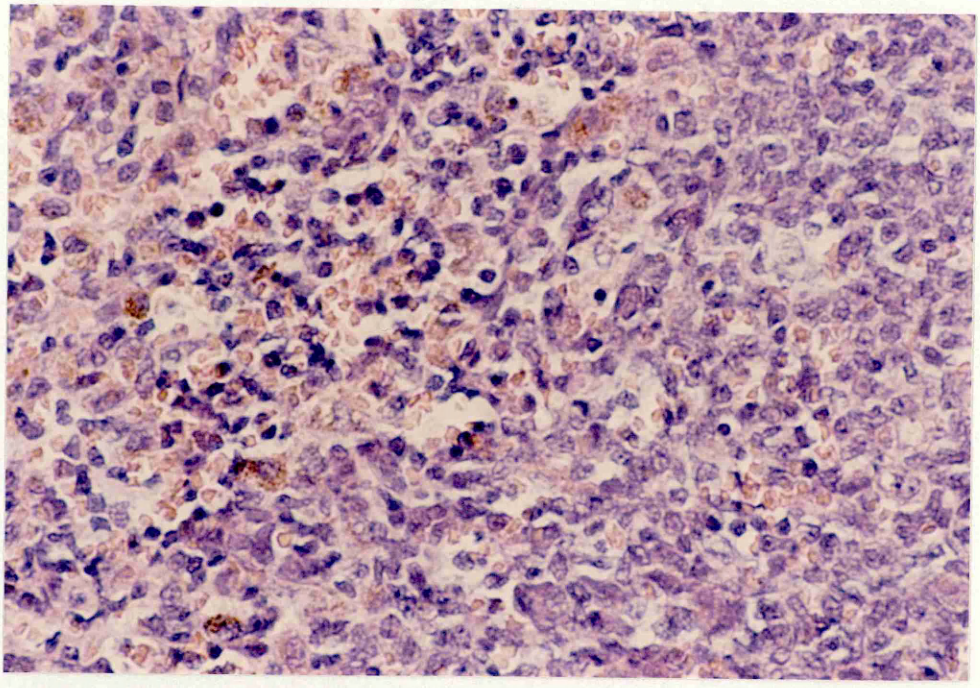

Fig. 19. Normal spleen. (x40)

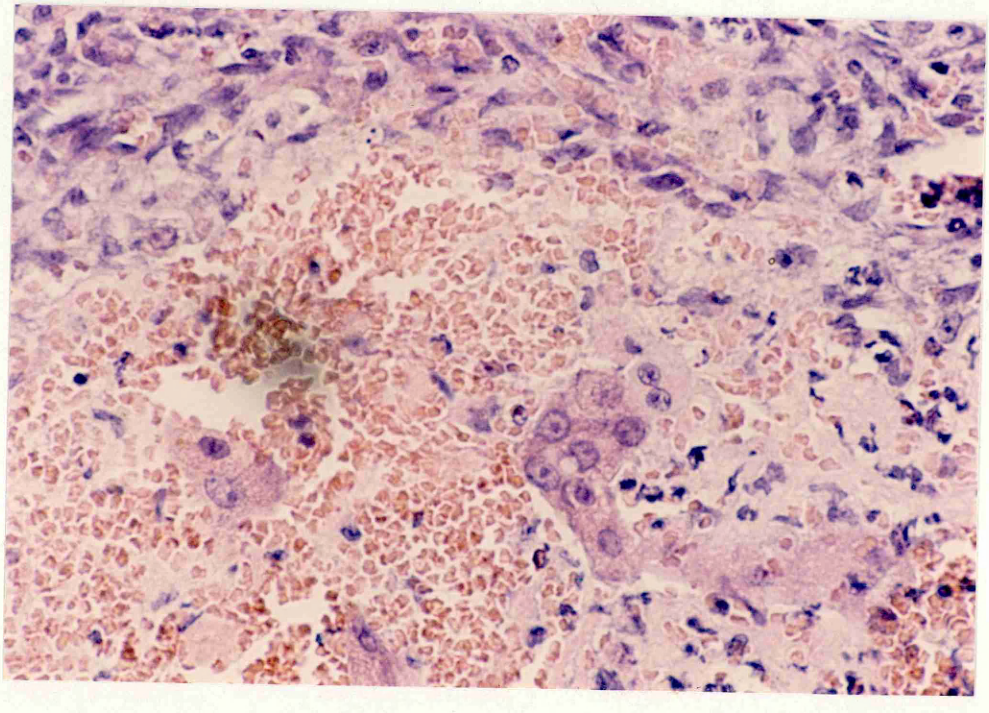

Fig. 20. Hepatocytes in spleen. (x40)

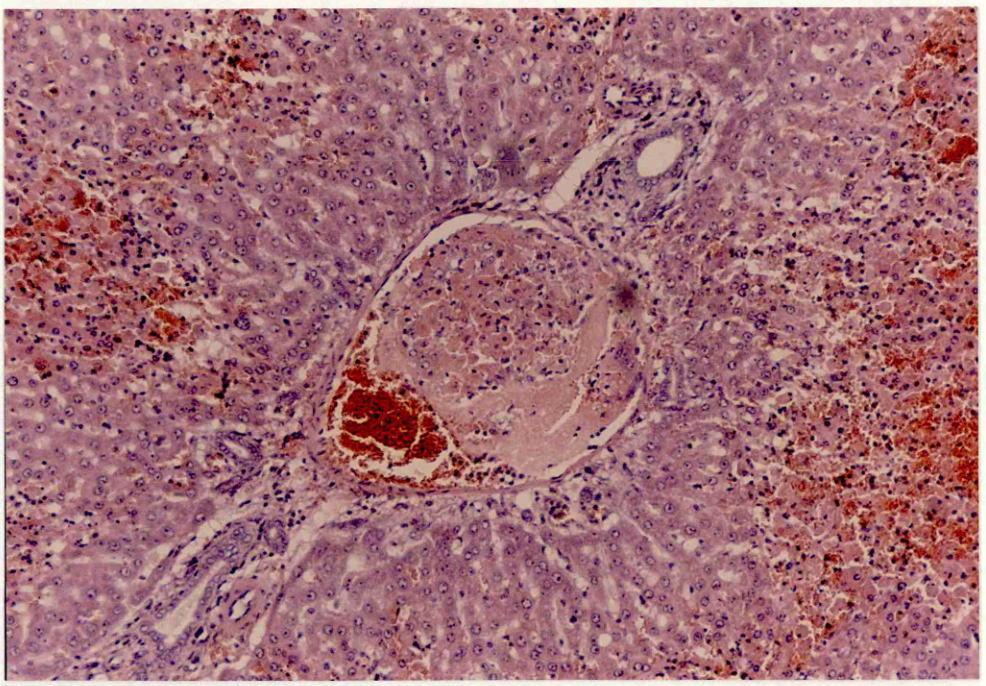

Fig. 21. Hepatocytes in portal vein after intrasplenic transplantation. (x10)

INTRAPERITONEAL HEPATOCYTE TRANSPLANTATION

Method

20 adult male Wistar rats weighing 300 - 440g each received a single intraperitoneal injection of DMNA at a dose of 50mg/kg to induce acute hepatic failure. 6 hours later under ether anaesthesia each rat received an intraperitoneal injection of hepatocyte suspension. In each case the volume of the suspension was 1ml but the number of contained hepatocytes varied. 5 rats received 3.94×10^7 hepatocytes with a viability of 83%; 5 were given 4.12×10^7 hepatocytes with a viability of 84%; 5 received 4.42×10^7 hepatocytes of viability of 78% and the other 5 were given 6.15×10^7 hepatocytes with a viability of 84%. The results for these rats were compared with a group of 4 rats each weighing between 280 and 340g which were given an intraperitoneal dose of 50mg/kg DMNA to induce acute hepatic failure and then 6 hours later received an intraperitoneal injection of HBSS+. In both groups monitoring blood samples were taken before induction of acute hepatic failure, prior to intraperitoneal injection of hepatocytes or HBSS+ and daily till death or sacrifice. All rats underwent postmortem examination with removal of the liver and any other relevant tissue for histological examination.

Results

2 rats in the hepatocyte group survived the episode of acute liver failure with sacrifice at 150 and 216 hours and the mean survival for the other 18 was 58 hours with an overall mean survival of 71 hours. Both of the surviving rats were in the group which received 3.94×10^7 hepatocytes but there was no significant difference in survival amongst the dose variations.

Of the rats given intraperitoneal HBSS+ there was a mean survival of 56 hours with the rats dying at 44, 53, 57 and 72 hours. There was no statistically significant difference in survival between the two group when analysed by the Mann-Whitney test.

Normotest (mean)

hours	0	6	24	48
hepatocytes	47%	43%	12%	6%
HBSS	34%	41%	5%	<5%

hours	72	96	144	216
hepatocytes	5%		44%	62%
HBSS	<5%			

ALT (mean)

hours	0	6	24	48
hepatocytes	57	509	5104	2055
HBSS	79	68	3360	4449

hours	72	96	144	216
hepatocytes	1320		98	29
HBSS	229			

AST (mean)

hours	0	6	24	48
hepatocytes	215	894	5342	10303
HBSS	346	137	2965	8010

hours	72	96	144	216
hepatocytes	7248	4275	319	92
HBSS	1913			

AP (mean)				
hours	0	6	24	48
hepatocytes	438	349	445	1637
HBSS	361	259	30	566
hours	72	96	144	216
hepatocytes	678	1475	1125	442
HBSS	578			

Bilirubin (mean)				
hours	0	6	24	48
hepatocytes	6	5	17	52
HBSS	11	18	10	51
hours	72	96	144	216
hepatocytes	61	61	12	5
HBSS	36			

Histology

All the livers in the hepatocyte group showed necrosis with a mean of 74% necrosis. Both the rats which were sacrificed still showed substantial liver damage with 54% and 89% hepatic necrosis. 6 of the livers had deposits of hepatocytes in the portal vein branches. (Fig. 22) 17 rats had deposits within their peritoneal cavities that had not been encountered in any of the previously studied groups. These were usually positioned subdiaphragmatically but could occur anywhere. Microscopy revealed them to contain hepatocytes which in about half the cases appeared to be viable cells (Fig. 23) although there were a lot of dead cells in all the deposits (Fig. 24).

The rats in the HBSS group showed a mean of 67% hepatocyte necrosis at death. There were no intraperitoneal deposits seen in this group.

When subjected to statistical analysis (Mann-Whitney) there were no significant differences between the groups with respect to normotest, ALT, AST, alkaline phosphatase or

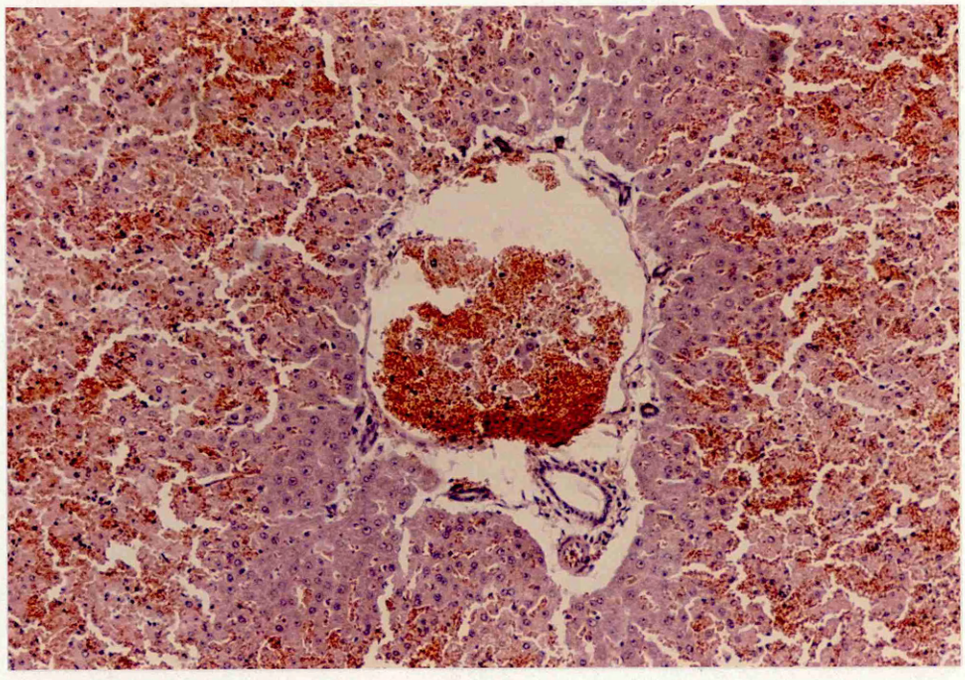

Fig. 22. Hepatocytes in portal vein after intraperitoneal transplantation. (x10)

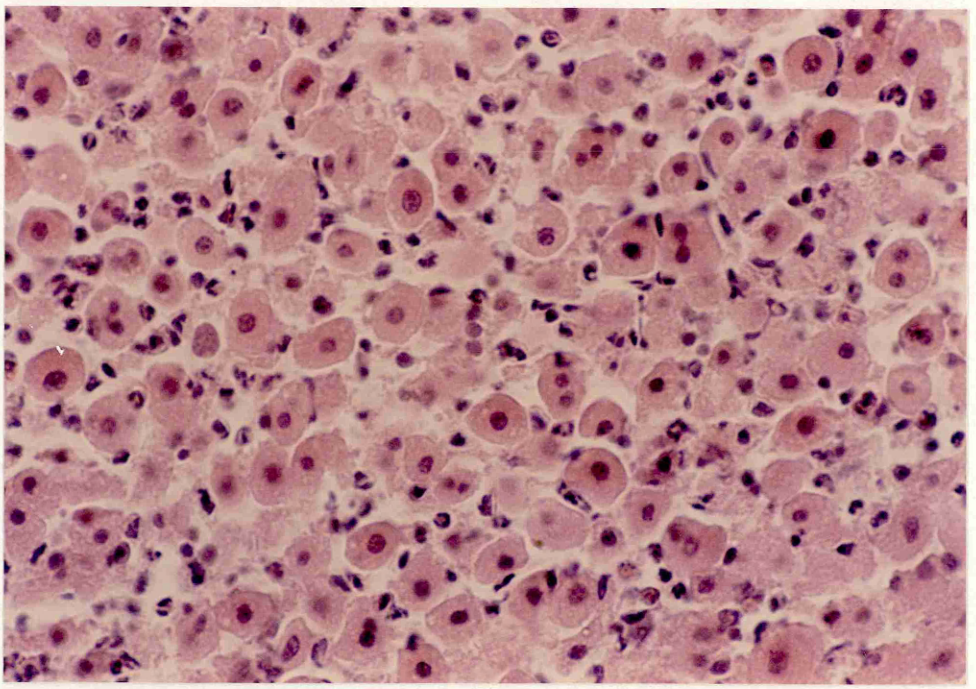

Fig. 23. Intraperitoneal deposits of live hepatocytes. (x40)

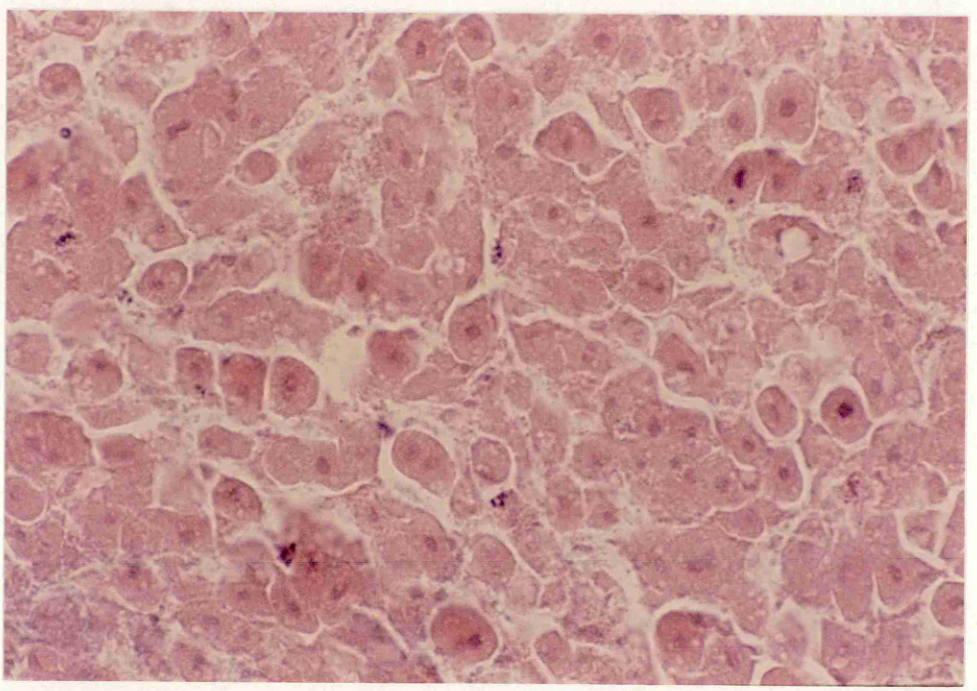

Fig. 24. Intraperitoneal deposits of dead hepatocytes. (x40)

bilirubin levels nor was there any difference between the extents of hepatic necrosis.

Conclusion

Despite 2 rats in the peritoneal hepatocellular transplant group achieving survival from the hepatotoxin induced liver failure there was no significant benefit from the hepatocytes administered by either route over the control groups in respect of coagulation, biochemical testing, survival or degree of histological liver damage. The long term survivors in the intrasplenic group without hepatic failure served to demonstrate that the procedure of intrasplenic injection itself was not detrimental to the rat and that the cause of death of the other rats could not be attributed to the technique.

Very few hepatocytes were evident in the spleens at death so there were certainly not enough functioning hepatocytes at this site to sustain the animal. There were however more hepatocytes, both ones which looked alive and dead ones, in the portal vein branches within the livers and they might have been able to contribute to the recovery of the rats. Of interest were the appearances of deposits of healthy looking hepatocytes within the peritoneal cavity as late as 72 hours after intraperitoneal hepatocyte transplantation. Previous reports of this technique have commented that hepatocytes had not been recovered from the peritoneal cavity (Sutherland 1977, Makowka 1980b).

In the above experiments hepatocellular transplantation by either the intrasplenic or the intraperitoneal site appeared to be no better than the non treatment control regimes at modifying the course or extent of the hepatic failure produced by DMNA in this model in the Wistar rat.

INTRAPERITONEAL ADMINISTRATION OF CYTOSOL

It has been suggested that in the treatment of acute hepatic failure it is not essential to supply intact, functioning hepatocytes to improve survival of the experimental animal (Makowka 1981) and that some other cells such as bone marrow cells produce the same results (Makowka 1980c). Cytosol prepared from homogenised liver of fetal or partially hepatectomised rats has also proved beneficial. Regenerating cytosol has been shown to be effective in improving the survival in rats in a chemotherapeutic model of acute liver failure (Miyazaki 1983). Cytosol from homogenised normal adult liver has been shown not to improve survival (Makowka 1981). Liver cytosol was produced by a different method, from a suspension of isolated hepatocytes from normal adult rats and this was administered in a previously unreported dosage regime.

Ten male adult Wistar rats weighing 320g - 390g with acute hepatic failure were treated with repeated intraperitoneal injections of cytosol produced from other similar rats and the results compared with rats in acute liver failure treated with daily intraperitoneal injections of saline.

Method

Cytosol was prepared from isolated hepatocyte suspension as described earlier. Acute hepatic failure was induced by the single intraperitoneal injection of 50mg/kg dimethylnitrosamine and 6 hours later treatment was

commenced with intraperitoneal cytosol or control injections according to the following regime.

Cytosol group - 2 rats daily injections of cytosol equivalent to the contents of $5.7x10^6$ hepatocytes per day, 2 rats injected daily with cytosol equivalent to the contents of $3x10^7$ hepatocytes per day, 4 rats injected every second day with cytosol equivalent to the contents of $3x10^7$ hepatocytes per injection and 2 rats injected every third day with cytosol equivalent to the contents of $3x10^7$ hepatocytes per injection.

Control group - 4 rats receiving daily injections of saline.

The cytosol and the control injections were all given intraperitoneally and each injection was of 1ml.

Blood samples were taken for clotting assessment and biochemical liver function testing before the induction of acute hepatic failure, 6 hours afterwards, before the first treatment injection and at daily intervals. At death or sacrifice all animals underwent postmortem examination with removal of the liver for histological examination.

Results

Mean survival for the cytosol treated group was 95 hours; that being 73 and 100 hours for the low dose daily injected rats, 100 and 48 hours for the high dose daily injected ones, 336(sacrificed), 48, 72 and 48 hours for the high dose every second day injections and 72 and 54 hours for the high dose every third day treated rats. The mean survival for the control rats was 46 hours.

Normotest (mean)

hours	0	6	24	48	72	96	148	194	240	336
group										
cytosol	40%	37%	7%	7%	8%	10%	22%	13%	70%	39%
saline	46%	32%	8%	4%						

ALT (mean)

hours	0	6	24	48	72	96	148
group							
cytosol	109	85	3626	3711	867	106	152
saline	80	63	1864	1312			

AST (mean)

hours	0	6	24	48	72	96	148
group							
cytosol	162	201	2768	10648	3470	1104	304
saline	194	104	1912	7282			

AP (mean)

hours	0	6	24	48	72	96	148
group							
cytosol	321	226	386	523	510	448	708
saline	518	406	420	539			

Bilirubin (mean)

hours	0	6	24	48	72	96	148
group							
cytosol	9	8	11	65	58	12	12
saline	10	15	27	21			

Postmortem

At death 2 of the rats in the cytosol group had intraperitoneal haemorrhage and the rats in this group had evidence of acute liver failure, except the rat which was sacrificed after recovery. The 9 rats with histological hepatic destruction had a mean of 71% liver necrosis.

1 of the rats in the saline group had postmortem evidence of intraperitoneal haemorrhage. All the control rats had liver necrosis with a mean of 67% hepatocyte destruction.

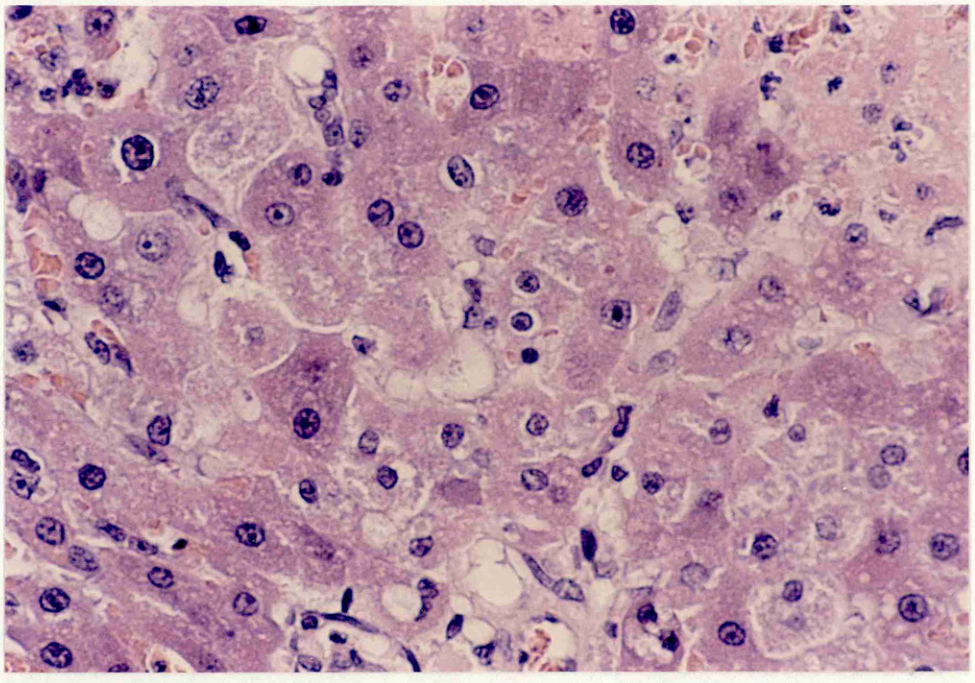

Fig. 25. Regenerating hepatocytes. (x40)

The 1 rat which survived in the cytosol group had signs of liver regeneration on histology (Fig. 25) and also areas showing bridging fibrosis.

Despite the apparent trend towards improved biochemistry at 24 and 48 hours in the cytosol group, these results failed to reach statistical significance (Mann-Whitney). There was no significant difference between the groups in respect of the extent of hepatocyte necrosis at death. The results do show an improvement in survival for the rats treated with intraperitoneal cytosol with a significant difference of $p<0.05$.

Conclusion

Other workers have claimed that cytosol prepared from non regenerating liver is ineffective at improving the survival of rats with acute liver failure (Schwarz 1985). This experiment has shown that hepatocyte cytosol prepared from isolated cell suspension and given in the regime described can produce long-term survivors in a model of liver failure with an otherwise 100% fatality. There was also a trend toward biochemical improvement in the cytosol treated group but it does not reach statistical significance within the limited numbers used.

2 of the rats in this experiment suffered intraperitoneal haemorrhage as a result of the repeated injections when they had abnormal blood clotting; this risk is not desirable in the experimental animal and would be unacceptable if the therapy was to be applied clinically. A safer means of administering the cytosol requires to be found.

Intraperitoneal hepatocyte cytosol from normal adult rats when administered in repeated doses improves the survival of rats with toxin induced acute hepatic failure.

REPEATED INTRAVENOUS INJECTIONS OF CYTOSOL

Twelve rats were treated by repeated intravenous injections of rat hepatocyte cytosol beginning 6 hours after the induction of acute hepatic failure. The results from these rats were compared with a control group of 12 rats which received intravenous injections of normal saline at the same times.

All the rats were adult male Wistar rats. Those in the cytosol group weighed between 275g and 400g and the saline controls from 280-370g. Cytosol was prepared as before.

Acute hepatic failure was induced by a single intraperitoneal injection of 50mg/kg dimethylnitrosamine. Six hours later the rats received intravenous injections of cytosol or saline and these injections were repeated daily for up to five doses. Blood samples were taken from each rat prior to the induction of acute hepatic failure and before each daily injection. Intravenous injections were given into, and blood samples taken from a tail vein using a 1ml syringe attached to a 25G "Butterfly" needle. From the blood obtained the clotting ability was assessed by "Normotest" and the biochemical liver function tests of alanine transaminase, aspartate transaminase, alkaline phosphatase and bilirubin levels were measured. Postmortem examinations with removal of the livers for histology were performed on all rats at death or sacrifice.

Results

9 of the rats which received cytosol injections survived the experiment with return of clotting and biochemistry to pre-liver failure levels. The survivors were sacrificed between 365 and 630 hours after the induction of acute liver failure. The other 3 animals in this group died at 48, 72 and 116 hours. All rats in the saline group died of acute hepatic failure between 48 and 95 hours.

Normotest (mean)

hours	0	6	24	48	72	96	120	144	192	sacr
cytosol	44%	36%	13%	7%	33%	28%	40%	34%	29%	58%
controls	43%	26%	8%	6%	10%					

ALT (mean)

hours	0	6	24	48	72	96	120	144	192	sacr
cytosol	79	64	1558	4287	1815	620	380	146	89	92
controls	76	53	1994	2554	844					

AST (mean)

hours	0	6	24	48	72	96	120	144	192	sacr
cytosol	215	148	1978	7792	2066	708	445	214	164	104
controls	192	150	2683	8518	4286	1619				

Alk phos (mean)

hours	0	6	24	48	72	96	120	144	192	sacr
cytosol	497	369	534	715	950	1106	995	792	666	444
controls	363	349	389	548	654	349				

Bilirubin (mean)

hours	0	6	24	48	72	96	120	144	192	sacr
cytosol	11	13	11	50	32	15	10	12	12	9
controls	13	7	11	35	39	15	10	12	12	9

Histology

The 9 surviving rats in the cytosol group showed no evidence of hepatocyte necrosis. (Fig. 26) The rat in this group dying at 48 hours had 53% hepatocyte necrosis, the one dying at 72 hours had 60% and the one dead at 116 hours showed 34% hepatocyte necrosis at time of death. In the saline group there was a mean hepatocyte necrosis measurement at the time of death of 56%.(Figs. 27-29)

Five of the survivors in the cytosol group demonstrated bridging fibrosis within the liver and 3 appeared to have dilated central veins within the liver lobules.

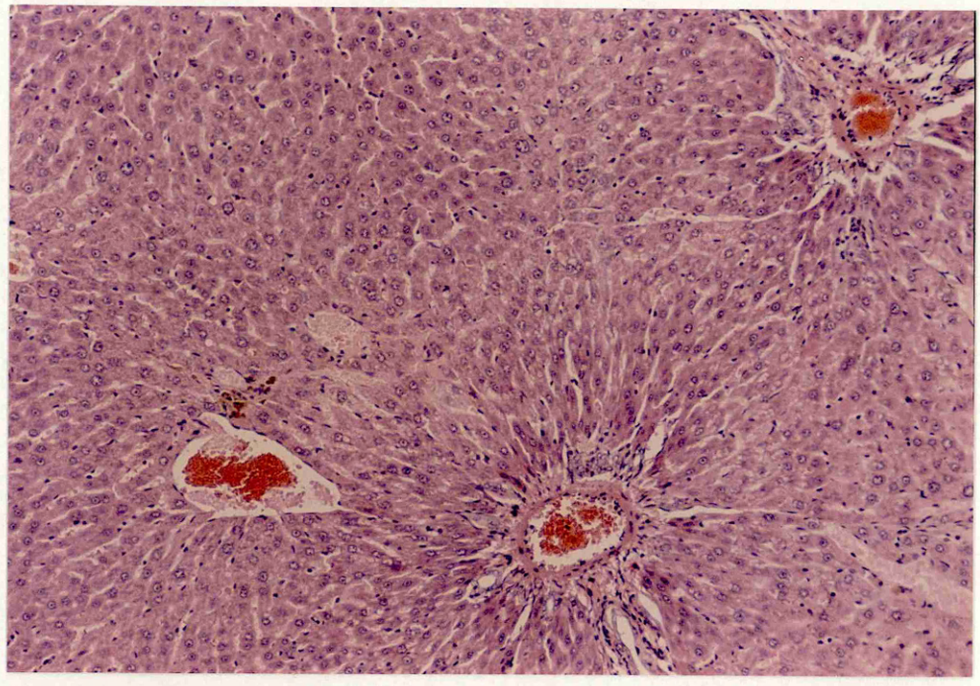

Fig. 26. Survivor after IV cytosol. (x10)

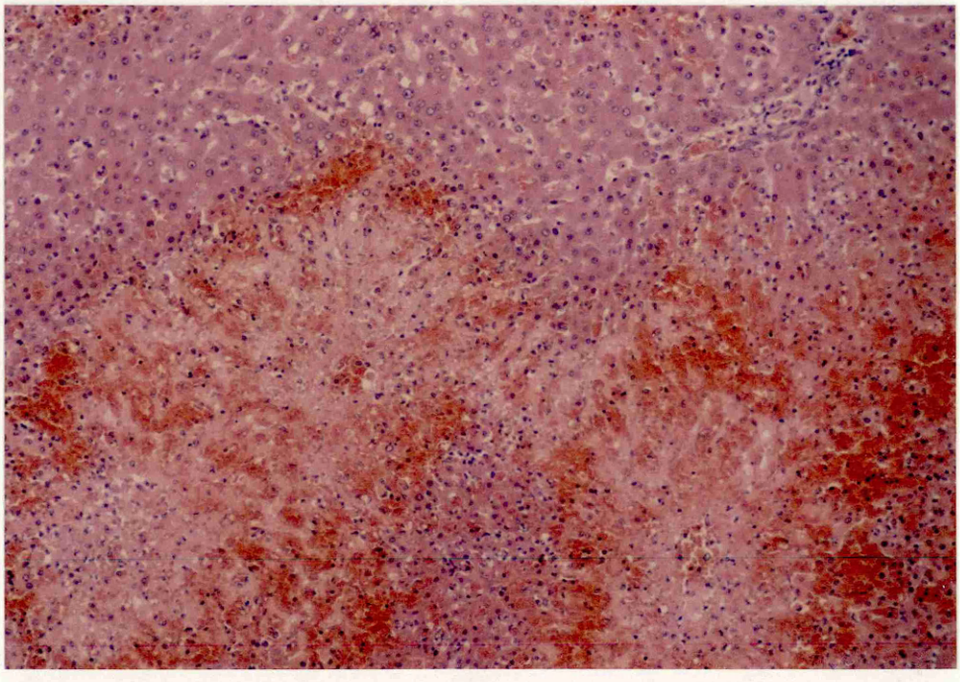

Fig. 27. Saline control. (x10)

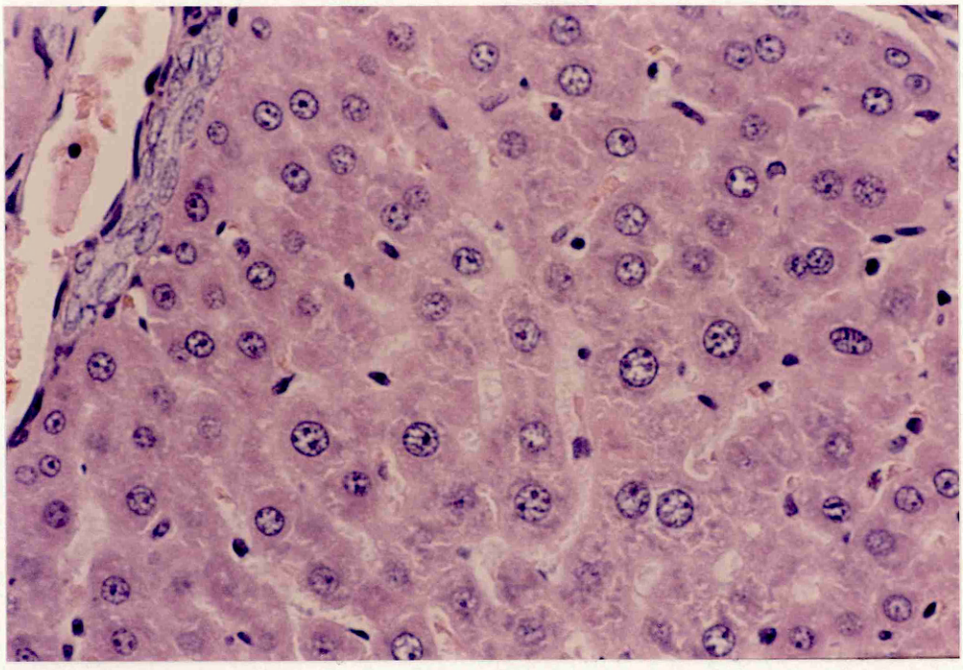

Fig. 28. Survivor after IV cytosol. (x40)

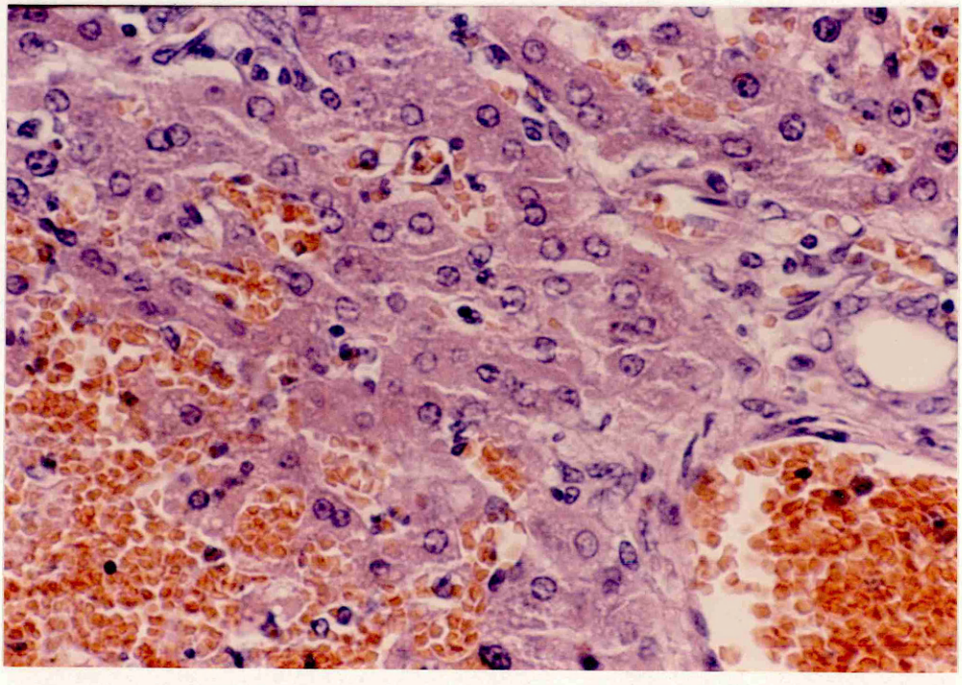

Fig. 29. Saline control. (x40)

EFFECT OF INTRAVENOUS PLASMA

4 rats were then treated with rat fresh frozen plasma in a similar treatment regime to that used above for cytosol and the results compared with the cytosol and control groups above. Adult male Wistar rats weighing between 270 and 310g were injected intaperitoneally with 50mg/kg of dimethylnitrosamine to induce acute hepatic failure. Six hours later each rat received an intravenous dose of 1ml of rat plasma. This had been obtained by bleeding 3 other rats, separating the plasma, filter sterilizing and freezing it. When required it was thawed, heated to 37^{o}C and administered. Blood monitoring was performed as before.

Results

2 rats died at 44 hours after the induction of liver failure and the other 2 survived, recovered from the hepatic failure and were sacrificed at 388 hours.

hours	0	6	24	48	72	96	144	192	sacr
Normotest	41%	34%	10%		12%	26%	30%	31%	41%
ALT	93	63	2109	5872		444	85	102	117
AST	182	121	1847	6000		631	117	102	82
AP	500	382	326	830		710	517	447	494
Bilirubin	14	10	27	11		12	12	7	10

Histology

The 2 rats which died had 70% and 75% hepatocyte necrosis and the 2 survivors had no hepatocyte necrosis at the time of sacrifice. (Figs. 30, 31)

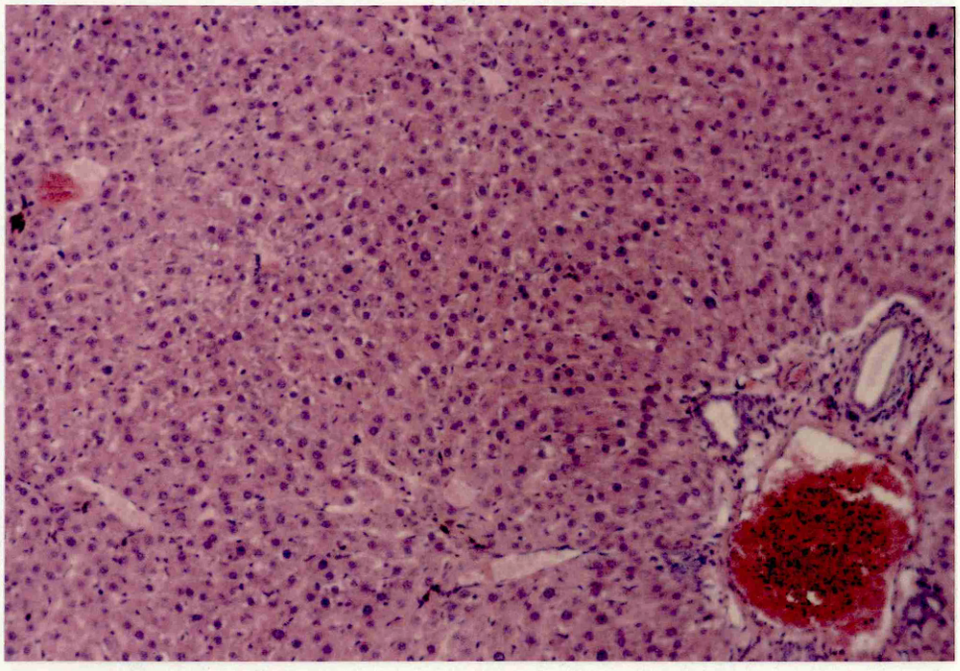

Fig. 30. Survivor after IV plasma. (x10)

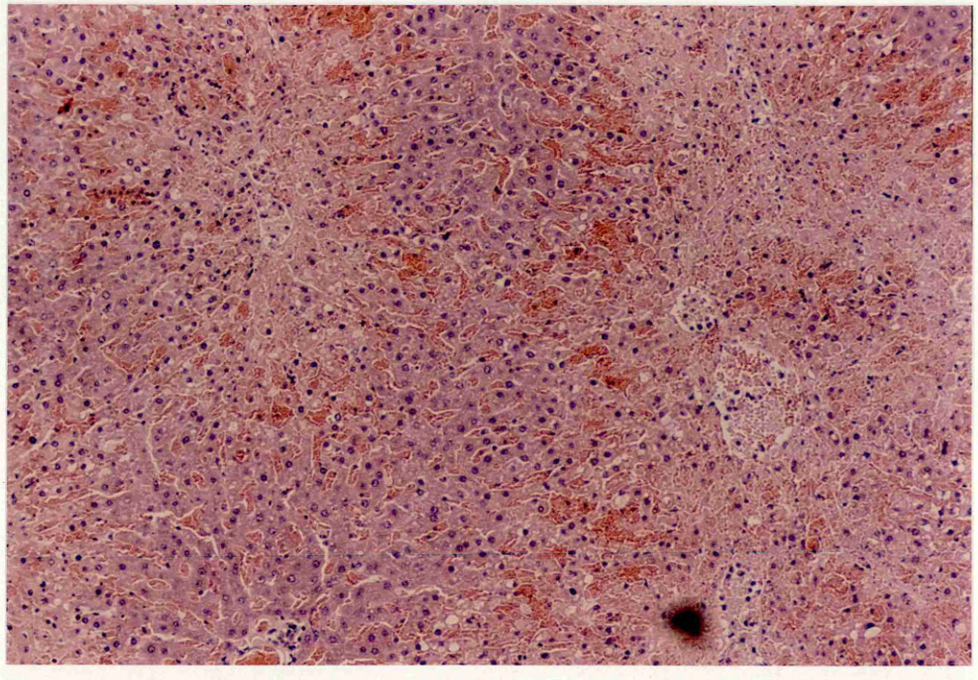

Fig. 31. Nonsurvivor after IV plasma. (x10)

ALTERATION IN CYTOSOL DOSAGE

Acute hepatic failure was induced as before in nine adult male Wistar rats weighing between 320 and 400g. Six hours later the rats were again anaesthetised and each rat was given an intravenous injection of cytosol which had been prepared from other adult male Wistar rats. Three rats received a concentration of cytosol equivalent to the contents of $1.02x10^7$ hepatocytes, three were given a cytosol with concentration equivalent to $1.9x10^7$ cells and three received a concentration of $3.42x10^7$ hepatocytes. The doses of intravenous cytosol were repeated daily maintaining the same concentration as the initial dose for each rat. All cytosol injections had a volume of 1ml. Blood samples for the measurement of Normotest and biochemical liver function tests were taken prior to the induction of acute hepatic failure, before each dose of cytosol and at regular intervals till sacrifice. Any rat recovering from acute hepatic failure was sacrificed when well. At death or sacrifice the rats were subjected to a postmortem examination with removal of the liver for histological review.

Results

One rat in each of the test groups died of acute liver failure, at 120 hours in the group given the lowest dose of cytosol, at 48 hours in the one receiving the intermediate dosage and at 72 hours in the rats given the highest concentration. All the surviving rats were sacrificed at 533 hours.

Normotest (mean)

hours	0	6	24	48	72	96	120	168	sacr
low dose	37%	45%	15%	8%		28%	44%	44%	37%
middle dose	40%	31%	14%	7%	23%	40%	37%	42%	50%
high dose	39%	41%	16%	9%	21%	41%	40%	44%	45%

ALT (mean)

hours	0	6	24	48	72	96	120	168	sacr
low dose	88	73	856			941	838	100	97
middle dose	78	57	350	4313	1715	748	202	85	120
high dose	84	63	708	4930	1985	1008	368	126	114

AST (mean)

hours	0	6	24	48	72	96	120	168	sacr
low dose	305	127	891			1270	1015	350	106
middle dose	143	122	980	8240	2126	925	355	200	100
high dose	227	116	830	7826	2212	987	410	410	103

Alk Phos (mean)

hours	0	6	24	48	72	96	120	168	sacr
low dose	453	416	491			916	897	295	453
middle dose	407	352	549	729	960	1232	765	242	389
high dose	423	372	478	698	852	1010	860	235	386

Bilirubin (mean)

hours	0	6	24	48	72	96	120	168	sac
low dose	8	3	10			28	21	20	5
middle dose	16	5	4	39	18	12	15	10	10
high dose	13	8	8	46	40	11	10	15	10

Histology

The surviving rats in each group showed no evidence of hepatocyte necrosis. The rat which died at 120 hours in the low dose group demonstrated 50% necrosis at time of death, the one in the middle dosage group which died at 48 hours showed 64% hepatocyte necrosis at time of death and the rat which died in the high dose group at 72 hours had 60% necrosis. The livers of the surviving rats in the middle and high dosage groups showed some bridging fibrosis and some of the livers, particularly in the low dose group, had dilated central veins.

ALTERATION IN TIMING OF THE FIRST DOSE OF CYTOSOL

Nine male Wistar rats weighing between 255 and 300g were injected intraperitoneally with 50mg/kg of dimethylnitrosamine to induce acute hepatic failure. The animals were divided into three groups; all to receive daily intravenous injections of rat hepatocyte cytosol to a maximum of five injections (4 in group 3). The animals in group 1 received their first dose at 6 hours after the induction of acute liver failure, those in group 2 at 12 hours after the dimethylnitrosamine and group 3 at 24 hours. The cytosol used in all groups was from the same donor rat and each dose contained the intracellular cytoplasmic contents of 1.9×10^7 hepatocytes. Blood samples were taken before each cytosol injection and at intervals afterwards till death or sacrifice. Postmortem examinations were performed on all rats with removal of the livers for histology.

2 rats were given DMNA as above but not given cytosol and they were sacrificed, one at 6 hours and the other at 24 hours, to see the amount of hepatocyte necrosis at these times after induction of acute liver failure.

Results

In group 1 one rat died of liver failure at 72 hours. Another rat in this group died of an undiagnosed cause while being anaesthetised at 216 hours. By this time in the experiment its clotting and biochemical liver function tests had returned to the normal range. All rats in group 2 recovered from the acute hepatic failure and were sacrificed. One rat in group 3 died at 72 hours of liver failure. All surviving rats in the three groups were sacrificed at 365 hours.

Normotest (mean)

hours	0	6	12	24	48	72	96	144	216	365
Group 1	36%	39%		4%	4%	12%	34%	38%	58%	70%
Group 2	29%		13%	5%	10%	35%	18%	32%	62%	51%
Group 3	52%			4%	18%	46%	38%	42%	47%	56%

ALT (mean)

hours	0	6	12	24	48	72	96	144	216	365
Group 1	189	103		4312		3165	364	138	172	142
Group 2	154		136	4410	2200	706	330	112	121	141
Group 3	129			4554	2467	683	320	162	152	125

AST (mean)

hours	0	6	12	24	48	72	96	144	216	365
Group 1	281	168		4704		3000	559	228	363	187
Group 2	221		312	6090	2607	852	375	139	185	135
Group 3	136			6915	3927	840	425	309	201	116

Alk Phos (mean)

hours	0	6	12	24	48	72	96	144	216	365
Group 1	551	562		700		902	1128	765	444	529
Group 2	619		600	740	962	1020	1088	629	639	564
Group 3	643			797	811	1013	1040	572	538	542

Bilirubin (mean)

hours	0	6	12	24	48	72	96	144	216	365
Group 1	25	10		28		56	10	10	10	10
Group 2	10		0	30	32	14	20	8	7	7
Group 3	3			32	42	20	11	10	10	10

Histology

None of the surviving animals showed any evidence of hepatocyte necrosis; neither did the rat in group 1 which died at 216 hours.

The rat in group 1 which died at 72 hours showed 70% hepatocyte necrosis at the time of death and the one which died at 72 hours in group 3 had 47% hepatocyte necrosis. The livers of the surviving rats again showed bridging fibrosis. In the untreated rats there was no evidence of hepatocyte necrosis at 6 hours (Fig. 32) but at 24 hours there was 60% necrosis (Fig. 33).

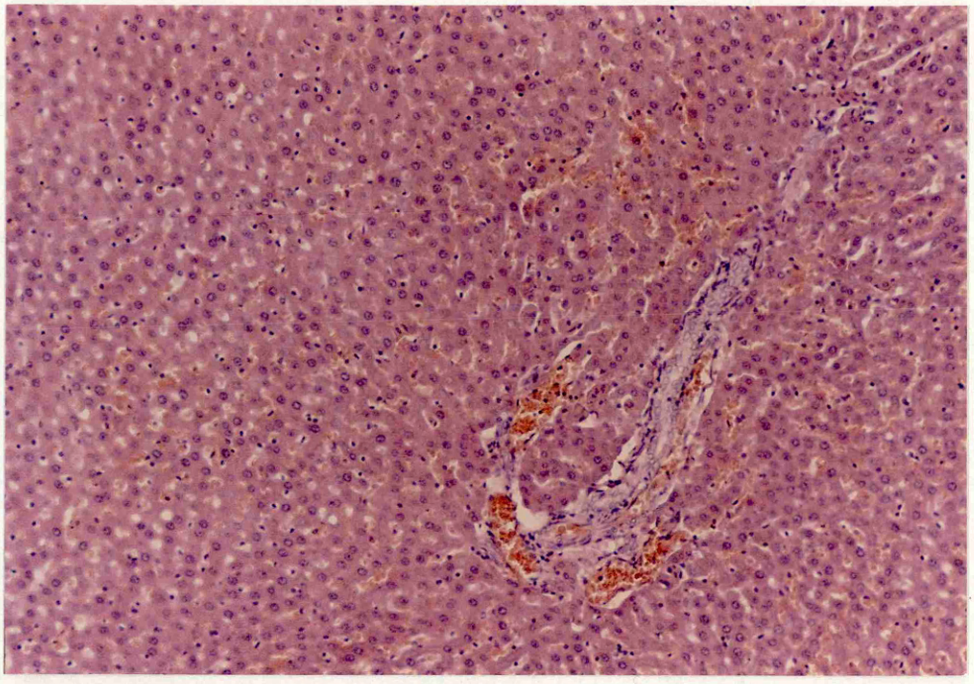

Fig. 32. 6 hours after DMNA. (x10)

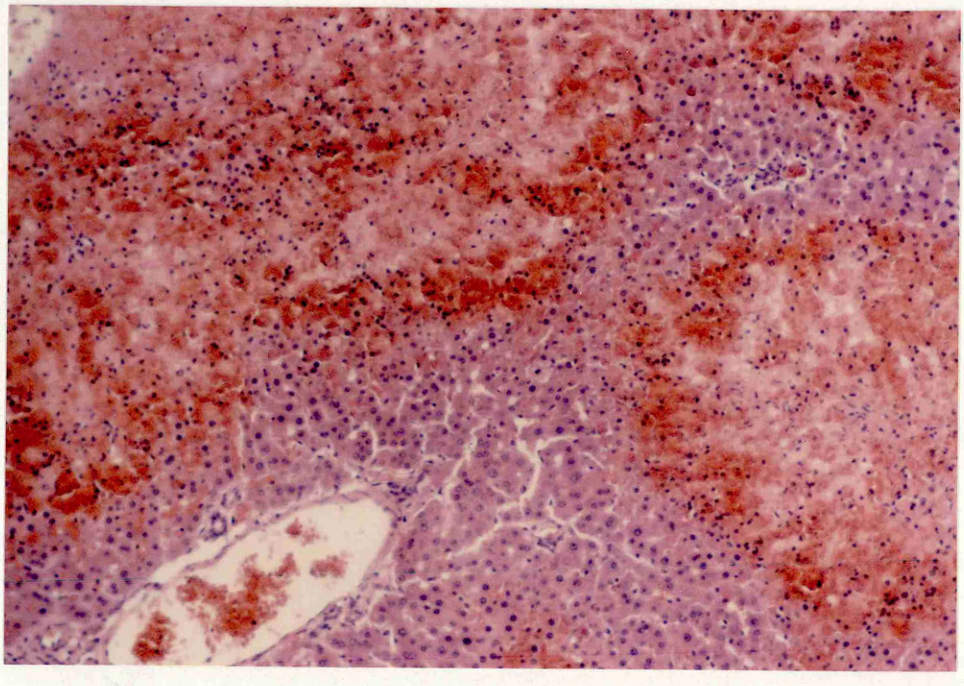

Fig. 33. 24 hours after DMNA. (x10)

CYTOSOL ADMINISTRATION AFTER 24 HOURS

To determine if hepatocyte cytosol administered in this regime was effective treatment if the first dose was given later than 24 hours after the induction of hepatic failure a further 10 rats were studied. These adult male Wistar rats weighing between 270 and 370g each received an intraperitoneal injection of 50mg/kg of dimethylnitrosamine to induce acute hepatic failure. 5 of the rats (group 1) were given their first dose of cytosol at 24 hours after the dimethylnitrosamine and the other 5 (group 2) received the first dose at 30 hours after the induction of hepatic failure. Each 1ml cytosol injection contained the cytoplasmic content of $3x10^7$ hepatocytes. The rats were monitored as previously. All surviving animals were sacrificed and postmortems with histological examination of the livers were undertaken.

In conjunction with this experiment 2 control animals were also studied. Both of these rats, weighing 280 and 360g, underwent induction of acute hepatic failure as above by a single intraperitoneal injection of dimethylnitrosamine. One rat received an intravenous injection of saline at 24 hours after the induction of acute hepatic failure and the other a similar saline injection at 30 hours.

Results

In group 1 (24 hours) 4 of the 5 animals died of acute liver failure at 48, 55, 60 and 140 hours and 1 survived to be sacrificed at 360 hours. All of group 2 (30 hours) died of liver failure at 31, 40, 40, 48 and 81 hours. The 24 hour saline control died of liver failure at 40 hours and the 30 hour control died at 30 hours.

Normotest (mean)

hours	0	24	30	48	72	96	168	216	312	360
Group 1	49%	13%		5%	27%	18%	35%	39%	43%	70%
Group 2	46%		4%	4%						
Control 24	51%	19%								
Control 30	39%		<5%							

ALT (mean)

hours	0	24	30	48	60	72	168	216	312	360
Group 1	80	1330		190	4398		70	90	135	105
Group 2	88		5985	6379		22				
Cont. 24	85	1115	>7500							
Cont. 30	65		4920							

AST (mean)

hours	0	24	30	48	60	72	168	216	312	360
Group 1	281	1540		7595	12185		85	155	95	85
Group 2	256		6525	11530		2016				
Cont.24	225	1355	>7500							
Cont.30	385		10290							

Alk Phos (mean)

hours	0	24	30	48	60	72	168	216	312	360
Group 1	319	310		498	751		410	360	390	452
Group 2	402		380	667		601				
Cont.24	380	385	624							
Cont.30	225		370							

Bilirubin (mean)

hours	0	24	30	48	60	72	168	216	312	360
Group 1	14	10		49	59		10	15	5	10
Group 2	10		25	38		58				
Control 24	10	21	27							
Control 30	15		53							

Histology

In Group 1 the three rats which died between 48 and 60 hours after the induction of acute hepatic failure showed hepatocyte necrosis of 64% of the liver. The rat dying at 140 hours had 14% hepatocyte necrosis and the rat which was sacrificed had no evidence of hepatocyte destruction. In Group 2 the mean amount of hepatocyte destruction was 72%. The 4 rats dying between 31 and 48 hours had hepatocyte loss of 74% and the one dying at 81 hours had 60% hepatocyte necrosis. The control animal receiving saline at 24 hours had 73% hepatocyte necrosis and the one receiving saline at 30 hours had 56% hepatocyte loss.

EFFECT OF RAT LIVER CYTOSOL ASSESSED AT THE TIME OF DEATH OF CONTROL RATS.

Acute hepatic failure was induced as before in a further eight male Wistar rats, weighing between 330g and 410g. The rats were divided into two groups and paired. One of each pair received an intravenous injection of rat hepatocyte cytosol at 6 hours after the induction of acute liver failure and at daily intervals and the other one was given intravenous saline at the same times. Upon the death of one of the pair the other one was sacrificed. Blood samples were taken before each treatment or control injection to monitor Normotest, and biochemical liver function tests. At death or sacrifice the rats underwent a postmortem with removal of the liver for histology and assessment of liver necrosis and for analysis of possible liver regeneration. Liver regeneration was assessed by counting the number of hepatocyte nuclei showing mitotic figures and expressing this as a percentage of the total number of nuclei within the same area.

Results

All of the control rats and one of the cytosol rats died at 72 hours and the remaining cytosol treated rats were sacrificed at this time. (One of the control rats had died a few hours earlier, considered to be at about 68 hours but its pair was sacrificed at 72 hours.)

Normotest (mean)

hours	0	6	24	48	72
cytosol	38%	39%	30%	<5%	5%
saline	38%	32%	9%	<5%	<5%

ALT (mean)

hours	0	6	24	48	72
cytosol	57	55	1762	4600	1631
saline	62	50	1096		1127

AST (mean)

hours	0	6	24	48	72
cytosol	192	131	2085	4850	3082
saline	148	122	2737		4097

Alk Phos (mean)

hours	0	6	24	48	72
cytosol	321	298	362	545	652
saline	292	239	345		583

Bilirubin (mean)

hours	0	6	24	48	72
cytosol	11	10	20	20	36
saline	12	10	10		57

Histology

In the cytosol treated rats the mean amount of hepatocyte necrosis was 51% (Fig. 34) and in the saline group it was 60% (Fig. 35). Signs of hepatic regeneration were seen in both groups but the rats in the cytosol group showed more regeneration than the controls. In the cytosol group the mean mitotic index measurement was 9.6% and in the cytosol treated group it was 13.3%. When analysed statistically (Mann-Whitney) this was a significant difference ($p<0.005$). The areas of regenerating cells in both groups can be seen in Figs. 36 and 37.

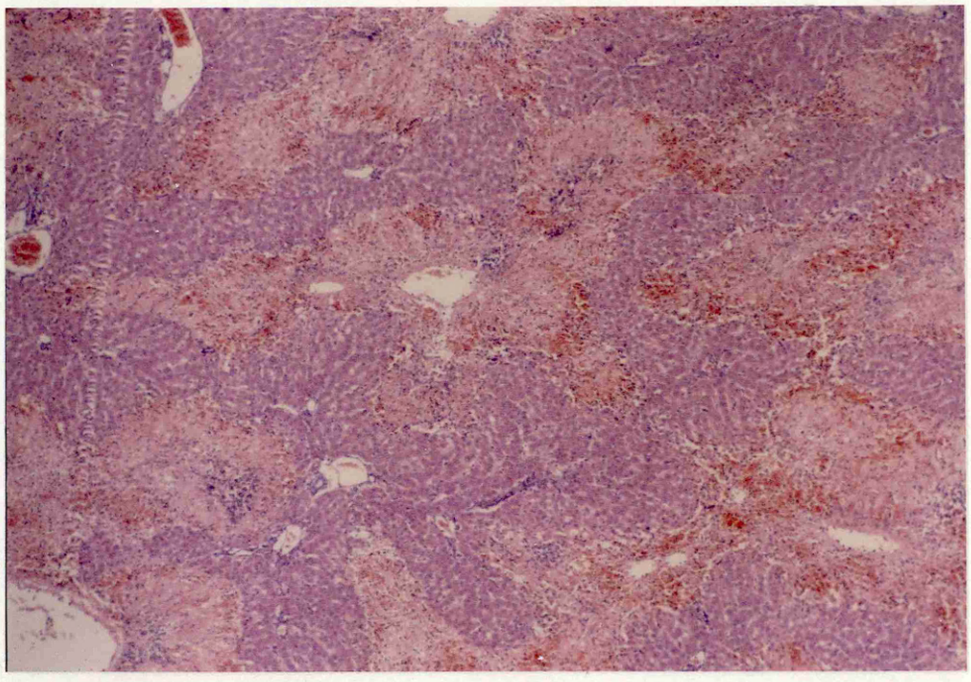

Fig. 34. IV cytosol, 72 hour sacrifice. (x4)

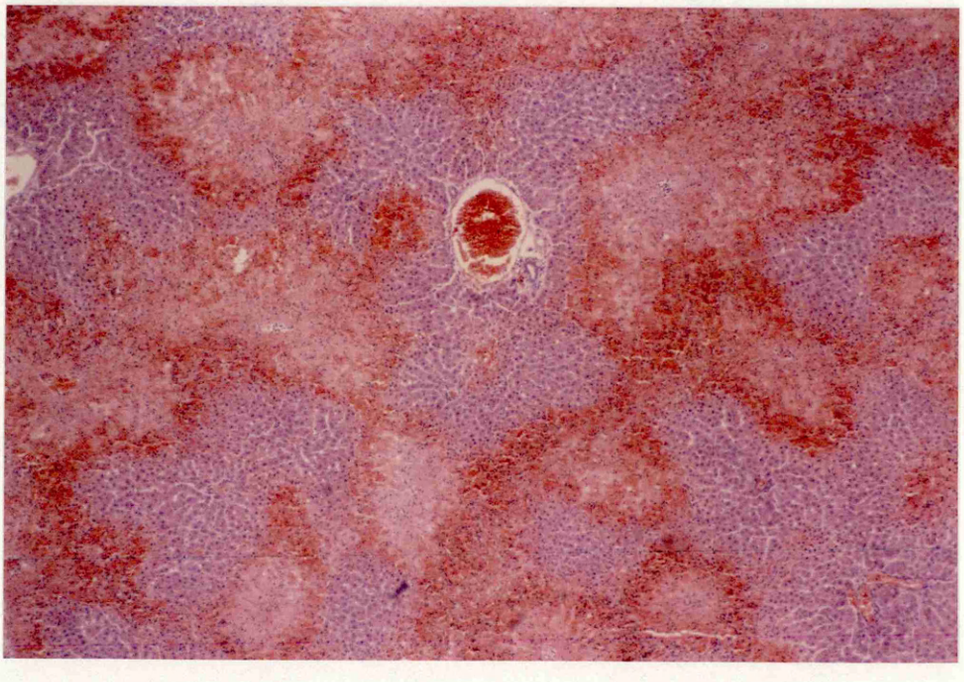

Fig. 35. IV saline, 72 hour death. (x4)

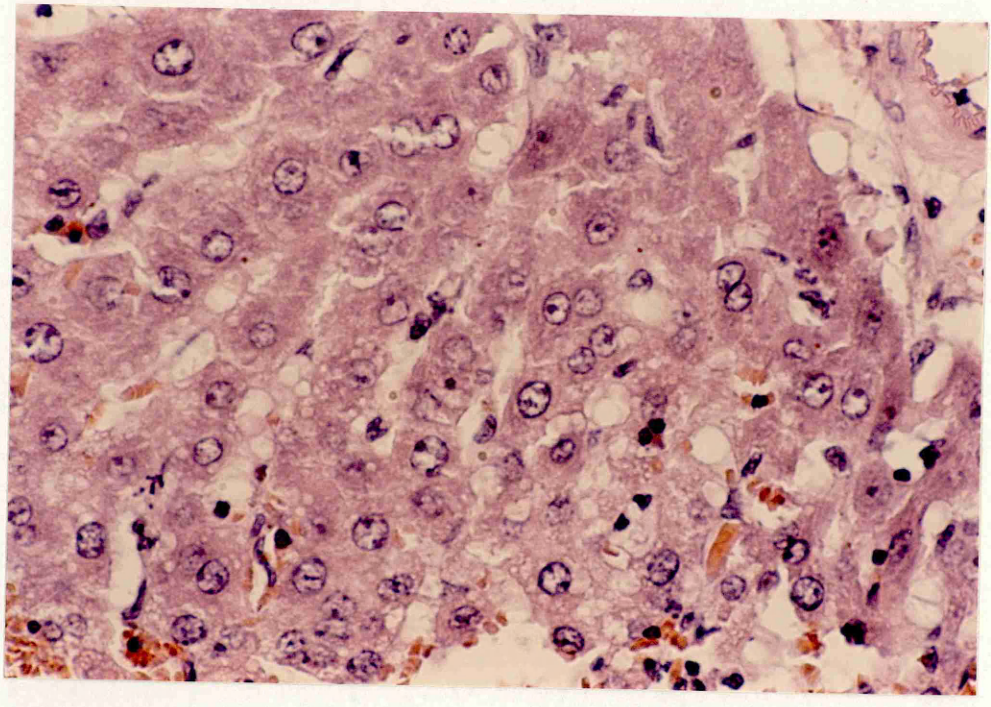

Fig. 36. IV cytosol, 72 hour sacrifice. (x40)

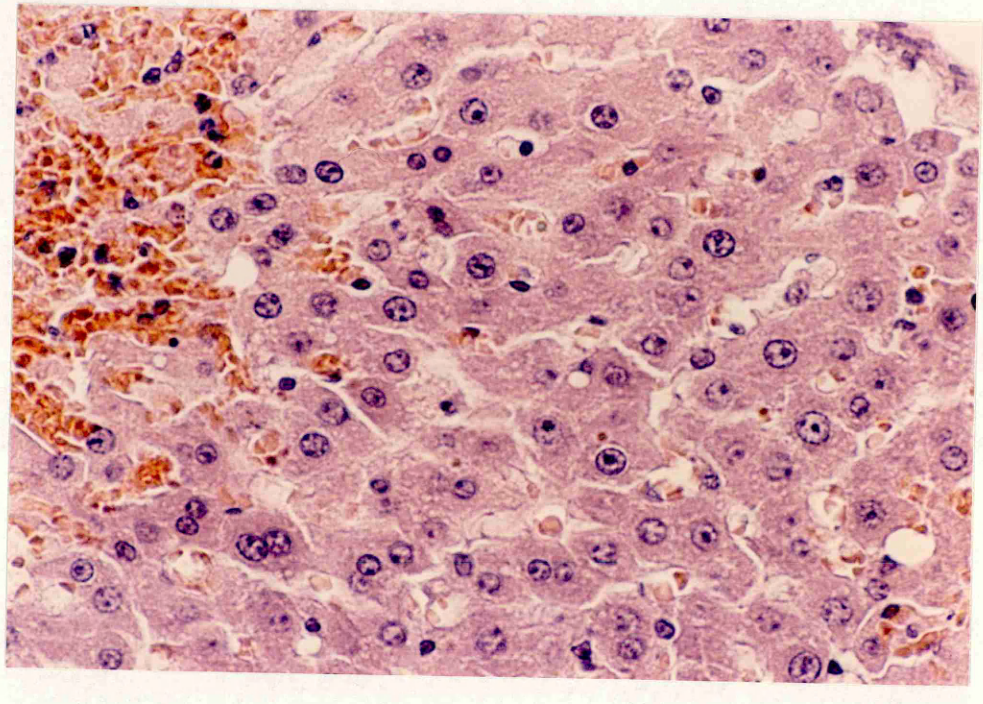

Fig. 37. IV saline, 72 hour death. (x40)

CONCLUSIONS

When subjected to statistical analysis there was a significant difference between the survival rates of the cytosol treated rats and the saline controls in the first experiment $p<0.005$. However, the biochemical and coagulation results showed no difference between the groups. The difference in the amount of hepatocyte necrosis observed was also very significant $p<0.001$. This reflects the longer survival since the sacrificed animals had no necrosis at postmortem.

When the plasma group was considered there was no statistically significant difference in survival between either the plasma and the earlier saline control group or between the plasma and the intravenous cytosol group. Neither were there any differences between the plasma group and either of the preceding groups in respect of biochemistry, coagulation or hepatocyte necrosis. This result raises the question as to whether the cytosol is acting purely as colloid and maintaining the intravascular volume or whether it has a stimulatory effect to regeneration on the hepatocytes.

The variations in cytosol dosage showed no differences amongst the groups in any of the results monitored, indicating that all of these doses were equally effective.

In the experiment giving the first dose of cytosol at different times it was seen that there were no differences

amongst the groups in relation to survival, biochemical measurement of liver function, coagulation results or the degree of liver necrosis. It was therefore evident that the cytosol was as effective when the first dose was given at 12 and 24 hours as it was when treatment was commenced at 6 hours after the induction of hepatic failure. When the timing of the first dose was extended after 24 hours there appeared to be a difference in survival between the rats treated from 24 hours compared with the rats where treatment was delayed till 30 hours. The difference did not reach statistical significance when assessed by the Mann-Whitney test ($p<0.07$). There was a statistically significant difference between these groups in the amount of hepatocyte necrosis seen ($p<0.05$). It therefore appears that cytosol is less effective if administered more than 24 hours after the induction of the liver failure.

When the animals were studied in the paired experiment there were no differences in the biochemistry or coagulation results between the groups. Neither was there any significant difference in the extent of hepatocyte necrosis recorded between the animals receiving cytosol and those given saline. There was, however, significantly more hepatocyte regeneration in the rats treated with cytosol. This suggests that cytosol is doing more than just maintaining volume and that it could be stimulating hepatocytes to regenerate at a faster rate than in the control animals.

SUMMARY

Rat hepatocyte cytosol administered intravenously in repeated doses improves the survival of rats with toxin induced hepatic failure and appears to do so by increasing the rate of regeneration of the surviving hepatocytes. It is effective when the first dose is given up to 24 hours after the induction of liver failure by which time hepatocyte necrosis is already established.

IN-VIVO PARTIAL CHARACTERISATION OF HEPATOCYTE CYTOSOL

The hepatocyte cytosol used in the previous experiments is a mixture of chemicals, cellular organelles and other components of the cytoplasm of the hepatocyte. It is therefore necessary to try to define which component or combination of factors is responsible for the improved survival of the rats treated with cytosol in the hepatotoxin-induced acute hepatic failure model.

In an attempt to separate different components cytosol was heated at two different temperatures. Another batch of cytosol was dialysed across a membrane allowing the passage of molecules smaller than 8,000 molecular weight.

Methods

Preparation of heat-stable fraction.

Cytosol was prepared as before but prior to sterilisation one batch, with an hepatocyte concentration of 3.0×10^7 per ml of cytosol, was heated at 56°C for 30 minutes. A second specimen of cytosol, with an hepatocyte concentration of 3.1×10^7 per ml was heated at 65°C for 15 minutes and a third cytosol (4.5×10^7 hepatocytes per ml) was also heated at 65°C for 15 minutes.

Preparation of dialysed cytosol.

Cytosol was prepared as previously but before filter sterilising it was put inside a dialysis membrane tube with a pore size of molecular weight cutoff of 6,000 - 8,000 ("Spectra/Por 1" membrane; Spectrum Medical Industries). It

was dialysed for 24 hours against Hank's balanced salt solution with calcium and magnesium, changing the dialysate twice during the process.

15 male adult Wistar rats weighing between 310 and 360g were given acute hepatic failure by an intraperitoneal injection of 50mg/kg of dimethylnitrosamine. 6 hours after this each rat received its first intravenous injection of modified cytosol, according to the following regime.

Group 1 - 3 rats - cytosol of 3.0×10^7 hepatocytes heated at $56^{\circ}C$ for 30 minutes.

Group 2 - 4 rats - cytosol of 3.1×10^7 hepatocytes heated at $65^{\circ}C$ for 15 minutes.

Group 3 - 2 rats - cytosol of 4.5×10^7 hepatocytes heated at $65^{\circ}C$ for 15 minutes.

Group 4 - 6 rats - dialysed cytosol.

Each rat then was given the same modified cytosol as its first injection at daily intervals to a maximum of 5 doses. Blood samples were taken from the rats' tail veins for Normotest and biochemical liver function testing prior to the induction of acute liver failure, before each dose of modified cytosol and at death. Postmortem examinations were performed on all animals and the livers removed for histological examination.

Results

All rats died of liver failure. Those in group 1 died at 96, 102 and 144 hours; in group 2 at 56 60 65 and 96 hours; in group 3 at 75 and 120 hours and in group 4, 2 die at 60 hours, 1 at 65, 1 at 70 and 2 at 90 hours.

Normotest (mean)

hours	0	6	24	48	60	72	96	102
Group 1	48%	26%	4%	4%		10%		13%
Group 2	36%	41%	6%	4%	4%			
Group 3	27%	40%	9%	4%		5%		
Group 4	31%	33%	8%	4%		4%		

ALT (mean)

hours	0	6	24	60	72	96	102	120
Group 1	95	68	3414		1770	116	415	
Group 2	75	62	2211	1271		250		
Group 3	68	76	2356		615			34
Group 4	76	95	607	2415	82	24		

AST (mean)

hours	0	6	24	60	72	96	102	120
Group 1	229	163	4325		1835	1690	1280	
Group 2	137	126	2563	5853		967		
Group 3	80	335	2628		3970			989
Group 4	162	182	844	8482	4415	1912		

A P (mean)

hours	0	6	24	60	72	96	102	120
Group 1	495	272	485		785	456	1015	
Group 2	295	153	352	483		636		
Group 3	303	217	232		395			722
Group 4	286	298	246	522	526	520		

Bilirubin (mean)

hours	0	6	24	60	72	96	102	120
Group 1	15	20	21		10	20	18	
Group 2	12	22	33	45		14		
Group 3	10	29	56		43			33
Group 4	22	18	15	35	71	40		

Histology

At death the rats in group 1 showed 60% hepatocyte necrosis, those in group 2, 56%, the ones in group 3 had 48% hepatocyte loss and the ones that were given dialysed cytosol (group 4) had 55% hepatocyte necrosis.

When these results were compared with those for the crude cytosol and control saline injections in chapter 9 and analysed statistically (Mann-Whitney) the coagulation, biochemical, survival and extent of hepatocyte necrosis

results were similar to those for the control saline injections. The heated and the dialysed cytosol tested here gave poorer results for survival and hepatocyte necrosis than the whole crude cytosol $p<0.05$.

Conclusion

The component of hepatocyte cytosol producing the improved survival of rats with acute hepatic failure would appear to be able to pass through a dialysis membrane of pore size 6,000 - 8,000 molecular weight. This suggests that it is a molecule possibly below 6,000 daltons but definitely smaller than 8,000 daltons. The active component is also destroyed by heating at 56°C for 30 minutes and 65°C for 15 minutes and therefore must be considered to be heat labile.

EFFECT OF RAT HEPATOCYTE CYTOSOL ON DNA SYNTHESIS IN RAT HEPATOCYTE CULTURE.

In the experiments described earlier it was seen that rat hepatocyte cytosol improved the survival of rats with hepatotoxin induced acute liver failure when administered intravenously in repeated doses. However, it was not possible to discern the mechanism for this. One possibility is that a component of the cytosol may have a stimulatory effect on DNA synthesis and so increase the regeneration rate of the surviving periportal hepatocytes to the extent that enough liver tissue is again functioning before the rat reaches a terminal stage of hepatic failure.

The effect of rat liver cytosol on DNA synthesis in rat hepatocyte culture was assessed to determine whether stimulatory effects could be demonstrated.

Materials and methods.

In three separate experiments hepatocyte suspensions were produced as previously described by collagenase perfusion of the liver of a Wistar rat. 3×10^5 hepatocytes were placed on each collagen coated cell culture plate. The cells were cultured in William's E medium (Williams 1974) without Arginine but with Ornithine (0.07mg/ml) and containing glutamine (2mg/ml), penicillin (100U/ml), streptomycin (100μg/ml), hydrocortisone (2μg/ml) and insulin (0.015U/ml). The medium was changed daily. At the beginning and at each medium change cytosol, EGF and TGFβ

were added according to the following scheme. Three plates were used in each group.

Experiment 1

a) control plates
   i) no addition
   ii) EGF (0.85nmol)
   iii) TGFβ (10ρmol)

b) test plates
   i) 20μl cytosol
   ii) 60μl cytosol
   iii) 5μl cytosol
   iv) 60μl cytosol + TGFβ (10ρmol)
   v) 60μl cytosol + EGF (0.85nmol)

The cytosol used in this experiment was from rat 72 with 1ml of cytosol prepared from 3.06×10^7 hepatocytes. The cells were cultured for 72 hours.

Experiment 2

a) control plates
   i) no addition
   ii) EGF (0.85nmol)
   iii) TGFβ (10ρmol)

b) test plates
   i) 5μl cytosol + EGF (0.85nmol)
   ii) 10μl cytosol + EGF "
   iii) 30μl cytosol + EGF "
   iv) 60μl cytosol + EGF "
   v) 5μl cytosol + TGFβ (10ρmol)
   vi) 60μl cytosol + TGFβ "
   vii) 5μl cytosol
   viii) 10μl cytosol
   ix) 30μl cytosol
   x) 60μl cytosol

The cytosol used here was obtained from rat 114 with 1.3ml of cytosol containing extract from 3.09×10^7 hepatocytes (2.38×10^7cells/ml). Cells were cultured for 72 hours.

Experiment 3

a) control plates
   i) no addition (a)
   ii) no addition (b)
   iii) EGF (0.85nmol) (b)

b) test plates
   i) 5μl cytosol (a)
   ii) 10μl cytosol (a)
   iii) 60μl cytosol (a)
   iv) 5μl cytosol (b)
   v) 10μl cytosol (b)
   vi) 60μl cytosol (b)
   vii) 5μl cytosol + EGF (0.85nmol) (a)
   viii) 10μl cytosol + EGF " (a)
   ix) 60μl cytosol + EGF " (a)
   x) 5μl cytosol + EGF " (b)
   xi) 10μl cytosol + EGF " (b)
   xii) 60μl cytosol + EGF " (b)

The cytosol used in this experiment was from rat 94 with 0.8ml of cytosol containing the cytoplasmic contents of $1.9x10^7$ hepatocytes ($2.4x10^7$cells/ml). In the plates marked (a) the cells were cultured for 48 hours and those marked (b) for 72 hours.

Cells were maintained in culture for 48 or 72 hours with daily medium changes and ^3H thymidine (1μcur/ml) added for the final 24 hours.

To halt growth and extract the DNA a modification of the method used by McGowan (1981) based upon that of Munro and Fleck (1966) was used. The cells on the plate were washed with PBS and cold (4^oC) 5% trichloroacetic acid (TCA) was added and the plates cooled to 4^oC. Two 1.5ml washes with 5% TCA were then performed followed by two 1.5ml washes with 95% ethanol. Solubilization was established by adding 1ml of 0.3N KOH for 1 hour at 37^oC. After this the plates were scraped and the cells transferred to a tube containing 1mg/ml bovine serum albumen (BSA). To this was added 400μl 20% TCA at 4^oC and left for at least 10 minutes. It was then centrifuged at 1000rpm for 1 minute at 4^oC and the supernatant removed and the cells washed with 1ml 5% TCA and centrifuged again. The resultant pellet was hydrolyzed in 200μl 5% TCA for 15 minutes at 90^oC and then centrifuged at 3000rpm for 15 minutes. From the supernatant 100μl was used in the diphenylamine assay to measure the DNA concentration and 40μl was put in 4ml of Optiphase X and the ^3H disintegrations per minute were measured in a scintillation counter.

DNA concentration was measured by the diphenylamine assay (Burton 1956) using 100μl of the liver extract in 5% TCA added to 400μl 0.5N perchloric acid (PCA) and 0.5ml of reagent. The tubes were stoppered and left to stand at room temperature overnight in the dark. Samples were placed in 2ml cuvettes and read in the spectrophotometer at OD 595. The results were read from a standard curve prepared from readings at 1μg/100ml TCA, 2μg/100ml, 3μg/100ml, 4μg/100ml, 5μg/100ml, 6μg/100ml and a blank of 100μl TCA.

Results

Experiment 1

Plates	^{3}H disintegrations / μg DNA 1	2	3	mean
C i)	31548	28777	32496	30940
ii) +EGF	155668	168709	161421	161932
iii) +TGFβ	11861	7800	13109	10923
T i) +20c	20057	16526	19627	18736
ii) +60c	19236	22087	19189	20170
iii) +5c	34608	27989	26117	29571
iv) +60c+TGFβ	15270	26607	4621	15499
v) +60c+EGF	197082	174985	172978	181681

Experiment 2

Plates	^{3}H disintegrations / μg DNA 1	2	3	mean
C i)	7512	8697	12256	9488
ii) +EGF	100103	99833		99968
iii) +TGFβ	6397	6612	9086	7365
T i) +5c+EGF	60380	56494	56950	57941
ii) +10c+EGF		44933	60714	52823
iii) +30c+EGF	25879	28598	27961	27479
iv) +60c+EGF	34639	30764	35797	33733
v) +5c+TGFβ	2406	2350	2234	2330
vi) +60c+TGFβ	3428	3889	3056	3457
vii) +5c	2679	2836	3918	3144
viii) +10c	24419	24262	22592	23757
ix) +30c	25135	36332	38023	33163
x) +60c	54368	52640	54260	53756

Experiment 3

Plates			^3H disintegrations / μg DNA 1	2	3	mean
C i)		(a)	28444	23437	26871	26251
ii)		(b)	16666	23287	34341	24765
iii)	+EGF	(b)	180828	190299	193921	188349
T i)	+5c	(a)	30227	31747	25242	29072
ii)	+10c	(a)	24280	22234	26969	24495
iii)	+60c	(a)	7372	24948	12704	15008
iv)	+5c	(b)	23162	23880	24390	23811
v)	+10c	(b)	24096	22592	19508	22065
vi)	+60c	(b)	20690	17767	12831	17096
vii)	+5c+EGF	(a)	180588	77007	175097	144231
viii)	+10c+EGF	(a)	162051	212774	166602	180476
ix)	+60c+EGF	(a)	111448	77831	104928	98069
x)	+5c+EGF	(b)	140959	148162	115031	134717
xi)	+10c+EGF	(b)	128970	170369	140700	146680
xii)	+60c+EGF	(b)	125762	141673	111019	126151

In experiment 1 the cytosol reduces the amount of DNA synthesized in a dose related pattern when compared with the control plates. The high concentration of cytosol however appears to lessen the inhibitory effect of TGFβ and not to interfere with the stimulatory effect of EGF.

In experiment 2 at the lowest dose of cytosol used it appears to have an inhibitory effect on DNA synthesis but as the concentration of cytosol increases there is a dose dependant stimulatory effect. When given with TGFβ the combination is more inhibitory than TGFβ alone. The cytosol is seen to have a dose dependant inhibitory effect when given with EGF but its negative effect on DNA synthesis is not sufficient to counteract the stimulation of cell growth produced by the EGF.

Again in experiment 3 the cytosol is seen to have an overall inhibitory effect on DNA synthesis. The phenomenon is still dose dependent and the rate of decline in cell

regeneration is more pronounced in the shorter experiments when the DNA was measured earlier after the administration of the cytosol. Cytosol was shown to reduce the effect of EGF but in the longer experiment the dose did not appear to be of such significance.

There is an inconsistency amongst the results of these experiments and the stimulatory effect seen in experiment 2 may be due to error. The measurements of DNA by the diphenylamine assay for the 10μl, 30μl and 60μl cytosol plates were abnormally low in this experiment. No explanation for this was evident but the experiment must be repeated before the result is accepted.

Since the plates incubated with cytosol in the other experiments showed inhibition of cell regeneration, and cytosol did not reverse the effect of TGFβ or enhance the effect of EGF, cytosol must be considered inhibitory to DNA synthesis.

On inspection of the cultured hepatocytes in each experiment there was an obvious difference in the cells between the plates which had been given cytosol and those which had not. The cells cultured with cytosol were seen to have rounded up compared to the control ones which retained a flattened appearance. (Figs. 38-42)

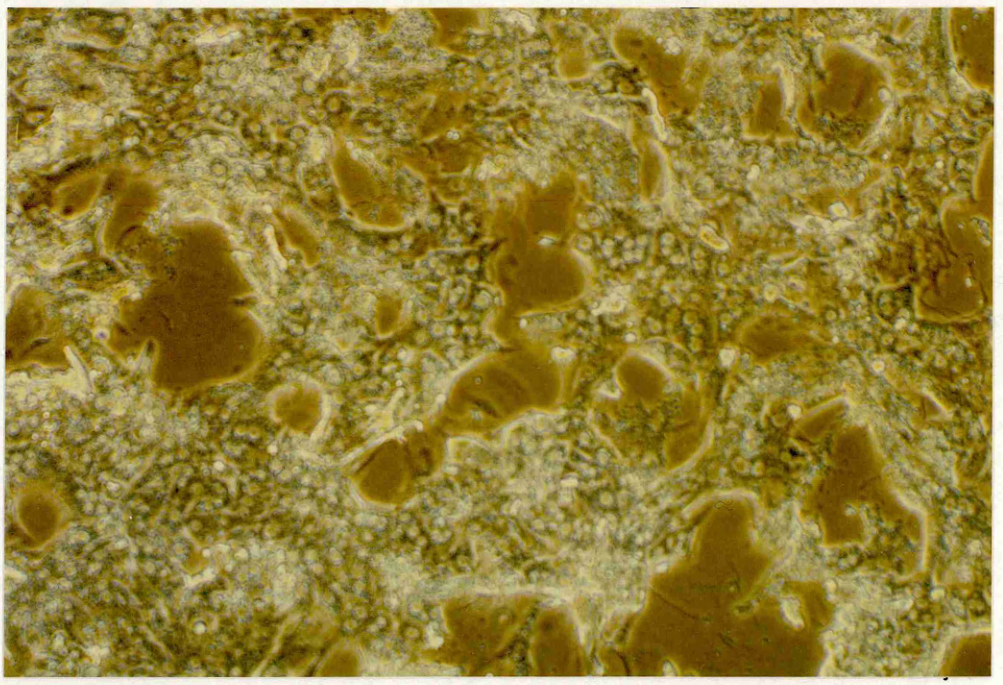

Fig. 38. Rat hepatocytes, collagen control.

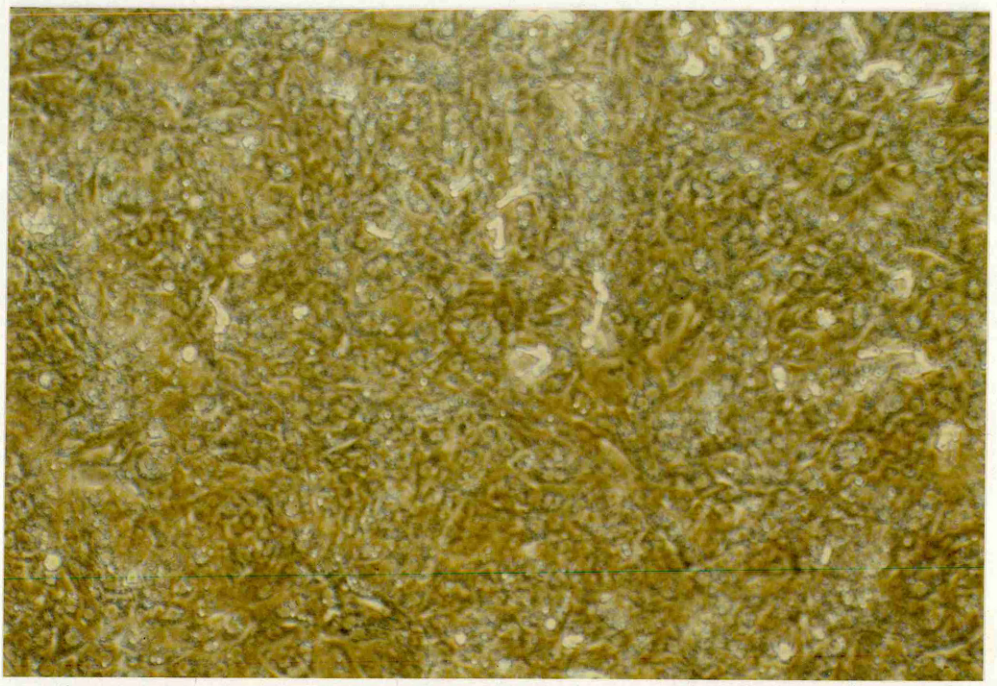

Fig. 39. Rat hepatocytes, control EGF.

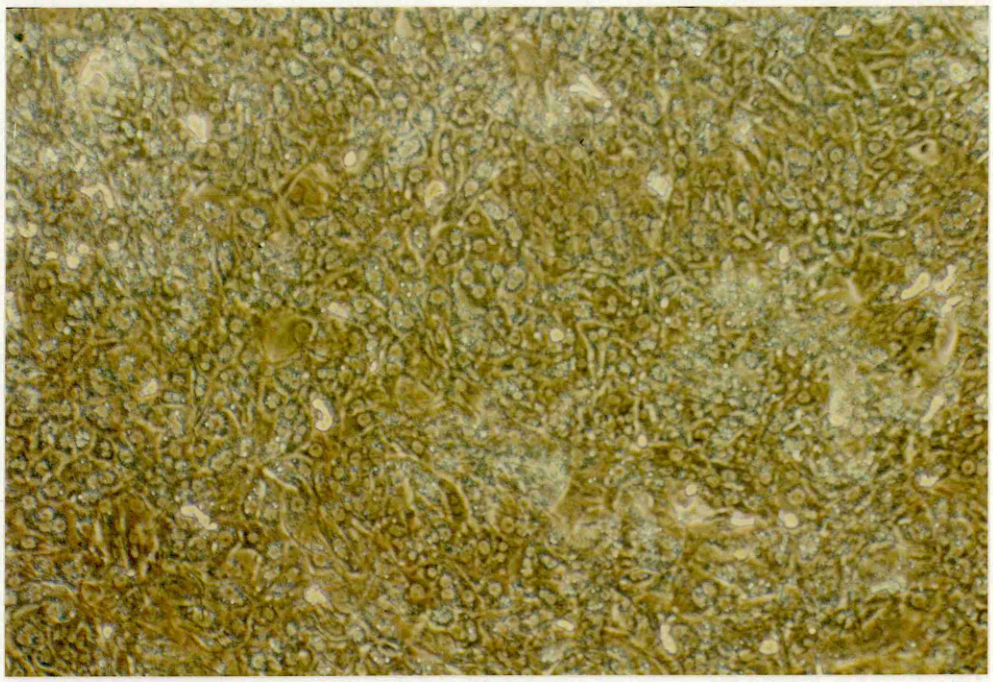

Fig. 40. Rat hepatocytes, 5μl cytosol + EGF.

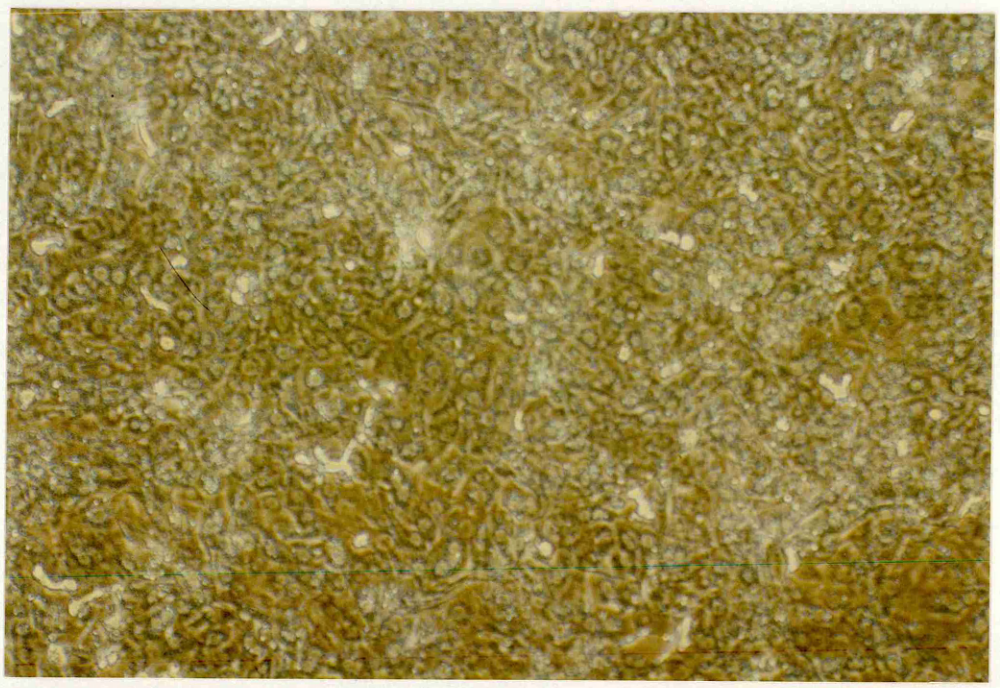

Fig. 41. Rat hepatocytes, 10μl cytosol + EGF.

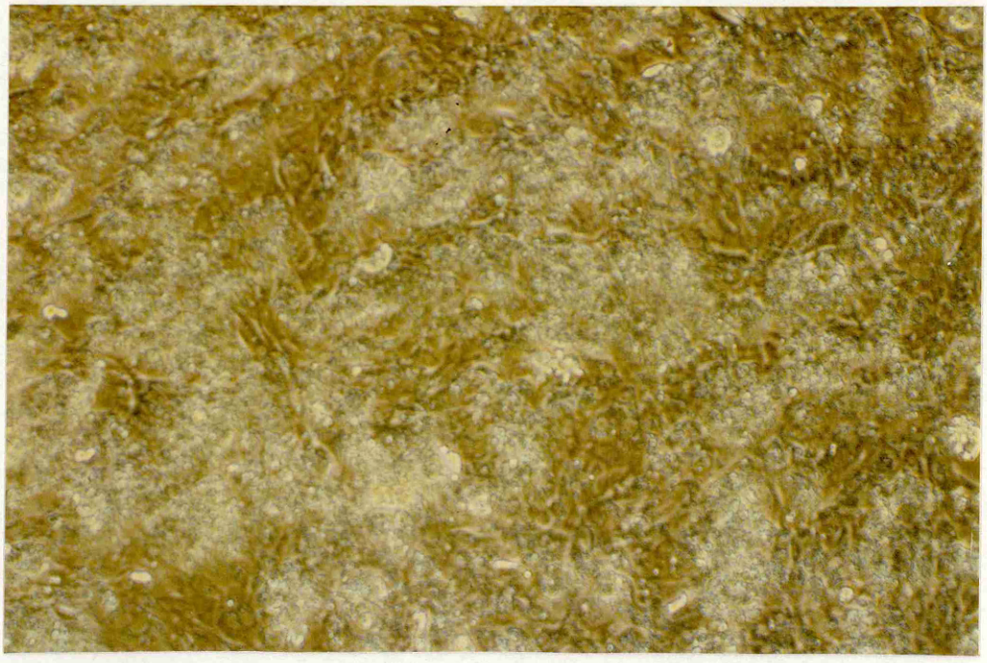

Fig. 42. Rat hepatocytes, 60μl cytosol + EGF.

Discussion.

In 1981 Makowka showed that normal adult rat hepatocyte cytosol had an inhibitory effect on DNA synthesis whereas cytosol prepared from regenerating rat liver was stimulatory in the hepatocyte culture situation. This he attributed to the switching on of "growth factors" by the stimulation to regeneration but assumed that in the normal resting situation the "factors" produced by the liver were inhibitory to cell division. The results from the above experiments could be explained in this way. It is of interest to note the more pronounced inhibition at an earlier time in experiment 3 since in an in vivo experiment in rats which had undergone partial hepatectomy normal adult rat cytosol was shown to have an initial inhibitory and then a stimulatory effect on liver DNA synthesis (Makowka 1983). Michalopoulos (1982) suggested that normal liver cytosol contained inhibitory and stimulatory factors and that the inhibitory ones predominated at higher concentrations. These experiments warrant repeating with ^{3}H incorporation at different times to see if the normal adult rat cytosol produced in this study does have a genuine effect upon cell regeneration or if its benefit to the survival of the rats in the hepatic failure model is dependant on some other mechanism of action.

The cell culture plate is a very controlled environment compared with the intact animal and it may be that cytosol though increasing regeneration in the in vivo experiments

requires other cofactors to be present for it to exert its effect and so it is ineffective when used alone.

The inhibition of DNA synthesis seen in these experiments suggests that the effect of cytosol encountered in the acute hepatic failure rat in not due to a direct stimulus to the hepatocyte causing an increase in the rate of hepatocyte regeneration.

IN-VIVO ASSESSMENT OF HUMAN HEPATOCYTE CYTOSOL IN ACUTE HEPATIC FAILURE IN RATS

To investigate if the substance responsible for improving the survival of rats with toxin-induced acute liver failure is specific for rat hepatocyte cytosol, cytosol produced from human hepatocytes was administered to rats using the same model of hepatic failure.

Materials and methods

Human hepatocyte cytosol was prepared by a modification of the method used in rats. A piece of human liver, weighing 138g, obtained from a multiorgan donor was placed on the perfusion platform and two hepatic vein branches were cannulated with 14G cannulae. The perfusion was begun with 1000ml Hank's balanced salt solution (HBSS) without calcium or magnesium, followed by 500ml HBSS with 0.5mM EGTA and then 400ml HBSS. After this the liver was perfused with an enzyme solution of 300ml HBSS containing 4ml 500mM calcium chloride, 150mg collagenase H, 300mg dispase, 150mg hyaluronidase and 15mg deoxyribonuclease which was recirculated at 65ml/min for 27 minutes. The liver was then cut and the hepatocytes in the centre shed and washed in HBSS with calcium and magnesium. The cells were then filtered through gauze and nylon mesh and the suspension centrifuged at 300rpm for 3 minutes. The supernatant was removed and the cells resuspended in HBSS with calcium and magnesium and centrifuged twice. Following final resuspension the volume of the cell suspension was made to 25ml. This cell suspension had a viability of 89% as measured by trypan blue exclusion and a cell concentration

of $2.12x10^7$ cells per ml. This was a total hepatocyte yield of $5.3x10^8$ cells in 25ml. This suspension was subjected to ultrasound bombardment in the soniprep to break down the cellular structure, at a power setting of 14 for 3 minutes. It was then ultracentrifuged at 40,000rpm for 90 minutes. The supernatant was removed, filter sterilised and dispensed into ampoules each of which contained a quantity of cytosol equivalent to the cytoplasmic contents of $2.65x10^7$ hepatocytes. This was stored at -20^oC until required.

Four adult male Wistar rats weighing between 300 and 325g were injected intraperitoneally with 50mg/kg of dimethylnitrosamine. Six hours later each rat was given an intravenous injection of 1ml human hepatocyte cytosol. The cytosol injections were repeated daily up to five doses. Blood samples were taken from the tail veins of the rats before the induction of acute hepatic failure, before each cytosol injection and at intervals afterwards. All animals surviving the hepatic failure were sacrificed and postmortems were performed on all rats at death or sacrifice with removal of the liver for histological examination.

Results

Two of the rats died of acute liver failure, at 75 and 116 hours after the induction of hepatic failure and the other two survived the experiment and were sacrificed when well at 388 hours.

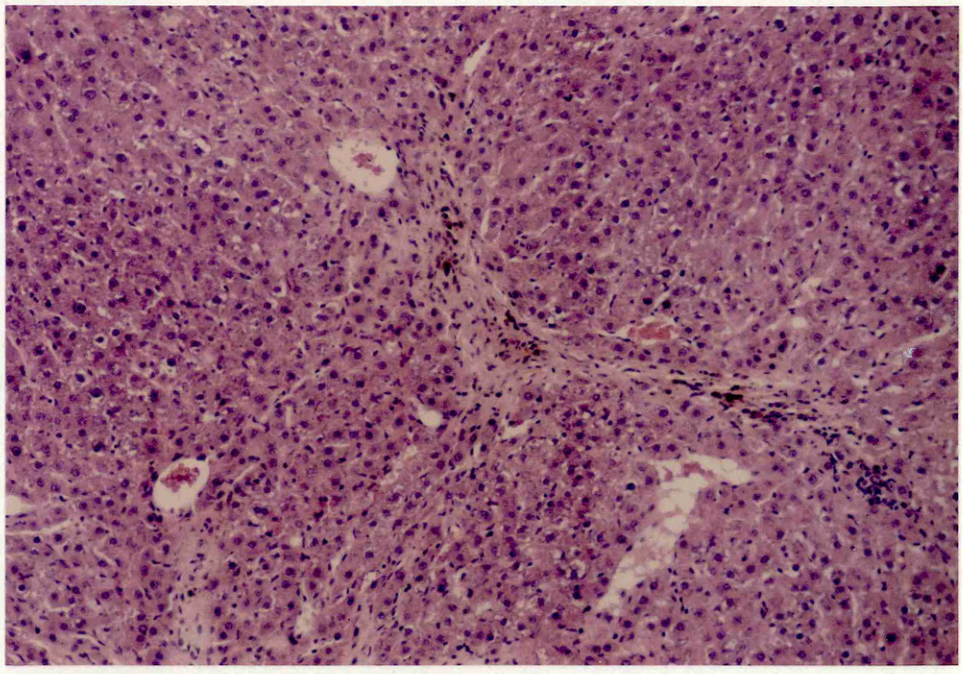

Fig. 43. Survivor after human cytosol. (x10)

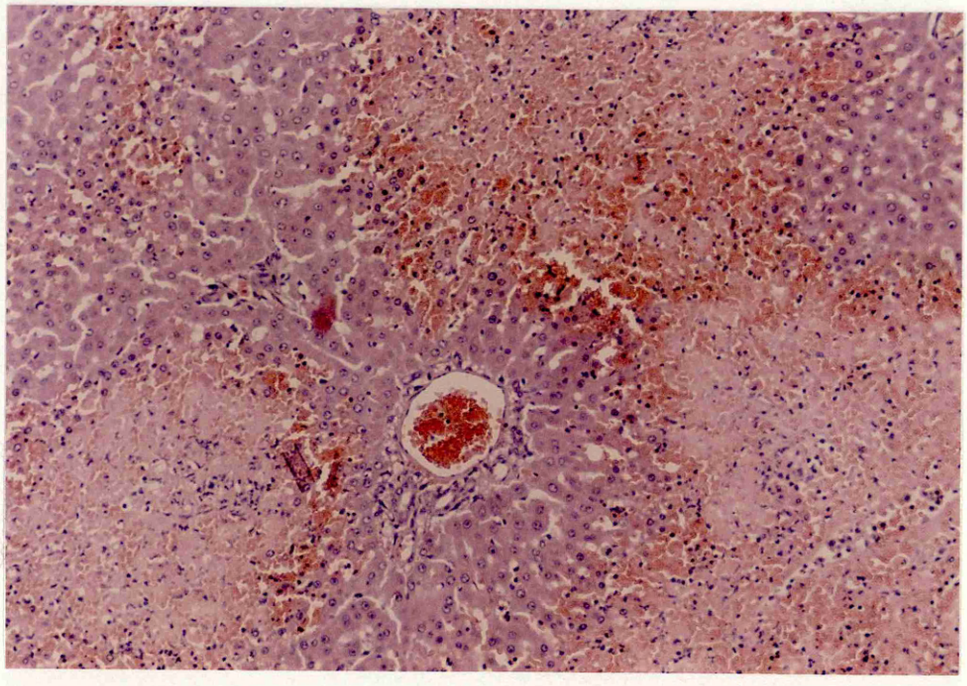

Fig. 44. Nonsurvivor after human cytosol. (x10)

Discussion

From this experiment it is seen that human hepatocyte cytosol contains a substance which is capable of successfully treating rats with toxin induced acute hepatic failure. This may be the same as the constituent of rat hepatocyte cytosol which produces the same result. The rats received repeated doses of the xenogeneic substance without exhibiting any overt signs of immunological reaction. There is obviously an antigenic potential from the foreign protein contained in the cytosol but it should be free of cell membrane antigens. These findings, however, can not be reversed to suggest that rat cytosol could safely be administered to humans.

A method has been described to prepare cytosol from human hepatocytes. When this is administered to rats with hepatotoxin induced acute hepatic failure it produces long term survival and recovery from the liver failure. The cytosol is easily prepared and can be stored frozen till needed. If it were to produce the same results in humans with fulminant liver failure this could be a beneficial addition to the treatment regime for some of these patients.

EFFECT OF HUMAN HEPATOCYTE CYTOSOL ON DNA SYNTHESIS IN HUMAN HEPATOCYTE CULTURE.

To investigate if any substance contained within the cytosol of human hepatocytes was capable of increasing the rate of DNA synthesis in human liver tissue the crude human hepatocyte cytosol was added to human hepatocytes in tissue culture.

Materials and methods.

Hepatocytes were obtained as in the previous chapter from pieces of human liver. The hepatocytes to be cultured were from the liver of a 50 year old adult male organ donor and the cells for hepatocyte cytosol production were from the liver of a ten year old brain dead boy. For cytosol production the cell suspension was suspended to a volume of 30ml in HBSS with calcium and magnesium and subjected to the soniprep at power 14 for 5 minutes and then ultracentrifuged at 40,000rpm for 90 minutes. The supernatant was removed and filter sterilised. Each 1ml of this cytosol contained the cytoplasmic contents of $3.518x10^7$ hepatocytes. For the cell culture there was a total yield of $4.7x10^7$ hepatocytes with a cell viability as assessed by trypan blue exclusion of 82%.

1.5ml of hepatocyte suspension containing 600,000 cells was placed on to each collagen coated cell culture plate and the hepatocytes were cultured as described earlier for rat hepatocytes. The medium was changed every 24 hours and at 0 hours, 24 hours and 48 hours cytosol was added to the test plates according to the following scheme.

control plates	i)	no addition
	ii)	EGF (0.85nmol)
	iii)	TGFβ (10ρmol)
test plates	i)	10μl cytosol
	ii)	10μl cytosol + EGF
	iii)	10μl cytosol + TGFβ
	iv)	60μl cytosol
	v)	60μl cytosol + EGF
	vi)	60μl cytosol + TGFβ

Three plates were included in each of the nine groups.

At 48 hours ^{3}H thymidine (1μcurrie/ml) was added to each plate and the growth stopped at 72 hours. DNA analysis was performed as described in the chapter about rat hepatocyte culture.

Results.

Plates	^{3}H disintegrations / μg DNA 1	2	3	(mean)
C i)	2125	3889	1822	2612
ii) +EGF	4071	4347	4905	4441
iii) +TGFβ	1340	1475	1952	1589
T i) +10c	770	879	561	736
ii) +10c+EGF	3474	2063	864	2134
iii) +10c+TGFβ	105	159	100	121
iv) +60c	500	386	378	421
v) +60c+EGF	808	302	153	421
vi) +60c+TGFβ	107	106	50	88

As can be seen from the above table human hepatocyte cytosol at both the doses used has an inhibitory effect on

DNA synthesis and this effect appears to be dose related with the higher dose producing the greater inhibition. At low dose the cytosol negates the stimulatory effect of EGF and at higher dose cancels all EGF effect completely. The cytosol effect is additive to the inhibitory effect of TGFβ.

The cells in the tissue culture looked different when they had been incubated with cytosol. By 48 hours the low dose cytosol plates (Figs. 45, 46) showed less cell adherence to the plate and the higher dosage cell demonstrated a rounded abnormal appearance (Fig. 47). At 72 hours the lower dose hepatocytes were very unhealthy (Fig. 48) and most of the cells in the higher dosage plates were dead (Fig. 49).

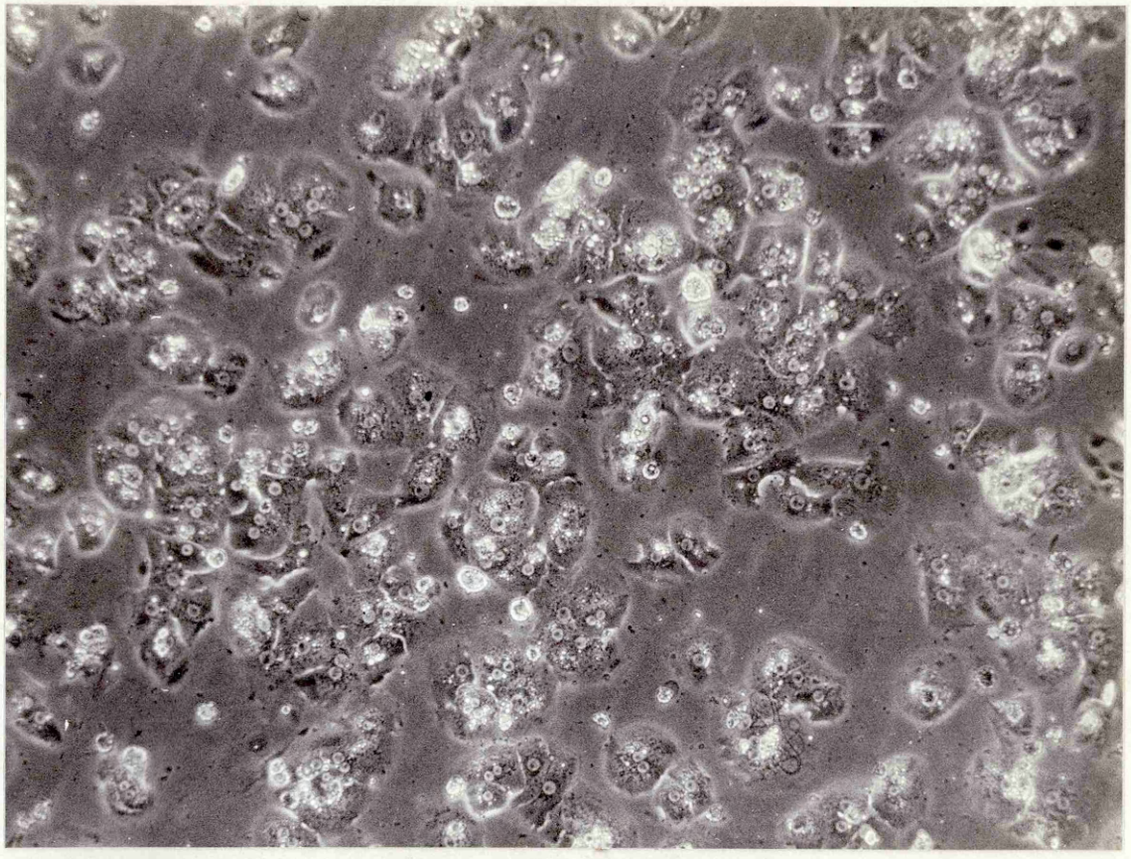

Fig. 45. Human hepatocytes, control

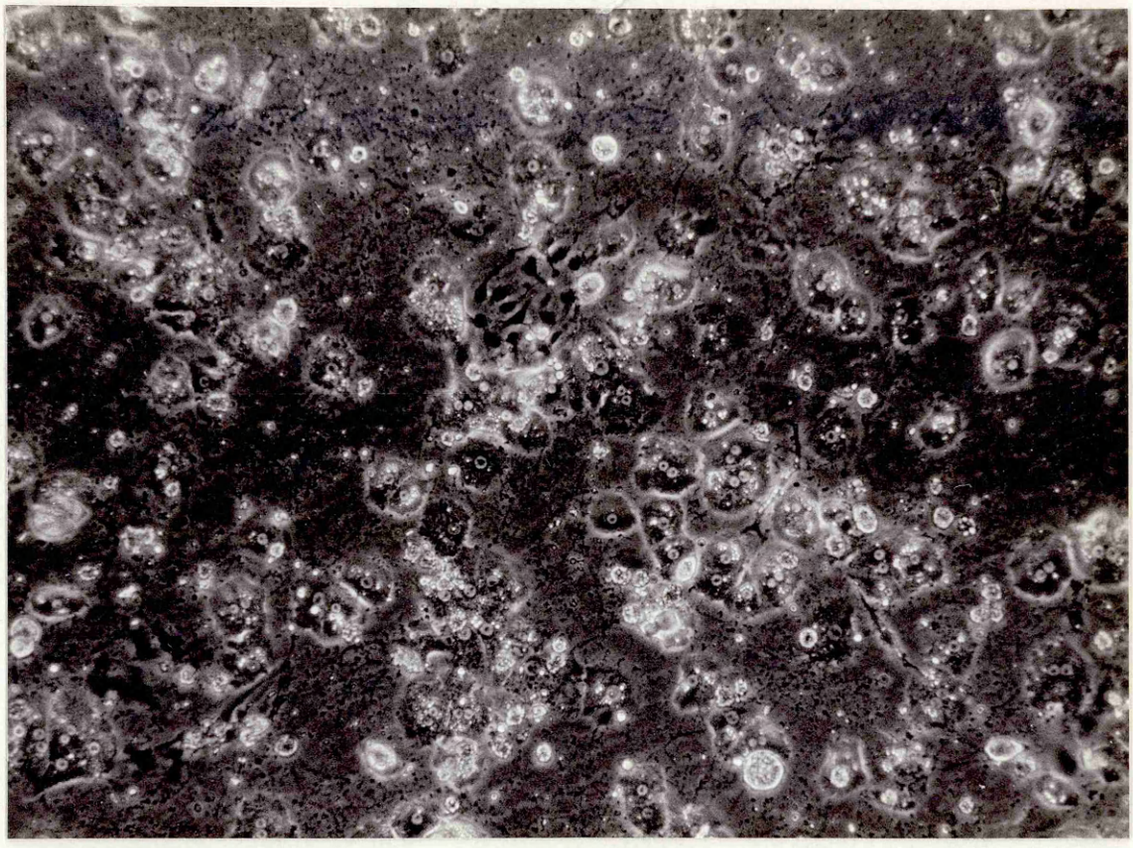

Fig. 46. Human hepatocytes, 30μl cytosol at 48 hours.

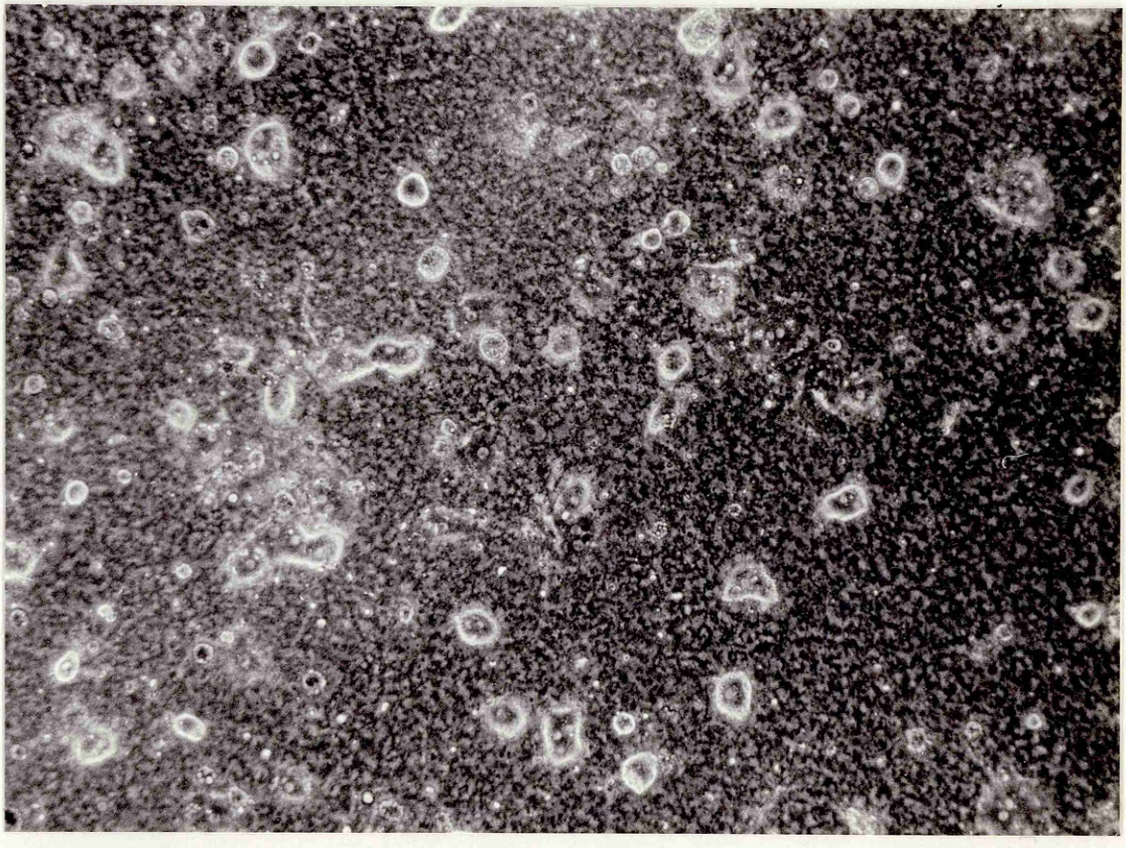

Fig. 47. Human hepatocytes, 60μl cytosol at 48 hours.

Fig. 48. Human hepatocytes, 30μl cytosol at 72 hours.

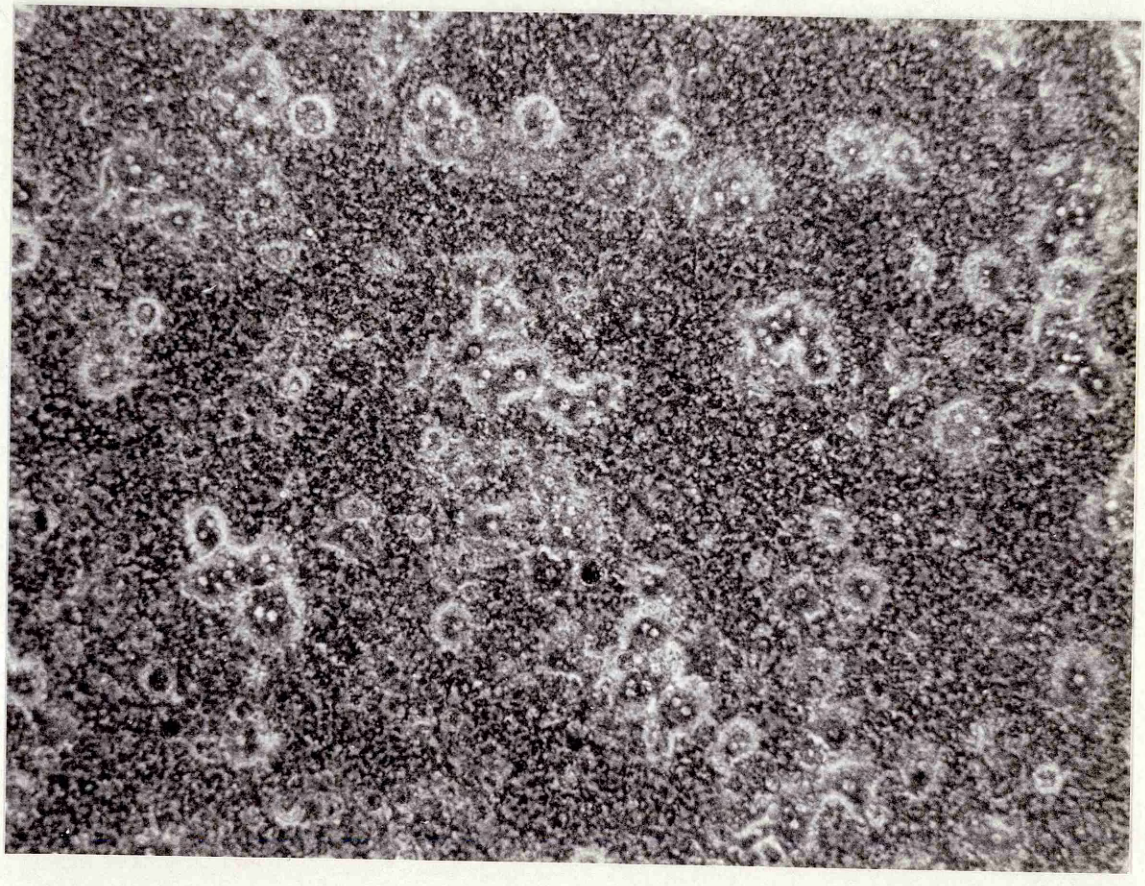

Fig. 49. Human hepatocytes, 60μl cytosol at 72 hours.

Discussion.

The results agree with the findings of Makowka(1981) using cytosol prepared from the liver of a normal adult rat in rat hepatocyte cell culture, when it was postulated that non regenerating liver produces an inhibitory factor to cell growth and division. The concentrations of cytosol used in this experiment are greater than the doses of cytosol given to the intact rats. Here the contents of $3.5x10^5$ and $2.0x10^6$ hepatocytes were given directly to $6x10^5$ cells compared with the contents of $3.0x10^7$ hepatocytes being given to an approximate liver cell population of $1.0x10^9$ cell after distribution throughout the body of the intact rat. Since the inhibitory effect appears to be dose dependant lower doses of cytosol may produce a different result in cell culture studies.

Cytosol does not appear to produce its effect on hepatocytes by encouraging regeneration when directly applied in controlled conditions but it could still promote cell growth and division by acting upon another substance or mechanism in the intact animal. The cytosol effect in the animal could be dependant upon regenerative factors circulating in the rat because of the liver failure or even by negating the effect of the growth inhibitory factors.

In 1983 Makowka demonstrated that although normal rat liver cytosol in rat hepatocyte culture produced an initial inhibition of DNA synthesis there was a significant increase in DNA synthesis at 31-40 hours. In the experiment

above the ^{3}H was given at 48 hours and the uptake measured at 72 hours so it is possible that the timing of the measurements needs adjustment, and that any positive effect of the cytosol was missed.

Human liver cytosol as assessed in this experiment has an inhibitory effect on DNA synthesis and cell regeneration in human hepatocyte culture.

DISCUSSION

Fulminant hepatic failure produces a considerable mortality (85%) despite numerous clinical treatments which have been employed over the last 20 years (Christensen 1984). To date only orthotopic liver transplantation offers a remedy which produces better survival figures than conventional medical therapy (Vickers 1988). Damaged liver is capable of regeneration and there is evidence of liver cell proliferation even in episodes of acute liver failure which prove fatal (Milandri 1980). It is therefore postulated that patients' lives could be saved if a) patients could be kept alive long enough for adequate liver function to recover, b) new liver is implanted or c) attempts are made to increase the rate of liver regeneration.

The clinical study undertaken in an acute liver failure referral unit showed that the largest single group of patients were those who had taken a deliberate overdose of paracetamol. This drug is converted in the liver to an arylating agent which is normally detoxified by conjugation with glutathione. However, if the dose of paracetamol is large enough to deplete the liver stores of glutathione there is extensive arylation of the intrahepatic macromolecules and the hepatocytes die. (Mitchell 1974) Cysteine or more commonly acetylcysteine, if given to patients who have taken enough paracetamol to be at risk of developing liver failure and at a time when it can counteract the paracetamol, is effective therapy in prevention of liver failure. The high incidence of grade IV

hepatic coma seen in the present study does not reflect the picture of paracetamol overdose seen in general medicine since patients had to have evidence of deranged hepatic function before admission to the unit. These patients usually presented late after the ingestion of the drug and were not eligible for acetylcysteine therapy.

The second large group was that of patients with viral hepatitis, most of these were considered to have non A non B hepatitis as a diagnosis of exclusion in patients who appeared to have a viral hepatitis but in whom screening for known viral aetiologies was negative. Hepatitis C serological testing was not available routinely at the time of the study. There were no patients with hepatitis A and this may be because this disease normally is self limiting with full recovery so that patients with this diagnosis were not referred to a specialized unit. Three patients had hepatitis B as the cause for their acute liver failure. The remainder of the patients had an assortment of aetiologies including acute Budd-Chiari syndrome, other drug reactions and post partum liver failure.

The overall mortality rate for patients reaching grade IV coma of 73% was low but reflects that in this series treatment was by medical management and by transplantation, with those patients in what have been reported as the poor prognosis groups being considered for transplantation. Of the patients taking an overdose of paracetamol and reaching grade IV coma the 75% mortality represents a waste of usually young life. Until recently liver transplantation

was not even considered in this group of patients because of the likelihood of further parasuicide attempts (Goldacre 1985). Three of our patients in this group were placed on the list for emergency liver transplantation but only in one was this undertaken because of the nonavailability of donor organs. In this patient who was transplanted the operation was probably performed too late.

In the viral aetiology and other groups, the decision to proceed to transplantation was usually because of continuing deterioration in the patient's clinical state. If a reliable method of identifying at the onset those patients who will not respond to medical management could be found this would help in the decision in whom and when to undertake liver transplantation.

O'Grady (1989) has published a new set of prognostic indicators which takes into account the underlying aetiology and gives an assessment on admission laboratory measurements and patient details. In this study's paracetamol group patients with an arterial blood pH <7.3, prothrombin time greater than 100 seconds and creatinine level above 300 have a poor prognosis. Our results would accord with this but in our patients a high admission bilirubin and being in coma on admission also have a poor prognosis. In O'Grady's study those patients at risk in the viral and other aetiologies group were aged less than 11 years or over 40 years, were jaundiced for more than 7 days before the onset of encephalopathy, had an admission bilirubin greater than 300 and a prothrombin time above 50

seconds. These factors were not significantly different between our survivors and non survivors. This difference may be a reflection of the numbers studied with many more patients over a longer time period in the Kings College Hospital series.

If these factors prove important in the prognosis of patients with fulminant liver failure they will be helpful in selecting patients who should be considered for transplantation. Even if this policy were to be followed there would still be a considerable number of patients who are at risk of dying from acute liver failure in whom transplantation would not be appropriate or for whom a donor liver could not be found. In these groups of patients there is a need for some other form of therapy to temporarily support the patient until the liver recovers.

Despite the reported successes of hepatocyte transplantation in the treatment of acute hepatic failure in laboratory animals (Sutherland 1977), the experiments reported here do not show any benefit to animal survival from hepatocyte transplantation by either the intrasplenic or the intraperitoneal route. This may have been because the model used was very severe with 100% mortality in the control animals. There were 2 survivors in the group of rats treated by intraperitoneal hepatocytes but the numbers studied were too small for any statistical difference to be demonstrated. In the spleens, of the intrasplenically injected rats at death, there were a few hepatocytes seen but there is no possibility that there was enough ectopic

liver tissue to function and keep the rat alive. Even the slightly more plentiful hepatocytes within the portal vein branches within the liver could not have been sufficient to maintain function.

In the rats which received intraperitoneal hepatocytes, hepatocytes were identified histologically in deposits within the peritoneal cavity and these had not been seen in experiments from other workers (Makowka 1980b, Sutherland 1977). This may be because these rats only survived for 72 hours after intraperitoneal injection of hepatocytes and that had the rat lived for longer these cells would have been destroyed. The implication of the presence of these cells and their possible future death could be important if it is accepted that intact hepatocytes are not responsible for improved survival in the animals with acute liver failure.

Since it has been shown by Makowka (1980c) that bone marrow cells are as effective as hepatocytes at treating acute liver failure in the experimental rat and by LaPlante O'Neill (1982) that the supernatant from hepatocyte cultures is equally effective, it is suggested that a substance from regenerating hepatocytes is what is producing the results seen. Further work implies that the substance(s) involved is organ specific but this does not explain the effect seen with bone marrow cells.

In the experiments described here attention was turned to the intracellular contents of the hepatocytes and a crude

preparation of cytosol was produced by an alternative method. All previous work using cytosol had employed the homoginization of pieces of liver and then centrifugation of the homogenate with removal of the supernatant for use. This preparation must contain the same proportion of contents of cells as the cell populations in liver tissue, i.e. approximately less than 70% hepatocytes and the other 30-40% a mixture of Kupffer cells and endothelial cells (Daoust 1958). The cytosol used in the presented experiments was made by centrifugation of a suspension of isolated hepatocytes and therefore contains less than 5% of nonhepatocytes. This may account for some of the differences between the results presented here and other previously published findings.

When cytosol prepared from the hepatocytes of normal adult Wistar rats was injected intraperitoneally into rats which had toxin induced acute liver failure the treated rats had a significant improvement in survival with recovery to near normal liver histology. This finding is contradictory to the work of Schwarz (1985) who has reported that although cytosol from regenerating hepatocytes, either post hepatectomy or weanling livers, produces improved survival, cytosol from normal adult (nonregenerating) liver, if anything inhibits regeneration of hepatocytes, and is detrimental in the treatment of hepatic failure. The other major difference in the methodology of the present experiment is the dosage regime with the cytosol being given by repeated injections. This method of administration resulted in intraperitoneal haemorrhage in 2 of the treated

rats and in 1 of the control group and it was therefore necessary to try a different route of administration which would be without this complication.

Since the cytosol did not contain any cellular particular matter it was thought that it could be given intravenously and for ease of administration a peripheral vein (tail vein) was chosen. If the factor responsible for the improved survival in the intraperitoneally injected rats was dependant on hepatotrophic factors produced within the nonhepatic splanchnic organs as work by Starzl (1980) had suggested, then peripherally administered cytosol might not be effective. The advisability of using this route would depend on whether the substance reached the liver or was metabolised or denatured in the lungs or other organs, whether it reached the liver in sufficient quantity considering the volume of distribution within the whole animal and if it was in fact acting directly upon the liver in the first place.

When the cytosol was given by repeated intravenous injection there was a marked improvement in survival with 9 of the 12 treated rats recovering from acute hepatic failure. However, the mechanism remains unclear. Since Magee's experiments in 1956 showed that all the DMNA was removed from the rat by 4 hours after dosage, it is unlikely that the cytosol is mopping up the hepatotoxin since the first dose is given 6 hours after the induction of liver failure. Chemicals within the cytosol could be supporting the liver and reducing the amount of toxins

which the liver is unable to metabolise circulating in the animal. This seems unlikely since this function should require intact functioning cells. It is more likely that a component of the cytosol stimulates regeneration of the remaining hepatocytes. This does not imply that it is acting directly upon the hepatic tissue but it could be producing its effect through another tissue or a hepatotrophic factor.

When the first dose of cytosol was given at 12 and 24 hours but not 30 hours after the induction of hepatic damage it was still effective at improving survival. If the recovery of the animals is due to stimulation of regeneration then it must be happening within the first 40 hours and initiated before 30 hours after the administration of DMNA.

The dosage of cytosol originally chosen was based on the number of hepatocytes usually transplanted ($3-4 \times 10^7$ cells) (Sommer 1979) and so each dose of cytosol was equivalent to the contents of that number of cells. The cytosol was however thought to be going into a much larger volume of distribution. Different doses of cytosol were tried and the two lower doses showed no difference from the original dose in respect of rat survival.

To try to show if the rat recovery was due to regeneration rats were paired, one to receive cytosol and one saline, and when one of the pair died the other was sacrificed. All the rats died or were killed at 72 hours, and there was no significant difference in amount of hepatocyte necrosis but

there was a significantly increased number of cells undergoing division in the cytosol treated rats. This suggested that cytosol was stimulating the hepatocytes to regenerate but the mechanism causing the increased regeneration and improved survival was not apparent.

Rat fresh frozen plasma did not improve the survival more than in the control group but neither was there a statistically proven difference between the survivals in the plasma and the cytosol treated groups. It is possible that if the liver is producing a substance which is capable of stimulating regeneration that this product could be secreted into the blood and be present in rat plasma. Alternatively the protein in the plasma may be beneficial in a similar way to the administration of fresh frozen plasma in humans. The histological findings of increased cell division in the paired experiment would suggest however that the cytosol is doing more than just maintaining intravascular volume. If the number of rats had been larger in the plasma experiment comparing the results to cytosol and control groups the result might have been statistically different.

When cytosol prepared in the same way from human hepatocytes was administered in a similar manner to the rat model it produced a significant improvement in rat survival and its effect was the same as that seen for rat cytosol. The component of the cytosol which is producing this effect is therefore not species specific but is certainly present in both rat and human hepatocyte cytosol. Terblanche in

1980 demonstrated that cytosol from regenerating dog liver was also effective at increasing liver regeneration.

The cytosol had been studied as a single entity but it is obviously a mixture of chemicals, mainly proteins, intracellular electrolytes with some electrolytes from the Hank's balanced salt solution in which the cells were suspended, and intracellular organelles. Attempts were made to try to characterise the active substance within the cytosol. Cytosol was heated at different temperatures for different times and none of the heated cytosols was effective at treating the acute liver failure in the animal model. The active component is therefore heat labile and could suggest that it is a peptide. When cytosol was dialysed across a small pore membrane against HBSS so that components from the HBSS would be retained but small molecules (smaller than 8,000 molecular weight) derived from the hepatocytes would be removed the modified cytosol was ineffective at treating acute hepatic failure. These findings suggested that the component of the crude cytosol which was responsible for the improvement in survival of the rat was a molecule of a size below 8,000 daltons and possibly below 6,000 daltons allowing for variations in the membrane.

To look for regeneration as shown by increased DNA synthesis the effect of cytosol added to rat hepatocytes in tissue culture was studied. The results of this series of experiments were difficult to interpret and this may be because of the different concentrations of cytosol used.

The cells were all cultured under identical conditions and other than the timing variation introduced into the third experiment a standard method was used throughout. The overall suggestion was that the crude cytosol did not increase DNA synthesis and in some instances was either inhibitory to cell regeneration or was causing cell toxicity and death. The culture plates receiving cytosol showed a difference in the appearance of the cells with the hepatocytes appearing more spherical rather than the flattened appearance seen in the control plates. Undoubtedly some change was happening in the hepatocytes and they were not showing increased regeneration.

These tissue culture experiments might suggest that the effect of cytosol administered to the animal model was not brought about by increasing hepatocyte regeneration or it could be that the cytosol could only cause an increase in regeneration by synergism with or by acting upon another substance. The results in tissue culture did not suggest synergism with epidermal growth factor which is a known hepatotrophic growth factor. The cells in the tissue culture plates should be more than 95% pure hepatocytes whereas in the animal model the cytosol was circulating throughout the animal. This raises the possibility that the mechanism of action may be indirect through other tissues and the product of this interaction could then be stimulatory to the liver.

Previous work by Starzl (1980), mainly in the dog, suggested that the liver could only be stimulated in the

presence of hepatotrophic factors derived from the nonhepatic splanchnic organs. In the dog with an Eck fistula if the extrahepatic organs including the pancreas and the spleen were removed the liver failed to regenerate. This was at the time attributed to substances from the pancreas (insulin and glucagon) being involved. This may be true but in the light of recent work with immunosuppressant agents it may have been an interaction with the reticuloendothelial system that is important.

Both cyclosporin (Kim 1988) and the new immunosuppressant FK506 (Francavilla 1989) have been shown to increase DNA synthesis in hepatocytes. The mechanism for this effect is as yet unknown but it has been suggested that the regeneration may be controlled or modified immunologically. It has been suggested that in normal situations the immune system limits cell growth but that this restricting effect is dependant on T cells whose function is inhibited by both FK506 and cyclosporin. It is possible that any immunological effect upon regeneration may also be operated by cytokines since both of the immunosuppressive drugs mentioned inhibit interleukin 2 production and function. Any involvement of the action of cytosol with the immune system is purely supposition but it would explain the apparent discrepancy between the in-vivo results reported here and those obtained from the in-vitro work.

Immunological stimulation could explain why the bone marrow cells transplanted by Makowka (1980c) were effective at treating rats with acute liver failure. Other workers

reporting studies looking at liver regeneration consider the identified hepatotrophic factors to be organ specific and site as evidence the fact that liver cytosol does not produce proliferation in the kidney (Starzl 1980). A hepatolymphatic relationship in the control of liver regeneration was suggested by Sakai (1975) when he postulated that an observed breakdown of lymphocytes 7-8 hours after partial hepatectomy might release a substance which was stimulatory to hepatocytes and lymphocytes. He showed also that partial hepatectomy reduces the recruitment of thymus cells but increases the number of B lymphocytes. Ohkawa in 1985 showed that regenerating rat liver cytosol improves reticuloendothelial function before it increases DNA synthesis supporting an immunologically mediated stimulus to liver regeneration.

Many substances have been suggested as hepatotrophic factors, some of which have been discounted but many have been identified, physically characterised, purified and the structure worked out. Proven factors stimulating liver regeneration include epidermal growth factor (EGF) (Cohen 1974), hepatic stimulator substance (HSS) (LaBrecque 1985, 1987, Francavilla 1988a), hepatocyte proliferation factor (HPF) (Schwarz 1985), hepatocyte proliferative stimulatory factor (HPSF) (Suemori 1988), transforming growth factor alpha (TGFα) (Fausto 1989, Tappin 1989), hepatopoietin A and hepatopoietin B (Michalopoulos 1984), liver growth factor (Diaz-Gil 1987), platelet associated factors (Strain 1981), insulin and glucagon (Leffert 1980), oestrogen (Francavilla 1988b) and prolactin (Buckley 1988). Amongst

these factors there is a marked similarity in size and three dimensional structure. Although some of them were originally described as larger molecules they have now been broken down and are now usually in the size range of 4,000 to 8,000 daltons with the majority having a molecular weight of about 6,000. Some of these purified factors have been given in animal models of acute hepatic failure and have improved the survival of the animals (Francavilla 1986).

SUMMARY

Although liver transplantation has improved the survival from fulminant hepatic failure for selected patients it is unfortunately a remedy which is not appropriate or possible for all patients suffering from this disease. It also involves difficult surgery in a critically ill patient and commits the survivor to a life time of immunosuppression with all its inherent morbidity. Alternative methods of supporting the failed liver or increasing its rate of regeneration require to be found.

In the series of experiments presented in this thesis it has been found that a component of normal adult cytosol prepared from a suspension of isolated hepatocytes administered by repeated intravenous injections is able to improve the survival of rats with an otherwise fatal toxin induced hepatic failure. The active substance is present in both rat and human liver cytosol and is demonstrated to be a heat labile molecule with a molecular weight below 8,000. This substance gave results better than those obtained for hepatocyte transplantation in this model of liver failure. It seemed to increase hepatocyte regeneration in the animal model but appeared to have no direct effect on liver regeneration when applied to hepatocytes in cell culture. If this technique of intravenous administration of normal hepatocyte cytosol could be applied to humans it might improve the prognosis for some patients with fulminant liver failure.

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APPENDIX

RAT USAGE

Rat No.	Procedure
1.	Blood sampling, liver and spleen histology.
2.	Blood sampling, unsuccessful liver perfusion.
3.	Hepatocyte isolation.
4.	Hepatocyte isolation.
5.	Hepatocyte isolation.
6.	Hepatocyte isolation.
7.	Hepatocyte isolation.
8.	Induction of acute hepatic failure, DMNA 40mg/kg.
9.	Induction of acute hepatic failure, DMNA 40mg/kg.
10.	Induction of acute hepatic failure, DMNA 40mg/kg.
11.	Hepatocyte isolation.
12.	Induction of acute hepatic failure, DMNA 50mg/kg.
13.	Induction of acute hepatic failure, DMNA 50mg/kg.
14.	Induction of acute hepatic failure, DMNA 50mg/kg.
15.	Induction of acute hepatic failure, DMNA 45mg/kg.
16.	Induction of acute hepatic failure, DMNA 45mg/kg.
17.	Induction of acute hepatic failure, DMNA 45mg/kg.
18.	Hepatocyte isolation.
19.	Induction of acute hepatic failure, DMNA 50mg/kg.
20.	Induction of acute hepatic failure, DMNA 50mg/kg.
21.	Induction of acute hepatic failure, DMNA 50mg/kg.
22.	Induction of acute hepatic failure, DMNA 50mg/kg.
23.	Induction of acute hepatic failure, DMNA 50mg/kg.
23(a)	Hepatocyte isolation.
24.	Acute hepatic failure, intrasplenic hepatocytes.
25.	Acute hepatic failure, intrasplenic hepatocytes.
26.	Died before hepatocytes given - discounted.
27.	Hepatocyte isolation.

28. Acute hepatic failure, intrasplenic hepatocytes.
29. Acute hepatic failure, intrasplenic hepatocytes.
30. Acute hepatic failure, intrasplenic hepatocytes.
31. Acute hepatic failure, intrasplenic hepatocytes.
32. Acute hepatic failure, intrasplenic hepatocytes.
33. Acute hepatic failure, intrasplenic hepatocytes.
34. Acute hepatic failure, intrasplenic hepatocytes.
35. Hepatocyte isolation.
36. Hepatocyte isolation.
37. Intrasplenic hepatocytes.
38. Intrasplenic hepatocytes.
39. Intrasplenic hepatocytes.
40. Intrasplenic hepatocytes.
41. Intrasplenic hepatocytes.
42. Acute hepatic failure, intrasplenic HBSS.
43. Acute hepatic failure, intrasplenic HBSS.
44. Acute hepatic failure, intrasplenic HBSS.
45. Acute hepatic failure, intrasplenic HBSS.
46. Acute hepatic failure, intrasplenic HBSS.
47. Acute hepatic failure, IP hepatocytes
48. Acute hepatic failure, IP hepatocytes
49. Acute hepatic failure, IP hepatocytes
50. Acute hepatic failure, IP hepatocytes
51. Acute hepatic failure, IP hepatocytes
52. Hepatocyte isolation.
53. Acute hepatic failure, IP hepatocytes
54. Acute hepatic failure, IP hepatocytes
55. Acute hepatic failure, IP hepatocytes
56. Acute hepatic failure, IP hepatocytes
57. Acute hepatic failure, IP hepatocytes

58. Hepatocyte isolation.
59. Acute hepatic failure, IP hepatocytes
60. Acute hepatic failure, IP hepatocytes
61. Acute hepatic failure, IP hepatocytes
62. Acute hepatic failure, IP hepatocytes
63. Acute hepatic failure, IP hepatocytes
64. Hepatocyte isolation.
65. Acute hepatic failure, IP hepatocytes
66. Acute hepatic failure, IP hepatocytes
67. Acute hepatic failure, IP hepatocytes
68. Acute hepatic failure, IP hepatocytes
69. Acute hepatic failure, IP hepatocytes
70. Hepatocyte isolation.
71. Hepatocyte isolation, cytosol production.
72. Hepatocyte isolation, cytosol production.
73. Acute hepatic failure, intraperitoneal cytosol.
74. Acute hepatic failure, intraperitoneal cytosol.
75. Acute hepatic failure, intraperitoneal cytosol.
76. Acute hepatic failure, intraperitoneal cytosol.
77. Acute hepatic failure, intraperitoneal cytosol.
78. Acute hepatic failure, intraperitoneal cytosol.
79. Acute hepatic failure.
80. Acute hepatic failure.
81. Acute hepatic failure, intraperitoneal cytosol.
82. Acute hepatic failure, intraperitoneal cytosol.
83. Acute hepatic failure, intraperitoneal cytosol.
84. Acute hepatic failure, intraperitoneal cytosol.
85. Hepatocyte isolation, cytosol production.
86. Acute hepatic failure.
87. Acute hepatic failure.

88. Acute hepatic failure, intravenous cytosol.

89. Acute hepatic failure, intravenous cytosol.

90. Acute hepatic failure, intravenous cytosol.

91. Acute hepatic failure, intravenous cytosol.

92. Hepatocyte isolation, cytosol production.

93. Hepatocyte isolation, cytosol production.

94. Hepatocyte isolation, cytosol production.

95. Acute hepatic failure, intravenous cytosol.

96. Acute hepatic failure, intravenous cytosol.

97. Acute hepatic failure, intravenous cytosol.

98. Acute hepatic failure, intravenous cytosol.

99. Acute hepatic failure, intravenous cytosol.

100. Acute hepatic failure, intravenous cytosol.

101. Acute hepatic failure, intravenous cytosol

102. Acute hepatic failure, intravenous cytosol.

103. Acute hepatic failure, intravenous cytosol.

104. Acute hepatic failure, intravenous cytosol 12hr.

105. Acute hepatic failure, intravenous cytosol 12hr.

106. Acute hepatic failure, intravenous cytosol 12hr.

107. Acute hepatic failure, intravenous cytosol 6hr.

108. Acute hepatic failure, intravenous cytosol 6hr.

109. Acute hepatic failure, intravenous cytosol 6hr.

110. Acute hepatic failure, intravenous cytosol 24hr.

111. Acute hepatic failure, intravenous cytosol 24hr.

112. Acute hepatic failure, intravenous cytosol 24hr.

113. Hepatocyte isolation, cytosol production.

114. Hepatocyte isolation, cytosol production.

115. Hepatocyte isolation, heated cytosol production.

115(a) Heated cytosol production.

116. Acute hepatic failure.

117. Acute hepatic failure.

118. Acute hepatic failure, IV heated cytosol.

119. Acute hepatic failure, IV heated cytosol.

120. Acute hepatic failure, IV heated cytosol.

121. Acute hepatic failure, intravenous cytosol.

122. Acute hepatic failure, intravenous cytosol.

123. Acute hepatic failure, intravenous cytosol.

124. Acute hepatic failure, intravenous cytosol.

125. Acute hepatic failure, intravenous cytosol.

126. Hepatocyte isolation, dialysed cytosol.

127. Hepatocyte isolation, heated cytosol production.

127(a) Heated cytosol production.

128. Acute hepatic failure, IV dialysed cytosol.

129. Acute hepatic failure, IV dialysed cytosol.

130. Acute hepatic failure, IV dialysed cytosol.

131. Acute hepatic failure, IV dialysed cytosol.

132. Acute hepatic failure, IV dialysed cytosol.

133. Acute hepatic failure, IV dialysed cytosol.

134. Acute hepatic failure, IV heated cytosol.

135. Acute hepatic failure, IV heated cytosol.

136. Acute hepatic failure, IV heated cytosol.

137. Acute hepatic failure, IV heated cytosol.

138. Acute hepatic failure, IV heated cytosol.

139. Acute hepatic failure, IV heated cytosol.

140. Acute hepatic failure.

141. Acute hepatic failure, IV cytosol 24 hrs.

142. Acute hepatic failure, IV cytosol 24 hrs.

143. Acute hepatic failure, IV cytosol 24 hrs.

144. Acute hepatic failure, IV cytosol 24 hrs.

145. Acute hepatic failure, IV cytosol 24 hrs.

146. Acute hepatic failure.

147. Acute hepatic failure, IV cytosol 30 hrs.

148. Acute hepatic failure, IV cytosol 30 hrs.

149. Acute hepatic failure, IV cytosol 30 hrs.

150. Acute hepatic failure, IV cytosol 30 hrs.

151. Acute hepatic failure, IV cytosol 30 hrs.

152. Plasma collection.

153. Plasma collection.

154. Plasma collection.

155. Acute hepatic failure.

156. Acute hepatic failure.

157. Acute hepatic failure, intravenous human cytosol.

158. Acute hepatic failure, intravenous human cytosol.

159. Acute hepatic failure, intravenous human cytosol.

160. Acute hepatic failure, intravenous human cytosol.

161. Acute hepatic failure, intravenous plasma.

162. Acute hepatic failure, intravenous plasma.

163. Acute hepatic failure, intravenous plasma.

164. Acute hepatic failure, intravenous plasma.

165. Acute hepatic failure, intraperitoneal saline.

166. Acute hepatic failure, intraperitoneal saline.

167. Acute hepatic failure, intraperitoneal saline.

168. Acute hepatic failure, intraperitoneal saline.

169. Acute hepatic failure, IV saline, 24hr sacrifice.

170. Acute hepatic failure, intravenous saline.

171. Acute hepatic failure, intravenous cytosol.

172. Acute hepatic failure, intravenous saline.

173. Acute hepatic failure, intravenous cytosol.

174. Acute hepatic failure, intravenous saline.

175. Acute hepatic failure, intravenous cytosol.

176. Acute hepatic failure, intravenous saline.

177. Acute hepatic failure, intravenous cytosol.

178. Acute hepatic failure, intraperitoneal HBSS.

179. Acute hepatic failure, intraperitoneal HBSS.

180. Acute hepatic failure, intraperitoneal HBSS.

181. Acute hepatic failure, intraperitoneal HBSS.

182. Acute hepatic failure, IV saline, 30hr sacrifice.

183. Hepatocyte isolation.

HEPATOCYTE ISOLATION

Rat No	H/C	Viability	Cells/ml	Mls	Tot.cells	Cytosols
3	H	72%	$7.2x10^6$	20	$1.42x10^8$	
4	H	80%	$1.63x10^7$	20	$3.26x10^8$	
5	unsuccessful					
6	H	92%			$4.9x10^8$	
7	H	82%	$1.12x10^7$	25	$2.8x10^8$	
11	H	75%	$1.64x10^7$	25	$4.1x10^8$	
18	H	89%	$2.56x10^7$	30	$7.68x10^8$	
23a	H	85%	$3.06x10^7$	20	$6.12x10^8$	
27	H	90%	$2.78x10^7$	20	$5.56x10^8$	
35	H	74%	$3.15x10^7$	20	$6.3x10^8$	
36	H	68%	$2.86x10^7$	20	$5.72x10^8$	
52	H	83%	$3.94x10^7$	20	$7.88x10^8$	
58	H	84%	$4.12x10^7$	20	$8.24x10^8$	
64	H	78%	$4.42x10^7$	20	$8.84x10^8$	
70	H	84%	$6.15x10^7$	20	$1.23x10^9$	
71	C	84%	$4.85x10^6$	20	$9.7x10^7$	$5.7x10^6$
72	C	87%	$2.40x10^7$	42	$1.0x10^9$	$3.06x10^7$
85	C	84%	$3.59x10^7$	20	$7.18x10^8$	$3.34x10^7$
92	C	88%	$3.28x10^7$	25	$8.2x10^8$	$3.42x10^7$
93	C	89%	$2.55x10^7$	20	$5.1x10^8$	$1.02x10^7$
94	C	93%	$3.25x10^7$	45	$1.46x10^9$	$1.9x10^7$
113	C	89%	$3.36x10^7$	25	$8.4x10^8$	$3.36x10^7$
114	C	88%	$2.05x10^7$	37	$7.6x10^8$	$3.09x10^7$
115	C	80%	$1.54x10^7$	20	$3.09x10^8$	$3.09x10^7$
115a	C	88%	$9.7x10^6$	15	$1.45x10^8$	$2.9x10^7$
126	C	88%	$2.95x10^7$	35	$1.03x10^9$	$3.43x10^7$
127	C	89%	$2.08x10^7$	30	$6.22x10^8$	$3.11x10^7$
127a	C	91%	$4.5x10^6$	100	$4.5x10^8$	$4.5x10^7$

SURVIVAL AND PERCENTAGE OF LIVER NECROSIS

Rat no.	survival (hrs)	% hepatocyte necrosis centre	periphery
DMNA 40 mg/kg			
8	sacrifice	0	0
9	48	81	39
10	sacrifice	0	0
DMNA 50 mg/kg			
12	48	97	36
13	48	88	53
14	48	93	29
DMNA 45 mg/kg			
15	sacrifice	0	0
16	122	49	49
17	sacrifice	0	0
DMNA 50 mg/kg			
19	41	95	73
20	43	90	75
21	42	90	55
22	42	79	56
23	43	79	53
AHF + IS hepatocytes			
24	48	77	49
25	48	62	54
26	6		
28	76	59	43
29	7	0	0
30	49	90	65
31	65	60	70
32	45	75	57
33	60	91	69
34	60	72	75
noAHF, IS hepatocytes			
37	sacrifice	0	0
38	sacrifice	0	0
39	sacrifice	0	0
40	sacrifice	0	0
41	sacrifice	0	0
AHF + IS sham injection			
42	48	80	76
43	48	73	60
44	24		
45	48	75	60
46	48	92	76

Rat no.	survival (hrs)	% hepatocyte necrosis	
		centre	periphery
AHF + IP hepatocytes			
47	48	78	66
48	216	54	42
49	60	67	69
50	150	89	62
51	70	69	58
53	62	75	69
54	62	91	72.
55	71	70	57
56	48	87	77
57	48	82	63
59	48	78	62
60	48	77	51
61	84	72	71
62	84	77	46
63	63	79	48
65	70	77	63
66	54	65	53
67	39	62	45
68	44	61	51
69	46	72	64
AHF + IP cytosol (71) daily			
73	73	68	40
74	100	62	61
AHF + IP cytosol (72) daily			
75	100	72	37
76	48	59	62
AHF + IP cytosol (72) every 2nd day			
77	sacrifice	0	0
78	48	86	81
81	72	75	55
82	48	79	64
AHF + IP cytosol (72) every 3rd day			
83	72	60	46
84	54	76	50
AHF controls			
79	72	62	33
80	95	55	48
86	72	74	51
87	90	40	25
116	72	64	55
117	48	87	44
155	72	60	34
156	72	70	52
AHF + IV cytosol (85) daily			
88	sacrifice	0	0
89	sacrifice	0	0
90	sacrifice	0	0
91	sacrifice	0	0

Rat no.	survival (hrs)	% hepatocyte necrosis	
		centre	periphery
AHF + IV cytosol (93) daily			
95	sacrifice	0	0
96	120	60	39
97	sacrifice	0	0
AHF + IV cytosol (94) daily			
98	48	65	62
99	sacrifice	0	0
100	sacrifice	0	0
AHF + IV cytosol (92) daily			
101	sacrifice	0	0
102	72	60	60
103	sacrifice	0	0
AHF + IV cytosol (94) daily after 12 hrs			
104	sacrifice	0	0
105	sacrifice	0	0
106	sacrifice	0	0
AHF + IV cytosol (94) daily after 6 hrs			
107	216	0	0
108	sacrifice	0	0
109	72	75	64
AHF + IV cytosol (94) daily after 24 hrs			
110	72	39	54
111	sacrifice	0	0
112	sacrifice	0	0
AHF controls			
116	72	64	55
117	48	87	44
AHF + IV heat treated cytosol daily			
118	140	58	43
119	102	79	47
120	96	78	58
AHF + IV cytosol (113) daily			
121	sacrificed	0	0
122	sacrificed	0	0
123	48	58	48
124	sacrificed	0	0
125	116	38	30
AHF + IV dialysed cytosol (126) daily			
128	65	66	56
129	90	68	66
130	70	65	25
131	60	73	49
132	90	58	56
133	60	52	28

Rat no.	survival (hrs)	% hepatocyte necrosis centre	periphery
AHF + IV heated cytosol (127,127a) daily			
134	56	50	52
135	60	78	80
136	96	49	38
137	65	72	32
138	120	62	38
139	75	48	42
AHF + IV cytosol (various) after 24 hrs and daily			
141	48	78	70
142	sacrificed	0	0
143	140	19	8
144	55	62	72
145	60	54	51
AHF + IV cytosol (various) after 30 hrs and daily			
147	31	79	71
148	40	80	69
149	48	78	74
150	81	64	56
151	40	76	68
AHF + IV saline at 24 and 30 hours			
140	40	81	65
146	30	58	54
AHF + IV human cytosol daily			
157	sacrificed	0	0
158	sacrificed	0	0
159	75	55	50
160	116	68	27
AHF + IV plasma daily			
161	sacrificed	0	0
162	44	76	74
163	sacrificed	0	0
164	44	75	64
AHF + IP saline daily			
165	48	70	54
166	48	67	58
167	44	82	84
168	44	56	66
AHF + IV saline and sacrifice			
169	24	63	57
182	30	62	52
AHF + IV saline daily			
170	72	55	47
172	72	78	48
174	68	69	52
176	72	70	60

Rat no.	survival (hrs)	% hepatocyte necrosis	
		centre	periphery
AHF + IV cytosol (various) daily and sacrifice			
171	72s	59	45
173	72s	44	54
175	72s	47	35
177	72	76	48
AHF + IP Hank's single			
178	53	82	62
179	57	70	69
180	44	70	58
181	72	52	69

MITOTIC INDICES

AHF + IV saline and death

Rat no.	mitotic figures	total no. of nuclei
170	8	143
	14	128
	10	95
	9	97
172	14	129
	7	122
	4	105
	9	113
174	19	162
	18	162
	16	163
	16	134
176	9	69
	17	145
	15	162
	12	112

AHF + IV cytosol and sacrifice

Rat no.	mitotic figures	total no. of nuclei
171	18	112
	14	101
	13	105
	18	110
173	13	107
	11	100
	13	83
	11	103
175	21	151
	10	95
	10	84
	12	83
177	19	146
	20	112
	16	120
	16	128

NORMOTEST RESULTS

DMNA 40 mg/kg

rat no.	8	9	10
hours 0	50%	43%	44%
6	38%	35%	42%
24	21%	22%	21%
48	7%	18%	12%
72	31%		31%
96	35%		47%
120	31%		35%
144	30%		39%
192	42%		47%
244			70%
292			35%
316			50%

DMNA 50 mg/kg

rat no.	12	13	14
hours 0	50%	45%	54%
16	23%	16%	23%
28		<5%	<5%

DMNA 45 mg/kg

rat no.	15	16	17
hours 0	41%	46%	46%
23	24%	10%	<5%
33	<5%	<5%	<5%
48		<5%	<5%
72	<5%	5%	<5%
105	62%	22%	34%
124	45%		50%
151	54%		50%

DMNA 50mg/kg

rat no.	19	20	21	22	23
hours 0	42%	46%	46%	42%	44%
18	6%	10%	7%	12%	18%
25		<5%	<10%	<5%	<10%
42		<5%	<5%	<5%	<5%

AHF + IS hepatocytes

rat no.	24	25	28	29
hours 0	37%	29%	46%	64%
6	27%	29%	27%	70%
24	<5%	<5%	9%	
30	<10%		<5%	
48	<5%	<5%	<5%	
56			<5%	
72			<10%	

rat no.	30	31	32	33	34
hours 0	35%	50%	46%	54%	70%
6	<5%	47%	20%	33%	<5%
21	<5%	<5%	<5%	14%	<5%
43	<5%	<5%	<5%		
49	<5%				

noAHF, IS hepatocytes

rat no.	37	38	39	40	41
hours 0	34%	38%	50%	45%	38%
20	30%	35%	25%	27%	45%
45	32%	50%	40%	100%	64%
68	54%	31%	40%	41%	34%
121	34%	50%	34%	34%	27%
164	31%	76%	86%	50%	50%
210	50%	58%	64%		64%
259	58%		64%		76%
308			46%		45%
356					46%

AHF + IS sham injection

rat no.	42	43	44	45	46
hours 0	54%	86%	45%	54%	63%
6	70%	50%	54%	78%	63%
24	31%	6%		9%	<5%

AHF + IP hepatocytes

rat no.	47	48	49	50	51
hours 0	31%	39%	54%	39%	76%
6	41%	58%	54%	56%	54%
30	<5%	9%	<5%	7%	<5%
48		<5%	<10%	<5%	<10%
72		6%		<5%	
150		47%		41%	
216		62%			

rat no.	53	54	55	56	57
hours 0	37%	45%	32%	42%	46%
6	47%	50%	32%	64%	34%
25		<10%	<10%	<10%	<10%
48	<10%		<10%	<5%	<5%

rat no.	59	60	61	62	63
hours 0	53%	50%	39%	60%	60%
6	41%	34%	46%	46%	37%
24	<10%	7%	10%	10%	5%
48	<5%	<5%	<10%	<5%	<10%

rat no.	65	66	67	68	69
hours 0	36%	58%	42%	70%	39%
6	53%	20%	31%	25%	34%
24	9%	<10%			<5%

AHF + IP cytosol (71) daily

rat no.	73	74
hours 0	31%	41%
6	70%	50%
24	9%	10%
72	<10%	<10%
96		<10%

AHF + IP cytosol (72) daily

rat no.	75	76
hours 0	30%	29%
6	21%	35%
24	7%	9%
48	10%	

AHF + IP cytosol (72) every 2nd day

rat no.	77	78	81	82
hours 0	35%	33%	34%	41%
6	35%	32%	35%	14%
24	8%	<5%	5%	<10%
48	5%		<10%	<5%
72	<5%			
96	13%			
148	22%			
194	14%			
240	70%			
336	39%			

AHF + IP cytosol (72) every 3rd day

rat no.	83	84
hours 0	76%	46%
6	58%	24%
25	<5%	8%
48	<10%	<10%

AHF control

rat no.	79	80	86	87
hours 0	35%	47%	44%	63%
6	31%	10%	28%	31%
24	<10%	<10%	<10%	14%
48	<10%	<10%	<10%	13%

AHF + IV cytosol (85) daily

rats no.	88	89	90	91
hours 0	53%	44%	46%	58%
6	35%	44%	35%	39%
24	9%	24%	21%	18%
48	9%	<10%	<5%	<10%
72	24%	21%	8%	15%
100	24%	16%	24%	29%
144	37%	41%	41%	47%
196	39%	41%	41%	27%
630	64%	70%	76%	76%

AHF + IV cytosol (93) daily

rat no.	95	96	97
hours 0	31%	37%	44%
6	41%	50%	44%
24	12%	18%	16%
48			9%
96	35%	<5%	44%
120	44%		44%
168	35%		32%
533	50%		44%

AHF + IV cytosol (94) daily

rat no.	98	99	100
hours 0	41%	39%	41%
6	12%	35%	47%
24	11%	13%	18%
48		7%	7%
72		20%	26%
96		35%	46%
120		35%	39%
168		44%	39%
533		50%	50%

AHF + IV cytosol (92) daily

rat no.	101	102	103
hours 0	41%	44%	32%
6	44%	50%	30%
24	16%	14%	16%
48	14%	<5%	9%
72	25%		18%
96	41%		41%
120	44%		37%
168	50%		39%
533	47%		44%

AHF + IV cytosol (94) daily after 12 hrs

rat no.	104	105	106
hours 0	34%	22%	30%
12	18%	<5%	17%
24	<5%	6%	<5%
48	21%	<5%	<5%
72	30%		39%
96	20%	5%	28%
144	39%	31%	26%
216	96%	44%	47%
365	54%	50%	50%

AHF + IV cytosol (94) daily after 6 hrs

rat no.	107	108	109
hours 0	41%	37%	29%
6	25%	41%	50%
24	<5%	<5%	<5%
48	<5%	<5%	
72	12%		
96	37%	31%	
144	44%	32%	
216	47%	70%	
365		70%	

AHF + IV cytosol (94) daily after 24 hrs

rat no.	110	111	112
hours 0	50%	47%	58%
24	<5%	<5%	<5%
48	29%	8%	
72	53%	39%	
96		34%	41%
144		44%	39%
216		47%	47%
365		54%	58%

AHF control

rat no.	116	117
hours 0	35%	43%
6	25%	27%
24	<5%	<5%
48		<5%

AHF + IV heat treated cytosol daily

rat no.	118	119	120
hours 0	37%	46%	60%
6	22%	23%	33%
24	<5%	<5%	<5%
48	<5%	<5%	
72	10%		
102	23%	5%	

AHF + IV cytosol (113) daily

rat no.	121	122	123	124	125
hours 0	48%	46%	43%	31%	48%
6	25%	27%	31%	45%	30%
24	<5%	14%	<5%	<5%	6%
48	<5%	<5%			<10%
72	18%	6%		<5%	18%
102	40%	9%		26%	
144	18%	20%		30%	
192	20%	20%		18%	
365	51%	48%		48%	

AHF + IV dialysed cytosol (126) daily

rat no.	128	129	130	131	132	133
hours 0	29%	20%	43%	26%	39%	33%
6	45%	34%	43%	43%	23%	11%
24	6%		<5%	9%		11%
48						<5%
72					<5%	

AHF + IV heated cytosol (127,127a) daily

rat no.	134	135	136	137	138	139
hours 0	29%	36%	43%	36%	29%	25%
6	34%	39%	54%	37%	43%	37%
24	<10%	<5%	8%	<5%	7%	12%
48	<5%	<5%			<5%	
72					5%	<5%

AHF + IV cytosol (various) after 24 hrs and daily

rat no.	141	142	143	144	145
hours 0	70%	43%	35%	64%	31%
24					13%
48	<5%		<10%	<5%	<5%
72		27%			
96		29%	6%		
168		35%			
216		39%			
312		43%			
360		70%			

AHF + IV cytosol (various) after 30 hrs and daily

rat no.	147	148	149	150	151
hours 0	41%	34%	64%	43%	58%
30	<5%				
48			<5%		

AHF + IV saline

rat no.	140	146	155	156
hours 0	51%	39%	29%	33%
6			29%	30%
24	19%		6%	9%
30		<5%		
48				<5%
72			16%	<5%

AHF + IV human cytosol

rat no.	157	158	159	160
hours 0	21%	31%	34%	76%
6	36%	33%	27%	26%
24	<5%	6%	11%	10%
48	<5%		<5%	<5%
72	9%	6%	<5%	<5%
96	43%	24%		<5%
144	24%	27%		
192	20%	29%		
312	31%	34%		
388	36%	39%		

AHF + IV plasma daily

rat no.	161	162	163	164
hours 0	43%	48%	43%	31%
6	30%	34%	59%	20%
24	<5%	15%	12%	
72			12%	
96	33%		29%	
144	34%		26%	
192	28%		34%	
312	31%		39%	
388	43%		39%	

AHF + IP saline daily

rat no.	165	166	167	168
hours 0	37%	70%	37%	39%
6	28%	36%	29%	33%
24	10%	6%	11%	<5%
48	<5%	<5%		

AHF + IV saline and sacrifice

	rat no.	169	182
hours	0	37%	46%
	6	36%	51%
	24	<5%	<5%
	30		<5%

AHF + IV saline

	rat no.	170	172	174	176
hours	0	36%	37%	39%	39%
	6	31%	36%	33%	29%
	24	13%	13%	5%	<5%
	48				<5%
	72	<5%	<5%		<5%

AHF + IV cytosol (various) and sacrifice

	rat no.	171	173	175	177
hours	0	34%	37%	41%	39%
	6	31%	36%	55%	36%
	24	10%	10%	6%	<5%
	48	<5%	<5%		
	72	<5%	<5%	7%	

AHF + IP Hanks single

	rat no.	178	179	180	181
hours	0	41%	37%	39%	20%
	6	37%	55%	43%	31%
	24		7%	<5%	<5%
	48		<5%	<5%	<5%
	72				<5%

ALT RESULTS

DMNA 40 mg/kg

rat no.	8	9	10
hours 0	100	103	100
6	40	74	71
24	51	91	141
48	300	2626	
72	869		1000
96	427		357
120	214		155
144	99		96
192	74		88
292			91
316			65

DMNA 50 mg/kg

rat no.	12	13	14
hours 0	88	35	40
16	154	114	329
28	2004	597	591

DMNA 45 mg/kg

rat no.	15	16	17
hours 0		54	61
23		1038	1340
48		252	
105			197
124			77
151	64		37

DMNA 50 mg/kg

rat no.	19	20	21	22	23
hours 0	49	60	67	52	54
18		1008	2934	668	257
25				2679	
42		328	2353	72	561

AHF + IS hepatocytes

rat no.	24	25	28	29
hours 0	43	91	132	67
6			127	110
48	315	725		
76			560	

rat no.	30	31	32	33	34
hours 0	53	40		239	58
6		48	50		
21				4062	
43			2673		
49	975				
65		4157			4290

noAHF, IS hepatocytes

rat no.	37	38	39	40	41
hours 0	55	53	70	51	72
20		553	313	277	962
45			184	90	222
68	92	105		90	89
121	54	54	48		49
164	46	91	39	54	58
210	35	59	39		62
259	39		50		50
308			41		51
356					42

AHF + IS sham injection

rat no.	42	43	44	45	46
hours 0	42	42	44	51	45
6	57	36	85	51	35
24	694		2136		
48		4660		6789	7558

AHF + IP hepatocytes

rat no.	47	48	49	50	51
hours 0	49	32	44	39	76
6	32	26	35	38	52
30		3950		2750	
48	2040				
70					2715
150		84		112	
216		29			

rat no.	53	54	55	56	57
hours 0	56	68	73	54	54
6	48	76	59	35	97
69			280		

rat no.	59	60	61	62	63
hours 0	75	64	65	51	52
6	63	63	47	44	37
24			1185	717	5128
48	3525	5805			185

rat no.	65	66	67	68	69
hours 0	69	119	55	26	21
6	123		120	34	8640
24	11480				7116
39			49		
45				17	2050
54		900			
70	2100				

AHF + IP cytosol (71) daily

rat no.	73	74
hours 0	92	112
24	728	1540
72	910	
100		25

AHF + IP cytosol (72) daily

rat no.	75	76
hours 0	135	136
6	95	
24	6705	2600
48		3705
100	187	

AHF + IP cytosol (72) every 2nd day

rat no.	77	78	81	82
hours 0		125	150	76
6	77		115	76
25		9460	721	
48		8280		48
72			1390	
148	152			

AHF + IP cytosol (72) every 3rd day

rat no.	83	84
hours 0	84	75
6		70
54		2810
72	304	

AHF control

rat no.	79	80	86	87
hours 0	112	90	64	60
6		68	42	45
24				462
48				6040
72	2360			
90		117		

AHF + IV cytosol (85) daily

rat no.	88	89	90	91
hours 0	69	68	72	65
6	56	56	56	60
24	3051	576	1190	1932
48	3270			
72	1008	2114		
100	294	516	580	608
144	88	130	99	153
196	84	100	89	111
630	54	96	78	102

AHF + IV cytosol (93) daily

rat no.	95	96	97
hours 0	100	84	80
6	80	70	68
24	775	625	1169
96		1030	852
120	332	1848	336
168			100
533	94		101

AHF + IV cytosol (94) daily

rat no.	98	99	100
hours 0	84	80	70
6	70	60	40
24	1784	475	390
48	4040	4760	4140
72		1948	1482
96		830	665
120		245	160
168		65	105
533		112	129

AHF + IV cytosol (92) daily

rat no.	101	102	103
hours 0	92	80	80
6	56	70	
24	785	500	840
48	5180		4680
72	2590	1425	1940
96	1062		955
120	356		380
168	120		133
533	108		120

AHF + IV cytosol (94) daily after 12 hrs

rat no.	104	105	106
hours 0	175	128	168
12			136
24			4410
48	2208	2073	2320
72			706
96	355		305
144	120	120	96
216	110	126	126
365	132	142	150

AHF + IV cytosol (94) daily after 6 hrs

rat no.	107	108	109
hours 0	140	279	148
6		111	95
24	4312		
72			3165
96	575	152	
144	140	135	
216	160	184	
365		142	

AHF + IV cytosol (94) daily after 24 hrs

rat no.	110	111	112
hours 0	130	120	138
24	4212	4896	
48	1833	3101	
72	532	834	
96		261	380
144		84	240
216		144	160
365		133	118

AHF control

rat no.	116	117
hours 0	145	75
6		65
24		3450
48		858
70	280	

AHF + IV heat treated cytosol daily

rat no.	118	119	120
hours 0	119	70	95
6	80	60	65
24	1449	4949	3855
72	1770		
96			116
102	415		

AHF + IV cytosol (113) daily

rat no.	121	122	123	124	125
hours 0	100	99	85	70	70
6	84	70	60	75	63
24	1255	504		3945	2562
48			4020		
102	300			645	
144	100	120		125	
192	65	66		77	
365	78	104		92	

AHF + IV dialysed cytosol (126) daily

rat no.	128	129	130	131	132	133
hours 0	88	66	91	70	63	
6			95	60	105	121
24				749		465
60				1065		3157
65	3024					
70			82			
90					24	

AHF + IV heated cytosol (127,127a) daily

rat no.	134	135	136	137	138	139
hours 0	55	96	65	84	50	85
6	35	90	49	75	75	77
24				2211	4191	511
56	568					
60		2605				
65				641		
75						615
96			250			
120					34	

AHF + IV cytosol (various) after 24 hrs and daily

rat no.	141	142	143	144	145
hours 0	90	80	85	70	75
24					1330
48	19				
55				3865	
60					4932
168		70			
216		90			
312		135			
360		105			

AHF + IV cytosol (various) after 30 hrs and daily

rat no.	147	148	149	150	151
hours 0	75	91	95	95	84
30	5985				
40		6423			9828
48			2885		
81				22	

AHF + IV saline

rat no.	140	146	155	156
hours 0	85	65	84	50
6			65	35
24	1115		3960	300
30		4920		
40	7500			
48				2720
72			15	722

AHF + IV human cytosol

rat no.	157	158	159	160
hours 0	90	40	85	75
6	65	49	50	75
24	780	1106	330	1245
48				4664
72		1603	83	
96	835	715		1310
116				1672
144	135	155		
192	145	95		
312	133	110		
388	136	122		

AHF + IV plasma daily

rat no.	161	162	163	164
hours 0	65	132	85	90
6	65	56	60	70
24	5698	378	250	
44		5680		6068
96	492		400	
144	90		80	
192	95		110	
312	120		98	
388	118		116	

AHF + IP saline daily

rat no.	165	166	167	168
hours 0	90	85	70	75
6	70			56
24	1309	2354	1030	
44			3508	
48	270	158		

AHF + IV saline and sacrifice

rat no.	169	182
hours 0	80	77
6	65	49
24	16	2057
30		4372

AHF + IV saline

rat no.	170	172	174	176
hours 0	75	50	55	70
6	60	45	50	45
24	615	825	400	2545
72	22	43	4345	198

AHF + IV cytosol (various) and sacrifice

rat no.	171	173	175	177
hours 0	50	39	90	55
6	45	55	75	51
24	845	885		3555
48				4600
72		18	519	4355

AHF + IP Hanks single

rat no.	178	179	180	181
hours 0	105	95	70	45
6	75	75	65	56
24		3360		
48			6332	
53	3445			
57		3570		
72				229

AST RESULTS

DMNA 40 mg/kg

	rat no.	8	9	10
hours	0	210	230	237
	6	99	139	147
	24	223	321	357
	48	785	10019	
	72	2002		1498
	96	857		495
	120	441		206
	144	193		142
	192	97		104
	292			201
	316			89

DMNA 50 mg/kg

	rat no.	12	13	14
hours	0	164	113	155
	16	491	431	893
	28	3200	2110	2575

DMNA 45 mg/kg

	rat no.	15	16	17
hours	0		104	109
	23		1904	2191
	48		293	
	105			283
	124			134
	151	167		88

DMNA 50 mg/kg

	rat no.	19	20	21	22	23
hours	0	94	82	95	122	92
	18		990	2110	952	282
	25				6985	
	42		6919	10870	8900	11264

AHF + IS hepatocytes

	rat no.	24	25	28	29
hours	0	95	86	86	62
	6	610	170	165	110
	48	5907	5214		
	76			3049	

	rat no.	30	31	32	33	34
hours	0	222	70	96	82	60
	6	180	132	162		150
	21				4098	
	43			10131	11385	
	49	6292				
	65		9196			9185

noAHF, IS hepatocytes

rat no.	37	38	39	40	41
hours 0	136	100	100	94	92
20		841	622	509	1526
45			320	172	480
68	290	391		229	158
121	153	137	79		74
164	107	265	84	176	124
210	91	153	91		195
259	96		180		133
308			80		104
356					107

AHF + IS sham injection

rat no.	42	43	44	45	46
hours 0	107	199	216	111	212
6	244	142	389	157	111
24	1387				
48	17168	11133			

AHF + IP hepatocytes

rat no.	47	48	49	50	51
hours 0	194	70	110	86	250
6	130	174	354	222	117
30		7150		3470	
48	10310				
60			8520		
70					8100
150		282		357	
216		92			

rat no.	53	54	55	56	57
hours 0	222	183	215	188	225
6	170	258	246	100	318
48				12915	9705
69			5190		

rat no.	59	60	61	62	63
hours 0	245	420	303	145	172
6	170	318	174	124	135
24			1540	1032	9000
48	15630	19140			5130
87			5450	3100	

rat no.	65	66	67	68	69
hours 0	207	440	170	140	312
6	410		410		12270
24					9860
39			145		
45				122	11940
54		11820			
70	9300				

AHF + IP cytosol (71) daily

rat no.	73	74
hours 0	116	136
24	763	1060
72	2060	
100		1189

AHF + IP cytosol (72) daily

rat no.	75	76
hours 0	120	200
6	145	
24	4490	2060
48		9915
100	1020	

AHF + IP cytosol (72) every 2nd day

rat no.	77	78	81	82
hours 0		125	145	204
6	238		150	230
25		7320	917	
48		17120		7280
72			5150	
148	304			

AHF + IP cytosol (72) every 3rd day

rat no.	83	84
hours 0	244	170
6		245
54		8275
72	4200	

AHF control

rat no.	79	80	86	87
hours 0	232	255	96	130.
6		224	133	120
24				651
48				8500
72	9850			
90		1619		

AHF + IV cytosol (85) daily

rat no.	88	89	90	91
hours 0	132	100	92	100
6	136	126	112	115
24	3984	920	1920	2376
48	7885			
72	1352	2345		
100	427	648	664	836
144	168	195	135	192
196	160	220	111	195
630	80	116	80	102

AHF + IV cytosol (93) daily

rat no.	95	96	97
hours 0	225	184	205
6	115	115	152
24	760	745	1169
96		1800	740
120	336	2345	364
168			350
533	88		125

AHF + IV cytosol (94) daily

rat no.	98	99	100
hours 0	152	110	168
6	161	110	96
24	1656	655	630
48	8860	7828	8032
72		2340	1912
96		990	860
120		495	215
168		115	285
533		94	106

AHF + IV cytosol (92) daily

rat no.	101	102	103
hours 0	216	160	305
6	108	125	
24	865	715	910
48			8583
72	2675	2010	1950
96	1044		930
120	375		445
168	240		580
533	98		108

AHF + IV cytosol (94) daily after 12 hrs

rat no.	104	105	106
hours 0	295	188	180
12			312
24			6090
48	2859	2421	2540
72			852
96	385		365
144	150	175	92
216	244	150	162
365	160	125	120

AHF + IV cytosol (94) daily after 6 hrs

rat no.	107	108	109
hours 0	200	396	248
6		180	155
24	4704		
72			3000
96	610	508	
144	195	261	
216	394	332	
365		187	

AHF + IV cytosol (94) daily after 24 hrs

rat no.	110	111	112
hours 0	125	116	168
24	7016	6813	
48	2373	5481	
72	835	844	
96		333	516
144		108	510
216		177	225
365		101	132

AHF control

rat no.	116	117
hours 0	275	180
6		190
24		3055
48		9300
70	4164	

AHF + IV heat treated cytosol daily

rat no.	118	119	120
hours 0	350	147	190
6	180	140	170
24	1827	6027	5120
72	1835		
96			1690
102	500	2061	

AHF + IV cytosol (113) daily

rat no.	121	122	123	124	125
hours 0	140	186	565	505	75
6	182	135	160	215	210
24	1560	819		4200	3486
48			7500		
102	375			740	
144	170	207		275	
192	155	111		126	
365	114	104		92	

AHF + IV dialysed cytosol (126) daily

rat no.	128	129	130	131	132	133
hours 0	132	242	175	133	126	
6			170	135	203	220
24				1043		645
60				6650		10234
65	8561					
70			4415			
90		908			2916	

AHF + IV heated cytosol (127,127a) daily

rat no.	134	135	136	137	138	139
hours 0	136	138	135	120	80	80
6	110	145	154	95	145	525
24				2563	4653	602
56	4755					
60		8595				
65				4210		
75						3970
96			967			
120					989	

AHF + IV cytosol (various) after 24 hrs and daily

rat no.	141	142	143	144	145
hours 0	460	280	140	120	405
24					1540
48	7595				
55				11240	
60					13130
168		85			
216		155			
312		95			
360		85			

AHF + IV cytosol (various) after 30 hrs and daily

rat no.	147	148	149	150	151
hours 0	330	133	245	465	105
30	6525				
40		13524			13566
48			7500		
81				2016	

AHF + IV saline

rat no.	140	146	155	156
hours 0	225	385	364	150
6			120	112
24	1355		3430	620
30		10290		
40	7500			
48				4890
72			1345	1786

AHF + IV human cytosol

rat no.	157	158	159	160
hours 0	185	155	190	240
6	150	203	135	140
24	1005	1050	390	1175
48				5797
72		1498	4755	
96	825	640		1300
116				2164
144	115	125		
192	195	105		
312	125	100		
388	84	108		

AHF + IV plasma daily

rat no.	161	162	163	164
hours 0	140	209	235	145
6	120	105	110	150
24	4378	749	415	
44		6000		6000
96	513		750	
144	95		140	
192	85		120	
312	85		70	
388	76		88	

AHF + IP saline daily

rat no.		165	166	167	168
hours	0	100	225	305	145
	6	125			84
	24	1330	3157	1250	
	44			6120	
	48	5670	10055		

AHF + IV saline and sacrifice

rat no.		169	182
hours	0	355	175
	6	180	154
	24		2728
	30		7292

AHF + IV saline

rat no.		170	172	174	176
hours	0	210	145	110	125
	6	145	115	100	126
	24	972	1000	6665	2310
	72		3085	9945	3357

AHF + IV cytosol (various) and sacrifice

rat no.		171	173	175	177
hours	0	120	120	395	135
	6	120	150	145	108
	24	1550	1275		3430
	48				4850
	72	2562	2985	2176	4530

AHF + IP Hanks single

rat no.		178	179	180	181
hours	0	105	510	390	380
	6	110	145	120	175
	24		2965		
	48			6000	
	53	7500			
	57		10530		
	72				1913

ALKALINE PHOSPHATASE RESULTS

DMNA 40 mg/kg

rat no.	8	9	10
hours 0	426	365	396
6	271	536	490
24	440	414	472
48	330	683	
72	720		672
96	699		907
120	754		692
144	797		538
192	550		450
292			554.
316			536

DMNA 50 mg/kg

rat no.	12	13	14
hours 0	212	527	481
16	312	323	343
28	435	420	491

DMNA 45 mg/kg

rat no.	15	16	17
hours 0		398	388
23		417	346
48		884	
105			664
124			587
151	578		460

DMNA 50 mg/kg

rat no.	19	20	21	22	23
hours 0	172	221	362	232	241
18		348	742	189	643
25				184	
42		551	657	500	409

AHF + IS hepatocytes

rat no.	24	25	28	29
hours 0	360	576	750	513
6			148	247
48	567	621		
76			709	

rat no.	30	31	32	33	34
hours 0	170	295		250	327
6		188	202		
21					831
43			749		
49	498				
65		1211			761

noAHF, IS hepatocytes

rat no.	37	38	39	40	41
hours 0	488	329	422	314	378
20		363	438	567	536
45			400	294	436
68	284	141		132	360
121	336	282	315		327
164	537	410	318	318	465
210	389	372	360		312
259	450		294		495
308			385		530
356					360

AHF + IS sham injection

rat no.	42	43	44	45	46
hours 0	360	358	555	372	309
6	147	252	198	236	231
24	204		408		
48	530	490		617	695

AHF + IP hepatocytes

rat no.	47	48	49	50	51
hours 0	417	390	456	350	320
6	188	285	213	100	375
30		531		308	
48	480				
70					747
150		606		1644	
216		442			

rat no.	53	54	55	56	57
hours 0	603	852	460	505	297
6	430	546	180	390	279
48				519	580
69			836		

rat no.	59	60	61	62	63
hours 0	510	545	384	575	392
6	415	204	327	385	440
24			525	417	605
48	809	751			653
87			2380		

rat no.	65	66	67	68	69
hours 0	441	270	455	304	308
6	245	675	290	706	264
24	225				507
39			503		
45				709	591
54		670			
70	683				

AHF + IP cytosol (71) daily

rat no.	73	74
hours 0	328	308
24	252	360
72	509	
100		492

AHF + IP cytosol (72) daily

rat no.	75	76
hours 0	355	48
6	180	
24	370	490
48		417
100	404	

AHF + IP cytosol (72) every 2nd day

rat no.	77	78	81	82
hours 0		440	300	372
6	308		270	225
25		464	378	
48		537		639
72			465	
148	708			

AHF + IP cytosol (72) every 3rd day

rat no.	83	84
hours 0	308	430
6		145
54		498
72	556	

AHF control

rat no.	79	80	86	87
hours 0	344	345	692	630
6		304	511	490
24				700
48				660
72	710			
90		349		

AHF + IV cytosol (85) daily

rat no.	88	89	90	91
hours 0	666	696	768	620
6	504	518	472	515
24	549	712	786	636
48	740			
72	1240	1057		
100	1323	1407	1312	1184
144	728	945	921	1041
196	712	1764	819	774
630	378	452	510	460

AHF + IV cytosol (93) daily

rat no.	95	96	97
hours 0	380	564	415
6	405	470	372
24	525	570	378
96		920	912
120	1080	711	900
168			295
533	403		503

AHF + IV cytosol (94) daily

rat no.	98	99	100
hours 0	516	335	371
6	301	390	364
24	656	545	445
48	630	832	724
72		992	928
96		1095	1370
120		740	790
168		420	65
533		383	395

AHF + IV cytosol (92) daily

rat no.	101	102	103
hours 0	308	445	445
6	364	380	
24	400	615	420
48	651		744
72	835	785	835
96	924		1095
120	725		995
168	168		301
533	374		398

AHF + IV cytosol (94) daily after 12 hours

rat no.	104	105	106
hours 0	565	624	668
12			600
24			740
48	1008	942	935
72			1020
96	1180		995
144	585	755	548
216	296	590	1032
365	612	537	543

AHF + IV cytosol (94) daily after 6 hrs

rat no.	107	108	109
hours 0	630	540	484
6		534	590
24	700		
72			902
96	1060	1196	
144	865	666	
216	420	468	
365		529	

AHF + IV cytosol (94) daily after 24 hrs

rat no.	110	111	112
hours 0	640	616	672
24	820	774	
48	852	770	
72	938	1088	
96		906	1174
144		528	615
216		486	590
365		575	509

AHF control

rat no.	116	117
hours 0	430	435
6		255
24		280
48		528
70	876	

AHF + IV heat treated cytosol daily

rat no.	118	119	120
hours 0	539	546	400
6	315	320	180
24	785	560	460
72	785		
96			456
102	810	1221	

AHF + IV cytosol (113) daily

rat no.	121	122	123	124	125
hours 0	415	300	345	505	385
6	280	300	300	90	336
24	470	231		545	511
48			724		
102	815			790	
144	735	675		570	
192	240	381		469	
365	452	424		552	

AHF + IV dialysed cytosol (126) daily

rat no.	128	129	130	131	132	133
hours 0	374	253	245	350	210	
6			285	285	182	440
24				252		240
60				357		581
65	434					
70			526			
90		518			522	

AHF + IV heated cytosol (127,127a) daily

rat no.	134	135	136	137	138	139
hours 0	290	356	320	312	275	330
6	165	225	7	215	210	224
24				352	253	210
56	544					
60		595				
65				311		
75						395
96			636			
120					722	

AHF + IV cytosol (various) after 24 hrs and daily

rat no.	141	142	143	144	145
hours 0	330	290	385	350	240
24					310
48	498				
55				794	
60					708
168		410			
216		360			
312		390			
360		452			

AHF + IV cytosol (various) after 30 hrs and daily

rat no.	147	148	149	150	151
hours 0	310	462	425	395	420
30	380				
40		693			723
48			583		
81				601	

AHF + IV saline

rat no.	140	146	155	156
hours 0	380	225	301	245
6			310	224
24	385		450	335
30		370		
40	624			
48				460
72			524	506

AHF + IV human cytosol

rat no.	157	158	159	160
hours 0	385	430	295	265
6	310	301	340	335
24	215	336	385	335
48				297
72		462	458	
96	570	725		1265
116				1010
144	480	585		
192	340	390		
312	490	430		
388	450	464		

AHF + IV plasma daily

rat no.	161	162	163	164
hours 0	345	649	470	535
6	325	483	365	355
24	198	420	360	
44		862		798
96	879		540	
144	670		365	
192	510		385	
312	545		399	
388	558		430	

AHF + IP saline daily

rat no.		165	166	167	168
hours	0	545	520	455	550
	6	370			441
	24	630	286	345	
	44			620	17
	48	500	527		

AHF + IV saline and sacrifice

rat no.		169	182
hours	0	415	273
	6	215	84
	24	454	88
	30		438

AHF + IV saline

rat no.		170	172	174	176
hours	0	345	310	235	290
	6	285	210	185	237
	24	336	320	380	
	72	432	575	865	459

AHF + IV cytosol (various) and sacrifice

rat no.		171	173	175	177
hours	0	400	303	250	330
	6	305	315	290	282
	24	355	365		365
	48				545
	72	206	461	560	1480

AHF + IP Hanks single

rat no.		178	179	180	181
hours	0	430	365	285	365
	6	370	230	290	147
	24		30		
	48			528	
	53	553			
	57		618		
	72				578

BILIRUBIN RESULTS

DMNA 40mg/kg

	rat no.	8	9	10
hours	0	10	10	10
	6	10	10	10
	24	10	10	10
	48	10	10	
	72	10		10
	96	10		10
	120	10		10
	144	10		10
	196	10		10
	292			10
	316			10

DMNA 50mg/kg

	rat no.	12	13	14
hours	0	10	10	10
	16	10	10	10
	28	22	43	55

DMNA 45mg/kg

	rat no.	15	16	17
hours	0		10	10
	23		10	10
	48		10	
	105			10
	124			10
	151	10		10

DMNA 50mg/kg

	rat no.	19	20	21	22	23
hours	0	10	10	10	10	10
	18		10	10	10	10
	25				10	
	42		31	10	38	30

AHF + IS hepatocytes

	rat no.	24	25	28	29
hours	0	10	10	10	10
	6	10		10	10
	48	22			
	72			38	

	rat no.	30	31	32	33	34
hours	0	10	10	10	10	10
	6	10	10	10		10
	21				10	
	43			10	20	
	49	27				
	65		22			30

noAHF, IS hepatocytes

rat no.	37	38	39	40	41
hours 0	10	10	10	10	10
20		10	10	10	10
45			14	10	10
68	10	10		10	13
121	10	11	10		10
164	10	10	10	10	10
210	10	10	16		10
259	10		10		10
308			10		10
356					10

AHF + IS sham injection

rat no.	42	43	44	45	46
hours 0	10	10	10	10	10
6	10	10	10	10	10
24	10		10		
48	27	47		44	70

AHF + IP hepatocytes

rat no.	47	48	49	50	51
hours 0	10	10	10	10	10
6	5	5	5	5	5
30		30		24	
48	56				
60			45		
70					65
150		5		18	
216		5			

rat no.	53	54	55	56	57
hours 0	5	5	5	5	5
6	5	5	5	5	5
48				28	44
69			85		

rat no.	59	60	61	62	63
hours 0	5	5	5	5	5
6	5	5	5	5	5
24			5	5	21
48	32	40			57
87		44	78		

rat no.	65	66	67	68	69
hours 0	5	5	5	5	5
6	5	5	5	5	5
24	5				26
39			43		
45				70	60
54		73			
70	52				

AHF + IP cytosol (71) daily

rat no.	73	74
hours 0	0	0
24	28	0
72	19	
100		13

AHF + IP cytosol (72) daily

rat no.	75	76
hours 0	0	48
6	15	
24	10	0
48		69
100	10	

AHF + IP cytosol (72) every 2nd day

rat no.	77	78	81	82
hours 0		0	15	10
6	0		0	15
25		16	10	
48		75		40
72			76	
148	12			

AHF + IP cytosol (72) every 3rd day

rat no.	83	84
hours 0	10	0
6		10
54		75
72	80	

AHF control

rat no.	79	80	86	87
hours 0	16	10	0	10
6		10	0	0
24				0
48				80
72	80			
90		45		

AHF + IV cytosol (85) daily

rat no.	88	89	90	91
hours 0	10	0	0	0
6	10	0	10	0
24	10	10	0	0
48	25			
72	16	21		
100	27	12	12	20
144	16	10	10	10
196	0	12	10	10
630	0	10	10	10

AHF + IV cytosol (93) daily

rat no.	95	96	97
hours 0	0	10	15
6	0	0	10
24	10	0	21
96		45	12
120	20	33	10
168			20
533			0

AHF + IV cytosol (94) daily

rat no.	98	99	100
hours 0	10	10	28
6	14	0	0
24	12	0	0
48	66	32	20
72		24	12
96		10	15
120		20	10
168		10	10
533		10	10

AHF + IV cytosol (92) daily

rat no.	101	102	103
hours 0	10	20	10
6	0	15	
24	0	15	10
48	56		36
72	25	61	35
96	12		10
120	10		10
168	10		21
533	10		10

AHF + *V cytosol (94) daily after 12 hrs

rat no.	104	105	106
hours 0	10	10	10
12			0
24			30
48	30	45	20
72			14
96	25		15
144	15	10	0
216	10	0	10
365	10	0	10

AHF + IV cytosol (94) daily after 6 hrs

rat no.	107	108	109
hours 0	30	36	10
6		10	10
24	28		
72			56
96	10	10	
144	10	10	
216	10	10	
365		10	

AHF + IV cytosol (94) daily after 24 hrs

rat no.	110	111	112
hours 0	0	0	10
24	28	36	
48	21	63	
72	14	26	
96		10	12
144		10	10
216		10	10
365		10	10

AHF control

rat no.	116	117
hours 0	20	10
6		15
24		10
48		46
70	32	

AHF + IV heat treated cytosol daily

rat no.	118	119	120
hours 0	10	10	25
6	20	20	20
24	14	10	40
72	10		
90			20
102	10	27	

AHF + IV cytosol (113) daily

rat no.	121	122	123	124	125
hours 0	20	21	20	10	10
6	21	15	10	45	14
24	35	10		20	10
48			83		
102	10			20	
144	10	10		25	
192	35	10		10	
365	10	16		10	

AHF + IV dialysed cytosol (126) daily

rat no.	128	129	130	131	132	133
hours 0	10	33	28	10	28	
6			10	10	42	10
24				10		20
60				43		28
65	35					
70			71			
90		43			36	

AHF + IV heated cytosol (127,127a) daily

rat no.	134	135	136	137	138	139
hours 0	20	10	10	10	10	10
6	25	20	35	10	20	28
24				33	77	35
56	46					
60		40				
65				49		
75						43
96			14			
120					33	

AHF + IV cytosol (various) after 24 hrs and daily

rat no.	141	142	143	144	145
hours 0	15	10	15	10	20
24	10				
48	49				
55				68	
60					50
168		10			
216		15			
312		5			
360		10			

AHF + IV cytosol (various) after 30 hrs and daily

rat no.	147	148	149	150	151
hours 0	10	10	10	10	10
30	25				
40		30			33
48			52		
81				58	

AHF + IV saline

rat no.	140	146	155	156
hours 0	10	15	28	20
6			10	10
24	21		10	10
30		53		
40	27			
48				15
72			24	22

AHF + IV human cytosol

rat no.	157	158	159	160
hours 0	10	10	10	20
6	10	7	10	10
24	10	7	14	10
48				55
72		35	24	
96	15	10		
116				22
144	10	10		
192	10	10		
312	10	10		
388	10	10		

AHF + IV plasma daily

rat no.	161	162	163	164
hours 0	15	22	10	10
6	10	7	5	20
24	33	28	20	
44		10		12
96	15		10	
144	10		15	
192	10		5	
312	10		10	
388	10		10	

AHF + IP saline daily

rat no.		165	166	167	168
hours	0	10	10	10	10
	6	20			10
	24	10	55	15	
	44			14	17
	48	19	34		

AHF + IV saline and sacrifice

rat no.		169	182
hours	0	10	10
	6	20	42
	24	35	66
	30		52

AHF + IV saline

rat no.		170	172	174	176
hours	0	10	10	10	20
	6	10	10	10	10
	24	10	10	10	10
	72	34	46	94	59

AHF + IV cytosol (various) and sacrifice

rat no.		171	173	175	177
hours	0	10	3	10	10
	6	10	10	10	10
	24	10	20		10
	48				20
	72	10	48	51	35

AHF + IP Hanks single

rat no.		178	179	180	181
hours	0	10	10	10	15
	6	10	20	10	35
	24		10		
	48			32	
	53	63			
	57		61		
	72				36

RAT DNA SYNTHESIS RESULTS

Experiment 1

Plates	^{3}H disintegrations / minute in 40μl			(mean)
C i)	6562	5410	7539	6503
ii) +EGF	54794	53987	53269	54016
iii) +TGFβ	1446	858	1442	1248
T i) +20c	5296	6379	6045	5906
ii) +60c	4655	6582	5066	5434
iii) +5c	9898	8005	7783	8562
iv) +60c+TGFβ	1527	1490	915	1310
v) +60c+EGF	76074	79094	72305	75824

Plates	DNA concentration in 100μl			(mean)
C i)	0.52	0.47	0.58	0.52
ii) +EGF	0.88	0.80	0.825	0.84
iii) +TGFβ	0.305	0.275	0.275	0.285
T i) +20c	0.66	0.965	0.77	0.76
ii) +60c	0.605	0.725	0.66	0.66
iii) +5c	0.715	0.715	0.745	0.725
iv) +60c+TGFβ	0.25	0.14	0.495	0.295
v) +60c+EGF	0.965	1.13	1.045	1.05

Plates	^{3}H disintegrations / μg DNA			(mean)
C i)	31548	28777	32496	30940
ii) +EGF	155668	168709	161421	161932
iii) +TGFβ	11861	7800	13109	10923
T i) +20c	20057	16526	19627	18736
ii) +60c	19236	22087	19189	20170
iii) +5c	34608	27989	26117	29571
iv) +60c+TGFβ	15270	26607	4621	15499
v) +60c+EGF	197082	174985	172978	181681

Experiment 2

Plates	^{3}H disintegrations / minute in 40μl			(mean)
C i)	3242	4798	6172	4737
ii) +EGF	68111	73038	12210	51119
iii) +TGFβ	2607	2457	3813	2959
T i) +5c+EGF	68810	72516	69684	70336
ii) +10c+EGF	44928	54981	62633	54179
iii) +30c+EGF	45952	56270	72130	58116
iv) +60+EGF	70650	58317	77737	68901
v) +5c+TGFβ	4099	4144	3665	3969
vi) +60c+TGFβ	5924	6859	5500	6094
vii) +5c	4790	5310	6738	5612
viii) +10c	4679	5231	2431	4113
ix) +30c	4816	8705	6373	6631
x) +60c	13679	11981	13652	13104

Plates	DNA concentrations in 100μl			(mean)
C i)	1.08	1.38	1.26	1.24
ii) +EGF	1.70	1.83	1.08	1.54
iii) +TGFβ	1.02	0.93	1.05	1.0
T i) +5c+EGF	2.85	3.21	3.06	3.04
ii) +10c+EGF	3.06	3.06	2.58	2.9
iii) +30c+EGF	4.44	4.92	6.45	5.27
iv) +60c+EGF	5.10	4.74	5.43	5.09
v) +5c+TGFβ	4.26	4.41	4.10	4.26
vi) +60c+TGFβ	4.32	4.41	4.50	4.41
vii) +5c	4.47	4.68	4.30	4.48
viii) +10c	0.48	0.54	0.27	0.43
ix) +30c	0.48	0.60	0.42	0.50
x) +60c	0.63	0.57	0.63	0.61

Plates	^{3}H disintegrations / μg DNA			(mean)
C i)	7512	8697	12256	9488
ii) +EGF	100104	99834		99968
iii) +TGFβ	6397	6612	9086	7365
T i) +5c+EGF	60381	56494	56950	57941
ii) +10c+EGF		44934	60714	52823
iii) +30c+EGF	25880	28598	27962	27479
iv) +60c+EGF	34639	30764	35797	33733
v) +5c+TGFβ	2406	2350	2235	2330
vi) +60c+TGFβ	3429	3889	3057	3457
vii) +5c	2680	2837	3918	3144
viii) +10c	24420	24263	22593	23757
ix) +30c	25136	36332	38023	33163
x) +60c	54368	52641	54261	53756

Experiment 3

Plates			^{3}H disintegrations / minute in 40μl			(mean)
C i)		(a)	6419	5743	5023	5728
ii)		(b)	3761	3902	4091	3918
iii)	+EGF	(b)	32050	41101	38127	37092
T i)	+5c	(a)	5943	5627	4963	5511
ii)	+10c	(a)	6655	5233	7131	6339
iii)	+60c	(a)	1878	5630	2990	3533
iv)	+5c	(b)	5900	6083	6449	6144
v)	+10c	(b)	6838	6630	6103	6523
vi)	+60c	(b)	6072	4870	3517	4819
vii)	+5c+EGF	(a)	58243	21853	58168	46088
viii)	+10c+EGF	(a)	60112	66563	58573	61749
ix)	+60c+EGF	(a)	48897	29625	35874	38132
x)	+5c+EGF	(b)	45462	53525	41556	46847
xi)	+10c+EGF	(b)	49090	48347	46741	48059
xii)	+60c+EGF	(b)	50305	60786	36881	49324

Plates			DNA concentration in 100μl			(mean)
C i)		(a)	0.56	0.62	0.46	0.55
ii)		(b)	0.56	0.42	0.30	0.43
iii)	+EGF	(b)	0.45	0.54	0.49	0.49
T i)	+5c	(a)	0.49	0.45	0.49	0.48
ii)	+10c	(a)	0.68	0.59	0.66	0.64
iii)	+60c	(a)	0.63	0.51	0.59	0.57
iv)	+5c	(b)	0.63	0.63	0.66	0.64
v)	+10c	(b)	0.71	0.73	0.78	0.74
vi)	+60c	(b)	0.73	0.68	0.68	0.70
vii)	+5c+EGF	(a)	0.80	0.71	0.83	0.78
viii)	+10c+EGF	(a)	0.92	0.78	0.88	0.86
ix)	+60c+EGF	(a)	1.09	0.95	0.85	0.96
x)	+5c+EGF	(b)	0.81	0.91	0.91	0.87
xi)	+10c+EGF	(b)	0.95	0.71	0.83	0.83
xii)	+60c+EGF	(b)	1.00	1.07	0.83	0.86

Plates			^3H disintegrations / μg DNA			(mean)
C i)		(a)	28444	23437	268712	26251
ii)		(b)	16666	23287	34341	24765
iii)	+EGF	(b)	180828	190299	193921	188350
T i)	+5c	(a)	30227	31747	25242	29072
ii)	+10c	(a)	24280	22234	26969	24495
iii)	+60c	(a)	7373	24948	12704	15008
iv)	+5c	(a)	23162	23880	24390	23811
v)	+10c	(a)	24096	22592	19508	22065
vi)	+60c	(a)	20690	17767	12831	17096
vii)	+5c+EGF	(a)	180588	77007	175097	144231
viii)	+10c+EGF	(a)	162051	212774	166602	180476
ix)	+60c+EGF	(a)	111448	77831	104928	98069
x)	+5c+EGF	(b)	140959	148162	115031	134717
xi)	+10c+EGF	(b)	128970	170369	140700	146680
xii)	+60c+EGF	(b)	125762	141673	111019	126151