TREATMENT MODALITIES IN EXPERIMENTALLY INDUCED ACUTE LIVER FAILURE

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TREATMENT MODALITIES IN EXPERIMENTALLY INDUCED ACUTE LIVER FAILURE

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To the memory of Professor D.L.Westbroek

To my wife Gea and my son Pieter Benjamin

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CHAPTER 1

GENERAL INTRODUCTION

Despite the availibility of a number of treatment modalities, the mortality associated with acute hepatic failure remains high. The syndrome of acute hepatic failure is rare (67,92) but the reported mortality rate ranges from 60 to almost 100% (4,67). The management of this hepatic syndrome is challenging but difficult, as the persisting high mortality rate indicates. The basic therapeutic approach to acute hepatic failure is still a combination of intensive care, monitoring, treatment of complications, artificial replacement or addiction of functions, and detoxification to prevent accumulation of the liver damage (72). Restoration of the patient's "internal milieu" is the main goal of the treatment, which is aimed at reducing the development of hepatic encephalopathy, the prevention of the cerebral edema which is the major cause of death (12), and the promotion of regeneration of the organ.

Liver regeneration is a process by which the liver restores damage it has undergone and which usually leads to normal functioning in all respects. Over 60% of the cells in the liver are hepatocytes. If part of the liver is excised in experiments done in rats, the hepatocytes in the remaining part soon show mitosis and the liver regains its normal size in approximately three weeks (26,45). If the hepatocytes are damaged by a nutritional deficiency, a toxic substance, or a viral infection, all of the mechanisms essential for the regeneration of functional hepatic tissue may be able to function in a harmonious way and thus restore the complicated normal architecture of the organ (31). The mechanism which determines the composition of the cell population of the liver is still obscure, and the restorative capacity of the organ is limited (12). When the number of damaged hepatocytes or the total hepatocellular damage exceeds a certain limit which has not yet been established exactly,

the mechanism required for regeneration is not triggered. Several factors are probably involved in the processes underlying regeneration of the liver (39,40,82,88). According to Leffert (40), there are seven possible regulators of rat liver regeneration. Koch and Leffert reported (33,40) that increases in the influx of Na^+ ions mediate rat hepatocyte proliferation. It has also been postulated that a Ca^{2+} flux induced by glucagon and insulin or insuline-like substances, is associated with elevated cAMP levels and thus triggers the liver regeneration. The possible regulators of rat liver renegeration involved can be divided in two main categories: the common regenerating stimulating factors i.e., the tissue-specific growth factors, and factors of hormonal origin (40,41,69), and the hepatotrophic factors which secretion is possibly regulated by the injured liver involved (10,20,22,53,61,81).

An injured liver that has undergone damage exceeding the critical threshold and is therefore unable to regenerate spontaneously, can sometimes be stimulated to regenerate by the administration of substances derived from donor liver tissue, as has been extensively reviewed (2,46,52,53,60,62-68,78,81,83,86,88,90). When the critical point has been reached and the process has become irreversible and will terminate in necrosis, the only solution is replacement of liver function by transplantation. There are also situations in which the injury itself is not irreversible but that the recovery time will be too long to permit survival. In the latter case treatment should be aimed at minimizing the process of destruction in the liver; in the first place to create sufficient time for the liver to regenerate and in the second place to stimulate the organ's remaining capacity to regenerate. However, replacement of the liver function by transplantation temporary or from an artificial device is still provisional (29,93).

It has been supposed that liver cells could functionally reach the "point of no return" and still have some degree of potency to regenerate if the appropriate stimulatory factors were brought into play (17,77).

1.1. Research on hepatocellular regeneration

Many attempts have been made to identify the underlying factors playing a key role in the regeneration of liver tissue (10,22,33, 37,38,81-83). Homogenates (9) of liver tissue and/or suspensions of liver cells injected intraperitoneally induced to an increase in the rate of mitosis in the liver, and the effect of homogenates of liver tissue of young animals was considerably greater than that of adult rats. Thier and Ravanti (89) reported that the critical factors were thermo-unstable and organ-specific. Investigations performed by Blomquist (9) showed that suspensions of regenerating liver tissue (collected 48 hours after 70% hepatectomy) and suspensions of normal liver tissue of young rats increased the mitotic rate in the liver after intraperitoneal administration. Marczek (53) investigated the effects of polypeptides isolated from intact and regenerating rat livers on thymidine incorporation. Intact liver yielded fractions not only inhibiting but also stimulating liver DNA synthesis in vitro. The DNA synthesis stimulating capacity of fractions derived from regenerating liver (collected 24 hr after partial hepatectomy) showed a considerable increase which was accompanied by an almost total loss of the DNA synthesis inhibiting properties of the fractions in question. Both inhibitory and stimulatory fractions were found to contain polypeptides with molecular weights in the range of 50-500 kD (20,37,61).

The studies concerning improvement of liver regeneration have pointed to the following factors as critical for stimulation of the process of liver regeneration: 1. the age of the donor rats when a suspension of normal liver tissue is administered; 2. the interval between the preparation of the extract of regenerating liver tissue and the time when it is administered; and 3. the time of day at which the extracts were prepared (10). Labreque and co-workers reported (37,38) that the time at which the extracts were prepared during the day was an essential parameter for the ability to demonstrate any stimulatory activity at all.

Thus, the research in this field has shown that a variety of factors play a critical role in the capacity of an experimental model to reveal how hepatotrophic factor(s) operate.

1.2. Experimental treatment modalities in acute hepatic failure

Several investigators have reported that treatment with isolated viable hepatocytes (15,48,50-52,62,79), or hepatocyte fractions deriving from normal or regenerating liver tissue (2,46,47,60,63-66,88) can improve the survival of rats suffering from toxin-induced acute hepatic failure. Stimulation of the regeneration of deteriorating liver tissue by factors secreted by transplanted liver cells seems to be the underlying mechanism here rather than the provision of temporary metabolic support.

These studies differed from those mentioned above, which were done in normal healthy rats rather than rats suffering from acute hepatic failure.

Makowka and co-workers reported that intraperitoneal implantation of syngenic, allogenic, and xenogenic hepatocytes reduced the mortality rate of rats suffering from toxin-induced liver insufficiency (50,52). Reduction of the mortality rate from 80-100% to 20-30% has been reported (51). Cytosol prepared from normal and regenerating liver tissue significantly enhanced the survival rate in rats with acute liver failure (20,46,47,60,63,64,88), whereas in other animals the same cytosol preparation significantly inhibited the hepatic regenerative response normally seen in rats 20-24 hr after partial hepatectomy. Baumgartner and O'Neill showed that cell-free supernatant of hepatocyte cultures improved the survival rate of rats suffering from acute hepatic failure (2,65,66).

1.3. Experimental liver insufficiency

For experimental purposes, liver injury can be induced in a variety of animal species by the administration of 2-deoxy-2-amino-D-galactose (d-galactosamine). This compound has proved to be highly suitable for use in studies on acute hepatitis (14). Galactosamine hepatitis has been accepted as a good experimental model, superior in several respects to models based on many other hepatotoxic agents such as tetrachlorcarbon, thioacetamide, and dimethylnitrosamine (14,68) for the following reasons: a good dose-effect relationship is obtained and the effect is restricted to the liver. No morphological or functional alterations have been observed in other organs after the administration of doses of galactosamine sufficient to cause liver lesions. The target selectivity of this amino sugar is due to the local abundance of metabolizing enzymes (14).

Figure 1.1 shows the metabolic pathway of galactosamine (14).

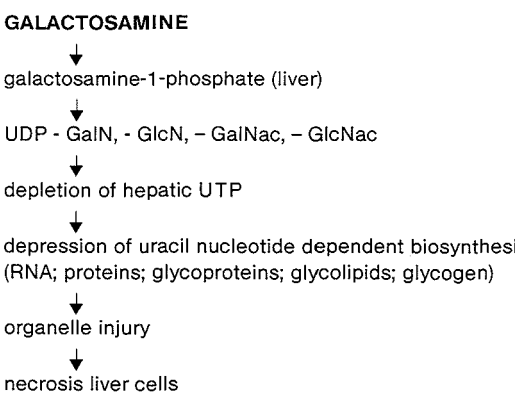

Fig. 1.1.
Pathway of galactosamine metabolism

Uridine-galactosamine	(UDP-GalN)
Uridine-glucosamine	(UDP-GlcN)
Uridine diphosphate N-acetyl-galactosamine	(UDP-GalNac)
Uridine diphosphate N-acetyl-glucosamine	(UDP-GlcNac)
Uridine 5′-triphosphate	(UTP)

During the first 30 min after administration of galactosamine to rats, the level of UDP-amino sugars rises. A marked change in the relative concentrations of the individual uracil nucleotides leads to a strong depression of the hepatic levels of UTP, UDP, UMP, UDP-glucose, UDP-galactose, and UPD-glucuronate. Galactosamine-induced UTP deficiency is highly selective. No reduction of pools of ATP, GTP, or CTP has been observed. This is in contrast to many other experimental conditions, where a deficiency of UTP is associated with a general depression of nucleoside triphosphates.

UTP deficiency depresses uracil nucleotide dependent biosynthesis, which terminates in organelle injury and cell necrosis. Injury to the hepatocyte plasma membrane is considered to be the best-defined cause of hepatocellular necrosis and may represent the terminal event of many mechanisms (68,76,77). Galactosamine induces structural and functional changes in rat liver plasma membranes, and these alterations have been associated with changes in the calcium content of the injured cells (17).

Several blood plasma parameters can be used for the quantitation of hepatocellular injury and necrosis. Typical hepatological laboratory findings include elevated levels of liver-specific enzymes and increased bilirubin concentrations in the blood plasma. Many of the coagulation factors present in serum are synthesized by the liver and have a short half-life value. The markedly prolonged prothrombin time as well as the associated hemorrhages indicate impaired function of the galactosamine-treated liver. The histological findings show a rapid loss of liver glycogen (14).

A single dose of galactosamine (1 mmol/kg = 215 mg/kg) induces light-microscopically detectable alterations which 4 hr later are characterized by acidophilic degeneration of the cytoplasm (14,76). Necrosis of single hepatocytes is evident 6 hr after the injection of galactosamine. After 24-26 hr, all of the signs of spotty necrotic hepatitis are found. Foci of hepatocellular necrosis are disseminated over the lobules, and the hepatocytes have been replaced by inflammatory infiltrates containing mainly segmented leukocytes,

lymphocytes, and plasma cells. Acidophilic degeneration and acidophilic bodies are frequently observed. The liver of animals surviving for 7 to 12 days after galactosamine administration shows normal histological features (14).

Evidence yielded by these studies led to the investigations reported in this thesis, which concerned the effect of the transplantation of hepatocytes or the administration of hepatocyte fractions, cytosol, or cell-free supernatant on the process of hepatocellular deterioration induced by galactosamine.

These studies were done in connection with the problems encountered in the treatment of acute hepatic failure. The effect of transplantation of hepatocytes and hepatocyte fractions on survival was studied in rats suffering from acute hepatic failure, as a step toward evaluation of the method for clinical use in patients with acute hepatic failure.

CHAPTER 2

MATERIAL AND METHODS

2.1. Animals

Male rats of the inbred Wag/Rij, BN/Rij, Wag/Cpb, and Lewis/Cpb strains and the outbred Cpb:Wu strain with a body weight between 220 and 290 g were used. The animals were fed ad libitum with standard laboratory pelleted rat diet throughout the experiments and had free access to water. They were kept at a constant temperature and given an artificial daylight cycle.

2.2. Induction of hepatic failure

Liver injury was induced by the administration of d-galactosamine [2,4].(SIGMA, St.Louis, Mo, USA)(d-GL). Galactosamine was dissolved in distilled water (200 mg/ml)(0,93 mol/l) and neutralized with 1N NaOH to pH 7.0. The solution was injected intraperitoneally immediately after preparation.

2.3. Uridine treatment

Galactosamine traps the essential cofactor uridine triphosphate (UTP)(32). With the appropiate dosage and schedule of administration, a lethal liver-cell necrosis can be induced with galactosamine. Administration of uridine together with or shortly after galactosamine gives protection against or reverses the UTP deficiency as well as the disturbed synthesis of RNA and protein (14,18,32,68,76,77) (Fig.1.1).

Uridine (BDH Chemicals, Poole, UK) was dissolved in distilled water (200 mg/ml) and 125, 500, 1000, or 2000 mg/kg (0.5-8.2 mMol) was injected intraperitoneally at various time-points (0, 6, 12, 18

and 24 hr) after galactosamine administration.

2.4. Isolation of hepatocytes

The two-step perfusion method used was a modification of the procedure described by Seglen (74). In short the rats were anesthetized with ether, the abdomen was prepared for surgery, and heparin (0.2 ml Thromboliquine^{R}, ORGANON, Oss, The Netherlands, 5000 IU/ml) was injected via the tail vein. The liver was exposed through a midline incision extended by transverse incisions to the left and right. The portal vein and infrahepatic caval vein were isolated. Loose ligatures were placed around the portal vein proximal to its junction with the pancreatico-duodenal vein and around the infrahepatic caval vein proximal to its junction with the renal veins. The portal vein was cannulated with a teflon catheter (Braunule^{R} Vasofix^{R} 16 G, B.BRAUN, Melsungen, W-Germany). The infrahepatic caval vein was transsected and perfusion of the liver with a Ca^{2+}-free salt solution (NaCl: 142 mmol; KCl: 6.7 mmol; HEPES: 10 mmol) at a constant flow of 20 ml/min and 37^{0}C was started. It appeared that for optimal perfusion of the liver, the outflow of the perfusion fluid must be led through the suprahepatic caval vein.

The chest was then opened and the suprahepatic caval vein cannulated with a teflon catheter, after which the loose ligature around the infrahepatic caval vein was tightened and the isolated liver was perfused for 10 min. Next, the perfusate was changed to a collagenase solution (50 ml Hanks' balanced salt solution containing: collagenase (SIGMA,IA) 25mg, $CaCl_2.2H_2O$ 27 mg, and HEPES (1 Mol) 2.5 ml, which was recirculated for 15 min under continuous gassing with oxygen and carbon dioxide. The liver was then removed and cut with a razor blade into small pieces which were brought into an Erlenmeyer flask provided with a stopper permitting continuous gassing. The cell suspension was incubated in a shaker (40 strokes/min) at 37^{0}C for 15 min, filtered through two nylon filters (100 and 250 nm; Monodur^{R}), and washed three times by centrifugation

at 20 g for 5 min each, in fresh Hanks' medium at 4^0C. Viability was always determined by The trypan blue exclusion test, i.e., the cells were scored according their ability to exclude trypan blue. The yield of hepatocytes from one liver varied from 200 to $300x10^6$ cells with a viability of 80 to 90 percent. Addition of hyaluronidase and DNAse to the perfusion fluid (5) did not improve the yield, confirming reports by other investigators. Of critical importance was immediate washout of blood after the start of the perfusion. This prompt evacuation of blood, which was demonstrated by the even white color of the liver, proved to be the most essential factor with respect to the yield and viability of the isolated hepatocytes.

Figure 2.1 shows a number of isolated hepatocytes. A darkly stained dead hepatocyte, a nucleolus, and a few healthy hepatocytes can be seen in this micrograph.

All glassware was siliconized (silicone solution, SERVA, Heidelberg, W-Germany). The final volume of the cell suspension was adjusted to give $20x10^6$ cells/ml for intraperitoneal injection and $40x10^6$ cells/ml for intrasplenic injection. After isolation, the cells were immediately injected intraperitoneally, via a teflon catheter.

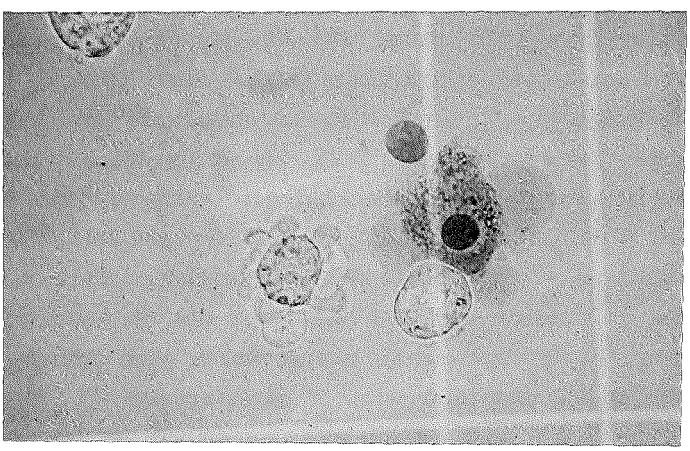

Fig. 2.1.
Micrograph showing a few hepatocytes after isolation and exposure to trypan blue to assess viability.

2.5. Hepatocyte culture

During the course of the experiments the viability of the hepatocytes was assessed several times with another test, as follows. After some of the cells in a given sample had been injected into the rats, part of the remaining portion was cultured for 24 hr according to standard techniques. These cell cultures were performed in collaboration with the Department of Cell Biology of Erasmus University (Dr.M.P.Mulder). The percentage of cell attachment at 4 hr was about 80 and the morphology of the cells 24 hr after plating, as observed with a phase-contrast microscope (Fig.6.4), showed whether the rats had received viable hepatocytes.

For the preparation of cell-free supernatant, isolated hepatocytes were cultured by plating ($2x10^6$ cells/plate) on 4 ml medium composed of RPMI/HEPES (Flow, 1460) containing 10% fetal calf serum, 20 uM dexamethasone, 10 uU/ml insulin, 0.8 mM $CaCl_2$, 50 ug/ml penicillin, and 50 ug/ml streptomycin. The culture dishes were placed in a water-saturated 95% O_2, 5% CO_2 atmosphere and the culture medium was changed at 4-hr intervals. The cell-free supernatant of the cultures which was stored at -80^0C, was used for studies on the effect on acute hepatic failure of the hepatotrophic factors secreted into the medium by the isolated hepatocytes during culture.

2.6. Implantation of hepatocytes into the spleen and abdominal cavity

Isolated hepatocytes ($20-30x10^6$) in suspension were implanted in the spleen by injection of a cell suspension ($40x10^6$ cells per ml) into the pulp via a 25-gauge needle, after occlusion of the celiac artery. The clamp was released five minutes after the injection.

Isolated hepatocytes ($40x10^6$ cells) were injected into the abdominal cavity as a cell suspension ($20x10^6$ per ml) was injected via a teflon catheter (Braunule^R Vasofix^R 16G).

2.7. Preparation of hepatocyte fractions

Livers of donor rats were excised and suspended immediately into medium (1:1, w/v) at 4^0C (medium: glucose 5%, HEPES 50 mM). The liver tissue was homogenized (Ultra-Turrax^R type TD 18-10), and 2 ml of this crude homogenate was injected intraperitoneally.

For the cytosol preparation, crude homogenate was centrifuged at 1000 g for 10 min and the supernatant was centrifuged at 100,000 g for 60 min (both steps at 4^0C). A 1-ml aliquot of the final supernatant was then injected intraperitoneally. The total protein content of 1 ml cytosol ranged between 60 and 80 mg, which is roughly the equivalent of $50x10^6$ cells. For the preparation of crude homogenates or cytosols of regenerating liver tissue, a suspension was made of the remaining part of the donor livers 24 or 72 hr after a 70% partial hepatectomy had been performed according to Higgins and Anderson (26).

2.8. Auxiliary partial liver transplantation (APLT)

Donor rats had the same body weight as the acceptor. Preparation of the donor for the operation was performed under ether anesthesia. The abdomen was opened by a midline incision. After a 70% hepatectomy had been performed and the common bile duct was ligated close to the liver hilus, the portal vein was dissected from surrounding structures as far as the superior mesenteric vein. Next, loops were placed around the vessels and around the portal vein proximal to the superior mesenteric vein as well as around the hepatic artery. The inferior caval vein was dissected and encircled at the segment between the right renal vein and the liver. Heparin was given intravenously (0.2 ml Thromboliquine^R, 5000 IU/ml).

Five minutes after the heparin injection all of the loose ligatures were tightened and the thorax was opened. The suprahepatic caval vein was dissected from surrounding structures and a ligature was placed as close to the heart as possible. The liver was removed

by cutting the diaphragm and the hepatic ligaments, taking care not to damage the liver capsule. The donor liver was placed on a glass tray which was put on ice. The infusion line was connected with a canule (Braunule^{R} Vasofix^{R} 22 G), which was inserted into the portal vein to establish a continuous saline infusion (0-4^{0}C at a height of 50 cm). The amount of saline needed to wash out the liver and make it suitable for transplantation was 250 ml.

Prior to surgery the acceptor rat was anesthetized with Thalamonal^{R} (a combination of Droperidol^{R} 2.5mg/ml and Fentanyl^{R} 0.05 mg/ml)(JANSSEN Pharmac, Goirle, The Netherlands) intramuscularly at a dose of 0.3 ml per 100 g body weight, and 0.05 ml Depomycine^{R} (GIST-BROCADES, Delft, The Netherlands) was given subcutaneously. The board to which the rats were fastened was kept at a temperature of 37^{0}C.

The abdomen was opened by a midline incision, and the aortic bifurcation including 1 cm of the right iliac artery was dissected. A Silastic^{R} (DOW CORNING, Brussels, Belgium) cannula (i.d. 0.51 - o.d. 0.94 mm) filled with a heparin solution (100 IU/ml) was fitted with a clamp and inserted into the right iliaca artery. Between the right and the left renal veins, the caval vein was freed of perivascular tissue. A section of the wall of the caval vein was isolated in a curved arterial clamp, and an aperture was cut in it. The cold liver graft was then placed in the abdominal cavity and the superior caval vein of the graft was to the recipient's caval vein anastomosed end-to-side with a running suture (9/0; DR 8 Mirafil^{R} blue, B.BRAUN, Melsungen, W-Germany). The portal vein of the graft was connected with the cannula, which was inserted into the right iliac artery and fixed in position with an encircling suture. In this way arterialization of the portal vein of the graft was achieved. Circulation was restored by releasing the clamp on the cannula. In some cases we evaluated changes in the morphology of the graft up to eight days after the transplantation. When an experiment required survival longer than 48 hr, the common bile duct of the graft was brought into the jejunum by the pull-through technique.

After circulation of the graft was established, 0.7 ml

bicarbonate (1000 mmol $HCO3^-$/l) was given intravenously every other hour during the first five post-operative hours (1). The incision was closed with Autoclips^R (BECTON DICKINSON, Parsipanny, USA). Most of the recipients developed severe coagulopathy, especially those given more then 500 mg/kg of galactosamine. Heating of the autoclips prevented blood loss from the abdominal muscles and the skin. Figure 2.2 showes the schematic drawing of the auxiliairy partial liver transplantation.

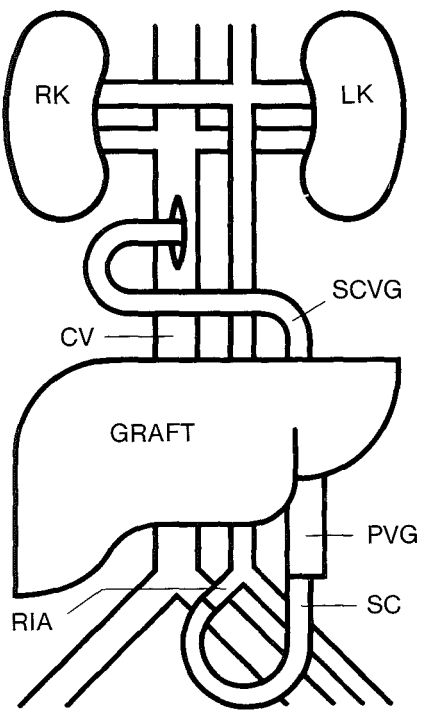

Fig. 2.2.
Schematic drawing of the anatomy after APLT

RK/LK = right/left kidney
CV = caval vein
SCVG = superior caval vein graft
RIA = right iliac artery
PVG = portal vein graft
SC = silastic cannula

Finally, 2.5 ml 5% glucose was given subcutaneously, and this was repeated five times during the first postoperative day. Twenty-four hours after the operation, a second dose of Depomycine^{R} was given.

2.9. Parameters

2.9.1. Biochemistry

The levels of serum glutamic-oxaloacetic transaminase (SGOT), serum glutamic-pyruvic transaminase (SGPT), bilirubin, and glucose were determined with standard laboratory equipment and techniques.

The bromsulfophtalein measurements were performed in a colorimeter (BECKMAN, Brea, California, USA) at 540 nm.

Antipyrine clearance was tested by high performance liquid chromatography (Beckman); Column (CHROMOPACK^{R}, Middelburg, The Netherlands), no: 28811, packing material: 10 RP 18 with mobile phase of methanol/water (55/45) at a flow rate of 2 ml/min (wave-lenght 254 nm).

The blood-gas measurements were performed with a automatic-blood-gas analyser (AVL System 945^{R}, Graz, Austria).

2.9.2. Histology

Samples were fixed in a 3.6% formaldehyde solution. The fixed tissue was dehydrated with ethanol and embedded in Paramat^{R}. Sections were cut to a thickness of 3 micron and stained with hematoxylin-eosin (HE). The Periodic Acid-Schiff (PAS) trichrome technique for polysaccharides and mucopolysaccharides was used in a few liver samples.

2.9.3. Function tests

Bromsulfophtalein (BSP) clearance test: A dose of 8 mg BSP (Bromthalein^{R}, E.MERCK, Darmstadt, W-Germany) per 100 g body weight injected via the tail vein gave maximum storage capacity of the liver of Wag/Rij rats (5). Blood was collected from the tip of the tail 2, 5, 10, and 20 min after the injection of BSP. The animals were not continuously anesthetized during the test, and received only brief ether sedation for the tail cut. A 20-ul aliquot of the plasma sample was added to 5 ml 0.5 N NaOH in 0.9% NaCl.

Antipyrine clearance test: Antipyrine in a dose of 100 mg per 1000 g body weight was injected via the tail vein and blood was collected from the tip of the tail 1, 2, and 4 hr after the injection. Ether sedation was given during the tail cut, because it is known that a short ether anesthesia does not significantly change antipyrine kinetics in rats (23). To 100 ul plasma, 200 ul acetonitril was added. After centrifugation the supernatant was stored at -20^{0}C. A 10 ul sample was chromatographed at room temperature.

2.10. Statistical analysis

The statistical significance of values obtained for effects on the survival rate were tested with the Kaplan-Meier method. This methods regards the cumulative survival times. The test starts with the total amount of used animals (100%). The number of rats which succumbed are counted at various intervals (0-10, 10-24, 24-34, 34-48, etc., up to and 168 hr) after galactosamine. The graph shows at each interval mentioned, the percentage of survival. The last interval for each survival course represents the percentage of surviving rats (28).

The statistical significance of values obtained for effects on regeneration in terms of biochemical parameters determined after a sub-lethal dose of galactosamine was tested with the Wilcoxon test (28).

CHAPTER 3

Dose-response relation of galactosamine in various rat strains

Before the investigation into the therapeutic effect of various treatment modalities on the course of experimentally induced acute hepatic failure could be started, a model was needed for evaluation of the selected forms of treatment. A variety of parameters are of importance in this respect, such as the dosage of the toxic substance to be used and the species and strain of the animals (3,7,24,25). The dosage is particulary important, and by preference should give a death rate of 80 to 90% (LD_{90}). The dose-response relation of galactosamine and the survival rate were first investigated in two strains of rats, BN and Wag/Rij. Later, the sensitivity to galactosamine of the Lewis, Wag/Cpb, and Cpb:Wu strains was also assessed. All of the dose-response data are reported in this chapter.

3.1. Dose-response relation and survival rate of galactosamine in Wag/Rij and BN rats

A dose response-relation for galactosamine and survival rate were estimated for the two strains of rats, Wag/Rij and BN, which have been used for many years in our laboratory for transplantation research. The serum levels of glutamic-oxaloacetic transaminase (SGOT), glutamic-pyruvic transaminase (SGPT), and bilirubin were determined in samples of blood taken from the tip of the tail 24 hr after the injection of galactosamine.

Table 3.1 shows the survival rate and some liver function parameters for Wag/Rij and BN rats given various doses of galactosamine. The Wag/Rij rats had higher transaminase levels and shorter survival than the BN rats ($p<0.001$). Twenty-four hours after the administration of 250 mg/kg galactosamine, these levels were even

higher in Wag/Rij rats than in BN rats given a ten times higher dose of galactosamine. These findings show that unlike Wag/Rij, the BN rat strain is relatively insensitive to the toxic effect of galactosamine and therefore not suitable for use as a model for hepatic failure induced by this toxic agent.

Table 3.1.
Survival of Wag/Rij and BN rats after the indicated intraperitoneal doses of galactosamine.

Rats	d-GL (mg/kg)	SGPT (u/L)	SGOT (u/L)	Bilirubin (umol/L)	Survival
Wag/Rij	0	52[a] ± 13	52 ± 5	< 2	4/4[b]
	250	836 ± 815	1023 ± 946	3 ± 1	4/4
	500	7433 ± 2126	9457 ± 2469	23 ± 3	3/4
	1000	4215 ± 470	9780 ± 1532	32 ± 13	0/4
	3000	6428 ± 1320	7865 ± 690	39 ± 8	0/4
BN	0	27 ± 3	56 ± 9	< 2	4/4
	1000	164 ± 90	355 ± 195	< 2	4/4
	3000	189 ± 90	378 ± 167	< 2	4/4

Tail blood was collected 24 hr after the injection of galactosamine for the determination of SGOT, SGPT, and serum bilirubin levels.
[a] Mean value ± S.D.
[b] Number of rats that survived/total number of rats

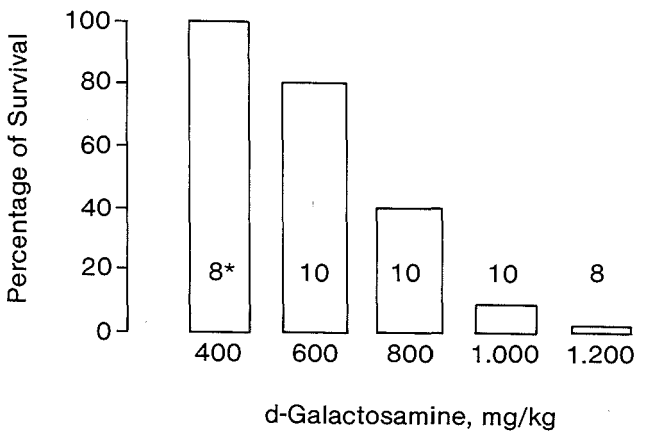

Fig. 3.1.
Percentage survival after intraperitoneal administration of the indicated doses of galactosamine

* Number of rats used per dose

The dose-response relation for galactosamine and the survival rate for the Wag/Rij rat strain was estimated in more detail to establish the LD_{90} value.

Figure 3.1 shows the relation between the dose of galactosamine and the survival rate in Wag/Rij rats. The LD_{90} dose was found to be 1000 mg/kg. Figure 3.2 shows the survival time distribution for Wag/Rij rats. The majority of the rats (64%) died 48 hr (second night) after receiving galactosamine.

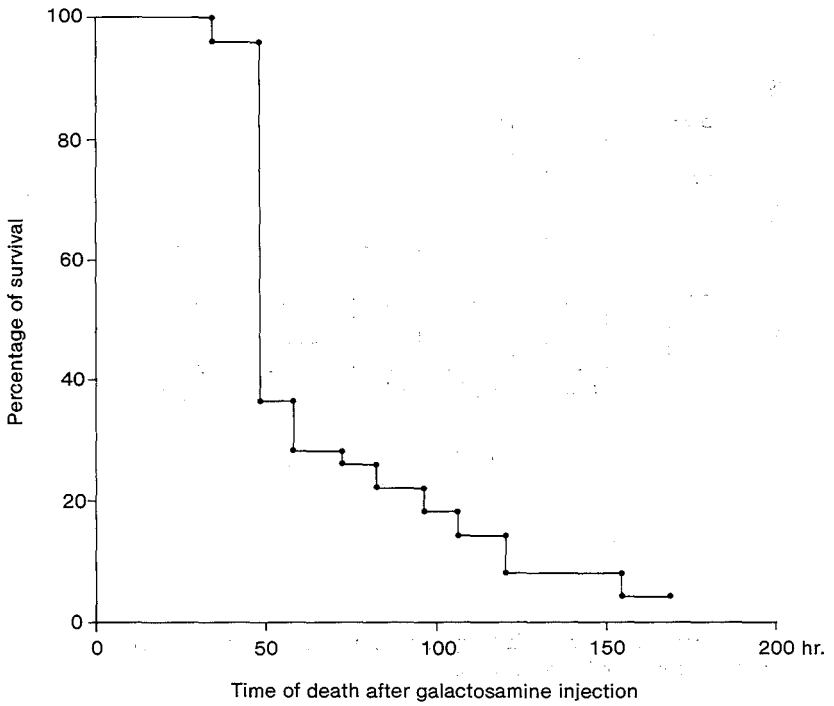

Fig. 3.2.
Course of survival time of rats which succumbed to the galactosamine poisoning (n=24).

3.2. Selection of a sub-lethal dose of galactosamine for Wag/Rij rats on the basis of liver function tests

In a later stage of this study we wanted to investigate the effect of different treatment modalities on deteriorating liver function. As parameter for liver function we used the clearance of bromsulfophthalein (BSP)(5) and antipyrine. After a sub-lethal dose of galactosamine both of these functions deteriorated and then recovered within a few days without treatment (30,32).

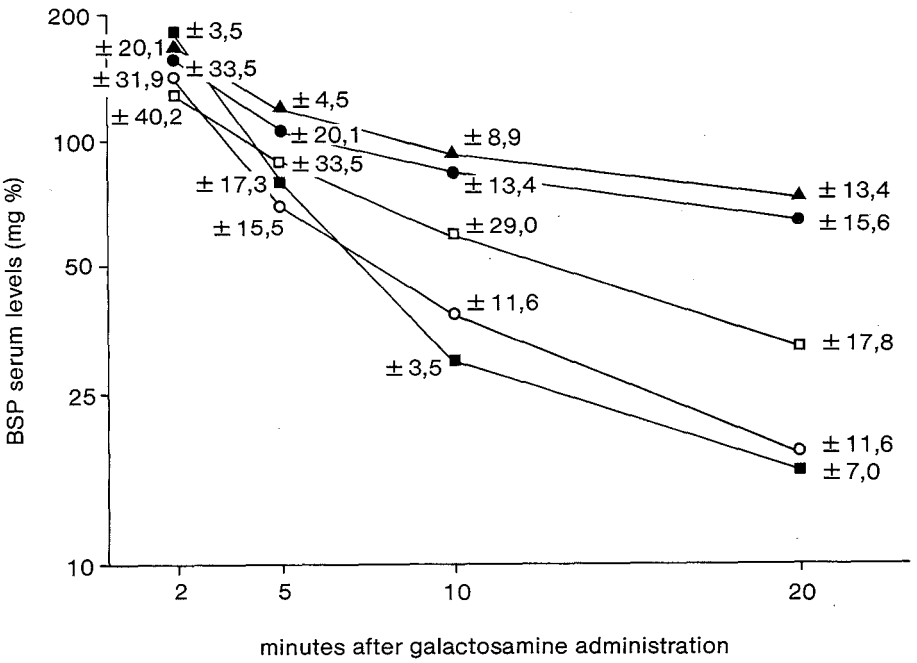

Fig. 3.3.
Clearance of bromsulfophthalein (BSP) as reflected by plasma levels in Wag/Rij rats given 8 mg per 100 g body weight, 48 hr after injection of 125, 250, 400, and 500 mg/kg galactosamine and saline. Blood was collected from the tip of the tail 2, 5, 10, and 20 min after the BSP injection (mean values ± S.D.).

Galactosamine was given in various doses:
125 mg/kg (n=5)(■)
250 mg/kg (n=5)(□)
400 mg/kg (n=5)(▲)
500 mg/kg (n=5)(●)
Control rats: saline (n=15)(o).

For selection of the appropriate sub-lethal dose of galactosamine, the BSP clearance test was performed in rats 48 hr after the administration of 0, 125, 250, 400, and 500 mg/kg galactosamine. From previous experiments on the induction of a spontaneously reversible liver damage we knew that a dose of about 500 mg/kg was the highest tolerable for Wag/Rij rats (6).

Figure 3.3 shows the BSP elimination values (given as mean $\pm$ S.D. in hours) determined 48 hr after administration of various doses of galactosamine. The lowest dose of galactosamine used (125 mg/kg) (clearance: half-life value (125 mg/kg) 5.8 $\pm$ 1.0) did not change the normal elimination curve significantly. The other three doses gave longer clearance rates, i.e., 9.0 $\pm$ 2.4, 17.2 $\pm$ 6.0, and 15.7 $\pm$ 4.7, for doses of 250, 400, and 500 mg/kg, respectively. The clearance rate was similar for 400 and 500 mg/kg galactosamine, but significantly shorter in rats given 250 mg/kg ($p<0.01$). On these grounds a dose of 400 mg/kg galactosamine was selected for the experiments on the deterioration of liver function.

3.3. Dose-response relation of galactosamine in Wag/Cpb, Cpb:Wu, and Lewis rats

The mean survival time of the Wag/Rij rats after galactosamine intoxication was 48 hr. Because this interval proved to be rather short for the present study, we determined the sensitivity of three other rats strains to galactosamine toxicity, i.e., WAG/Cpb, an inbred WAG strain with a genetic composition differing from that of WAG/Rij (3); Lewis, a strain which has been used by other investigators for the induction of acute hepatic failure (46-52, 84-86); and Cpb:Wu, an outbred Wag strain (Wistar).

Table 3.2 shows the survival of these rats after various doses of galactosamine. The differences in their sensitivity to galactosamine are significant. The Wag/Cpb strain proved to be significantly less sensitive to galactosamine than the Wag/Rij strain and was therefore discarded. The sensitivity for galactosamine of the Cpb:Wu strain was comparable to that of the Wag/Rij strain. Since the

Cpb:Wu is an outbred strain, it is suitable for experiments with cytosol but not for experiments involving transplantation of isolated hepatocytes.

The sensitivity of the Lewis strain was comparable to that of the Wag/Rij strain.

Figure 3.4 shows the survival-time distribution for the Lewis and Cpb:Wu strains and, for comparison, the Wag/Rij strain. The Lewis rats showed the same mortality pattern as the Wag/Rij rats. Most of the Cpb:Wu rats (73%) died 72 hr after galactosamine administration, i.e., one day later than the death of most (64%) of the Wag/Rij rats.

For the experiments on liver function recovery we selected a dose of 1500 mg/kg galactosamine for the Lewis and 1000 mg/kg for the Cpb:Wu rats. Figure 3.5 shows the course of antipyrine clearance in the Cpb:Wu strain after a sub-lethal dose (1000 mg/kg) of galactosamine, and gives the course of the half-life values of antipyrine in the Lewis strain given a sub-lethal dose (1500 mg/kg) of galactosamine.

Table 3.2.
Survival rate and the SGOT (mean, U/l) levels in four rat strains (Wag/Cpb, Cpb:Wu, Lewis, Wag/Rij) after various doses of galactosamine (0, 500, 1000, 2000, and 3000 mg/kg).

	Wag/Cpb		Cpb:Wu		Lewis		Wag/Rij	
d-GL mg/kg	Survival	SGOT (u/L)	Survival	SGOT (u/L)	Survival	SGOT (u/L)	Survival	SGOT (u/L)
0	4/4[a]	94[b]	4/4	40	4/4	117	4/4	52
500	4/4	740	4/4	840	4/4	1142	3/4	9457
1000	3/4	954	1/4	1641	4/4	820	0/4	9780
2000	4/4	1042	1/4	3355	1/4	1640	-	-
3000	4/4	1317	0/4	2578	0/4	2455	0/4	7865

Tail blood was collected 24 hr after the injection of galactosamine for the determination of SGOT levels.
[a] Number of rats which survived/total number of rats
[b] Mean values

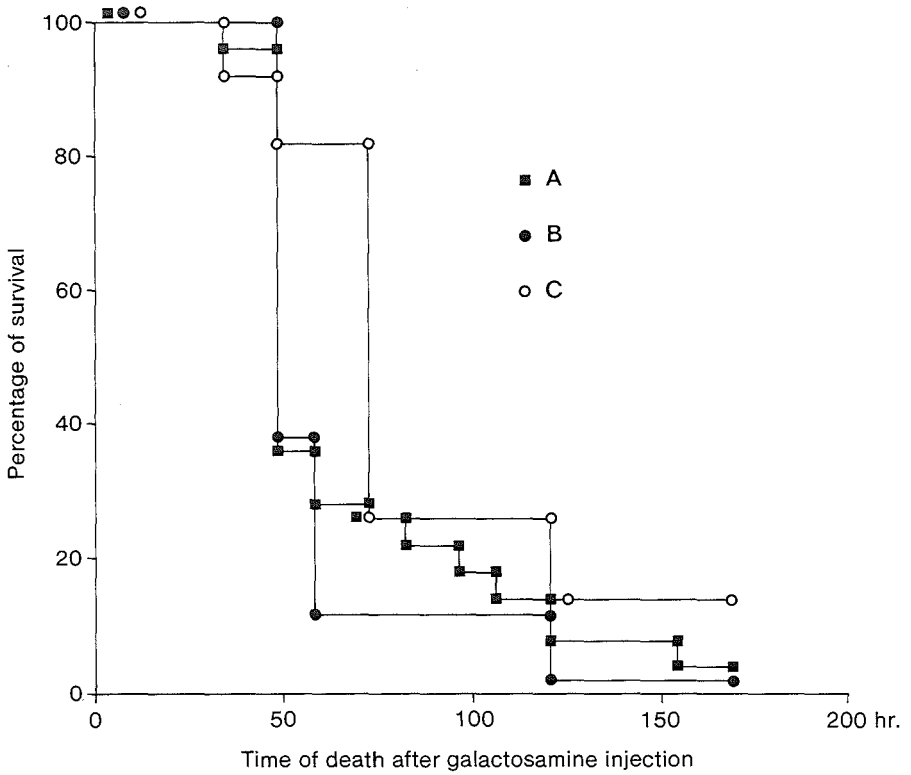

Fig. 3.4 A-C.
Course of the survival time of animals which succumbed to galactosamine poisoning.

A: Wag/Rij rats (n=24)
B: Lewis rats (n=10)
C: Cpb:Wu rats (n=10)

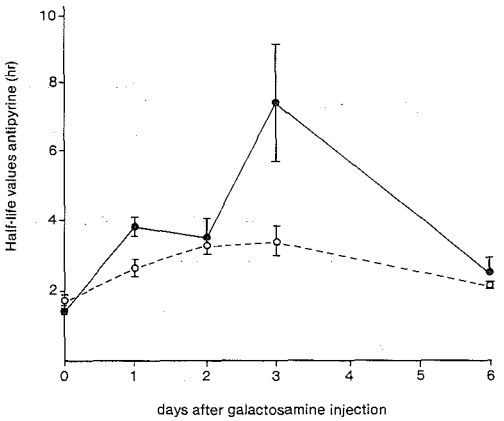

Fig. 3.5.
Course of antipyrine half-life values in Cpb:Wu rats after administration of 1000 mg/kg galactosamine and Lewis rats after administration of 1500 mg/kg galactosamine (values are mean and standard deviation).

——— : Cpb:Wu rats (n=10)
– – – : Lewis rats (n=10)

3.4. Conclusions

The results of the studies in rats of the BN, Wag/Rij, Wag/Cpb, Cpb:Wu, and Lewis strains showed that BN and Wag/Cpb were not sensitive to galactosamine, using the survival as parameter. Further analysis of the survival-time data for the Wag/Rij, Cpb:Wu, and Lewis strains showed that the majority (ca.60%) of the Wag/Rij and the Lewis rats died 48 hr after galactosamine administration and 73% of the Cpb:Wu rats at 72 hr.

For the survival studies the lethal dosage of galactosamine chosen for Wag/Rij rats was 1000 mg/kg. For the liver function studies in the Wag/Rij, Lewis, and Cpb:Wu strains the sub-lethal dose was put at 400, 1500, 1000 mg/kg galactosamine, respectively.

CHAPTER 4

Effects of different treatment modalities on the survival rate of Wag/Rij rats

Positive effects of transplantation of viable isolated hepatocytes, administration of homogenate or cytosol of normal or regenerating liver tissue, or administration of cell-free supernatant of hepatocyte cultures on the survival rate in galactosamine hepatitis have been reported, as discussed in the introduction of this thesis (2,15,20,46-52,63,64,83-86,90). These investigators considered that the evidence pointed strongly to the existence of a trophic factor that stimulates regeneration in such a way that significantly more rats survive after the administration of a hepatotoxic substance in a dosage causing so much cell injury that the majority of the animals die (LD_{90}). We investigated various therapeutic modalities to evaluate their effect on the survival rate of rats suffering from acute hepatic failure. In all of the experiments mentioned in this chapter, 1000 mg/kg galactosamine was administered intraperitoneally to induce lethal damage to the liver.

4.1. Treatment with hepatocytes injected into the abdominal cavity

The effect of transplantation of intact hepatocytes on the survival rate of rats after the intraperitoneal administration of a lethal dose of galactosamine (1000 mg/kg) was investigated first. At various intervals (6, 12, and 24 hr) after administration of galactosamine, viable hepatocytes ($40x10^6$) were injected into the abdominal cavity. Different rats were used for each interval. A group of control rats received the same volume of medium alone 24 hr after the injection of galactosamine.

Figure 4.1 shows the survival-time distribution for rats given

$40x10^6$ isogenically isolated hepatocytes 6, 12, or 24 hr after the galactosamine injection. No significant differences were observed between the groups given hepatocytes and the controls which received only saline.

4.2. Treatment with hepatocyte fractions

4.2.a. The effect of the administration of crude homogenate prepared from normal liver tissue on the survival rate after galactosamine intoxication was studied. Crude homogenate was injected intraperitoneally into all rats in the experimental group (n=12) 12 and 24 hr after the injection of galactosamine. Control rats received only the same volume of saline administered twice after the galactosamine injection (n=7).

4.2.b. The same experiment was performed with crude homogenate of regenerating liver tissue. One group of rats (n=15) was treated twice (12 and 14 hr after galactosamine poisoning) with crude homogenate prepared from liver tissue of donor rats which had undergone a 70% hepatectomy 72 hr earlier. The interval of 72 hr was chosen on the basis of the results of the study performed by Terblanche et al.(88). A second group (n=13) was treated with crude homogenate prepared from liver tissue of donor rats which had undergone a 70% hepatectomy 24 hr earlier. The interval of 24 hr was chosen on the basis of the findings made in several studies performed in rats (41,53,61,64). Control rats received only the same volume of saline administered twice after the galactosamine injection (n=6).

Figure 4.2 shows the survival-time distribution for the rats given a crude homogenate of normal tissue (group B), a crude homogenate of regenerating liver tissue prepared 72 hr after partial hepatectomy (group C) or 24 hr after partial hepatectomy (group D), in all cases 12 and 24 hr after the injection of galactosamine. Group A, the control group, received saline after the galactosamine injection.

A significant difference ($p = 0.01$) was found between the group

treated with crude homogenate (C and D) of regenerating liver tissue and the group treated with saline instead of homogenate. All of the treated rats died during the second day.

4.2.c. The same experiment was performed with cytosols of normal liver tissue and regenerating liver tissue. Figure 4.3 shows the survival-time distribution for rats given the cytosol at various intervals after the galactosamine injection. Cytosol prepared from regenerating liver tissue sampled 24 hr after a 70% partial hepatectomy, was injected 12 and 24 hr after the galactosamine injection (group B). In group C cytosol was administered 12 and 24 hr after galactosamine but the cytosol had been prepared 72 hr after 70% hepatectomy. Group D received cytosol of normal liver tissue 12 and 24 hr after galactosamine administration. Group A (the controls) received only the same volume of medium 12 and 24 hr after galactosamine. In this experiment none of the treatment modalities improved the survival rate significantly.

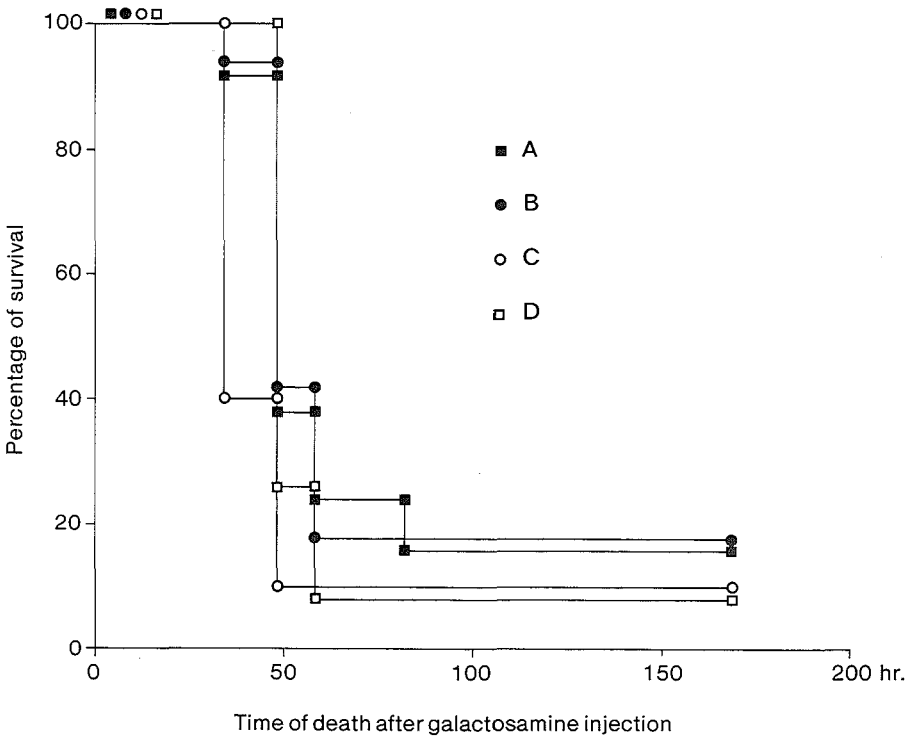

Fig. 4.1 A-D.
Results obtained with hepatocytes administered intraperitoneally, expressed as the distribution of survival times of rats given 1000 mg/kg galactosamine.

A: Rats given saline 24 hr after galactosamine (n=12)
B: Rats given 40 x 10^6 hepatocytes 24 hr after galactosamine (n=17)
C: Rats given 40 x 10^6 hepatocytes 12 hr after galactosamine (n=10)
D: Rats given 40 x 10^6 hepatocytes 6 hr after galactosamine (n=15)

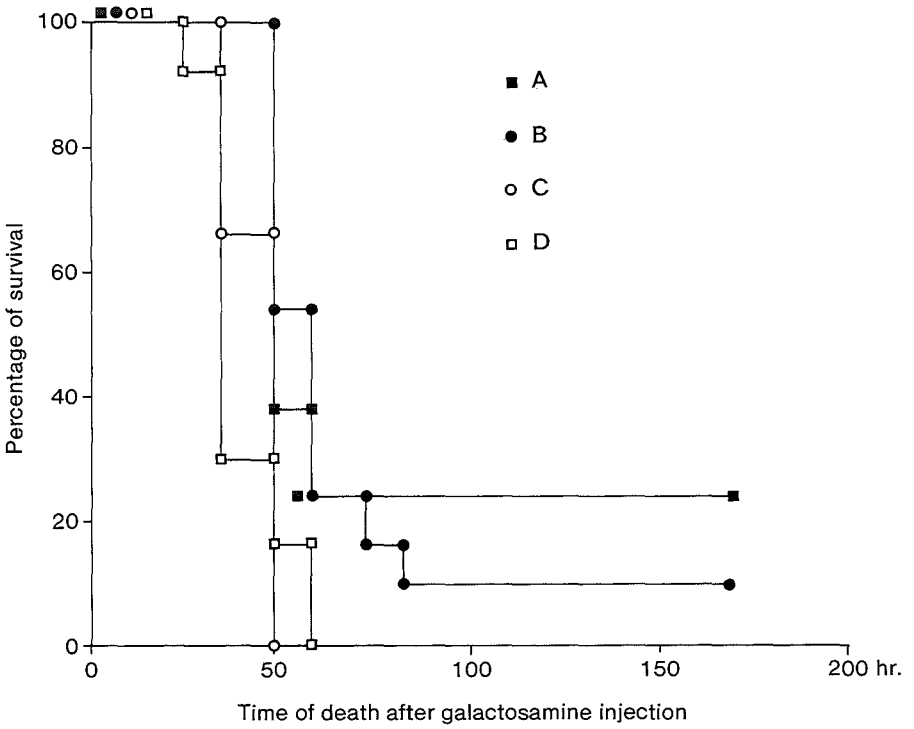

Fig. 4.2 A-D.
Results obtained with liver homogenate injected intraperitoneally, expressed as the distribution of survival times of rats after administration of 1000 mg/kg galactosamine.

A: Rats given saline 12 and 24 hr after galactosamine (n=13)
B: Rats given crude homogenate of normal liver tissue 12 and 24 hr after galactosamine (n=12)
C: Rats given crude homogenate of regenerating liver tissue prepared 72 hr after 70% hepatectomy in the donor and administered 12 and 24 hr after galactosamine (n=15)
D: Rats given crude homogenate of regenerating liver tissue prepared 24 hr after 70% hepatectomy in the donor and administered 12 and 24 hr after galactosamine (n=13)

4.3. Treatment with cell-free supernatant

The effect was investigated of the administration of cell-free supernatant prepared from liver cell cultures 24 hr after plating, on the survival rate after an injection of galactosamine. One group of rats (A) was given intraperitoneally 2 ml of this culture medium 12 and 24 hr after galactosamine poisoning. The control group (B) received the same volume of medium alone at the same time.

Figure 4.4 shows the survival-time distribution for rats given cell-free supernatant from hepatocyte cultures. No significant difference was found between the group treated with cell-free supernatant and the group given normal medium.

4.4. Treatment with hepatocytes implanted in the spleen

Implantation of liver cells in the spleen 24 hr after administration of 1000 mg/kg galactosamine led to severe bleeding problems. All rats died within three hours after receiving hepatocytes (Fig.4.5 A). Injection of saline into the spleen 24 hr after 1000 mg/kg galactosamine had no influence on the normal survival rate (Fig. 4.5 B). Problems of the same kind developed when we injected hepatocytes in the spleen 12 hr after galactosamine administration. All rats died within five hours after implantation (Fig.4.5 C). Implantation of cells in the spleen 6 hr after galactosamine did not lead to coagulation problems but the rats still died sooner (Fig.4.5 D) than those given saline and hepatocytes in the spleen (Fig.4.5 E). There was no mortality after implantation of hepatocytes in the spleen of normal rats. Implantation in the spleen has also been reported by others to give rise to severe clotting disorders (54).

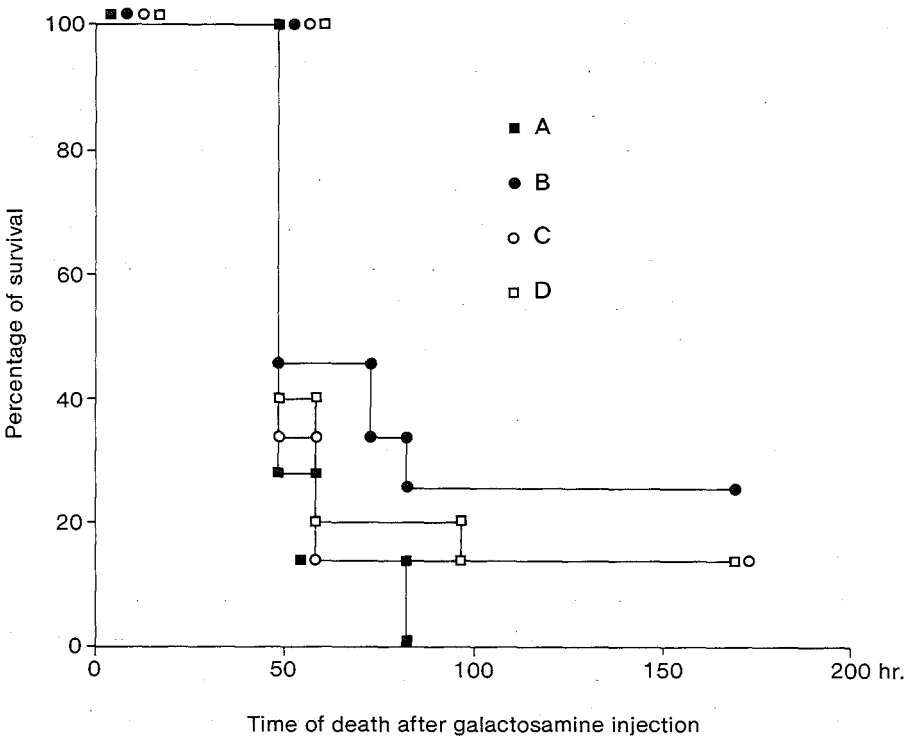

Fig. 4.3 A-D.
Results of treatment with cytosol administered intraperitoneally, expressed as the distribution of survival times of rats after administration of 1000 mg/kg galactosamine.

A: Rats given saline 12 and 24 hr after galactosamine (n=7)
B: Rats given cytosol of regenerating liver tissue prepared 24 hr after 70% hepatectomy in the donor and administered 12 and 24 hr after galactosamine (n=15)
C: Rats given cytosol of regenerating liver tissue prepared 72 hr after 70% hepatectomy in the donor and administered at 12 and 24 hr after galactosamine (n=15)
D: Rats given cytosol of normal liver tissue 12 and 24 hr after galactosamine (n=15)

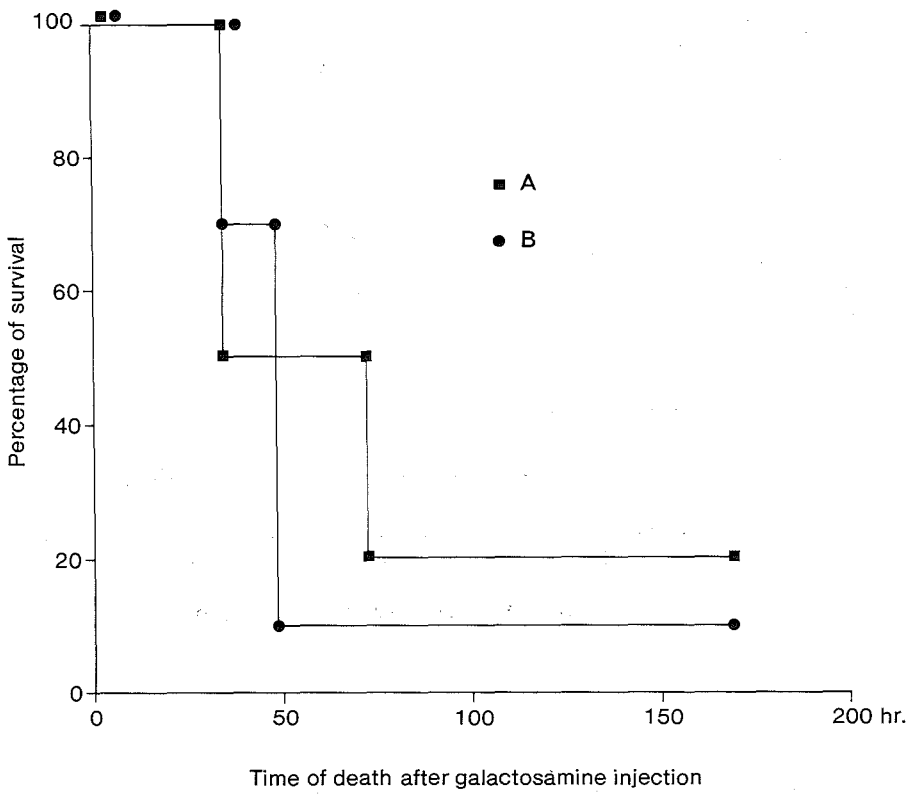

Fig. 4.4 A and B.
Distribution of survival times of rats treated intraperitoneally with cell-free supernatant after 1000 mg/kg of galactosamine.

A: Rats given normal culture medium 12 and 24 hr after galactosamine (n=10)
B: Rats given cell-free supernatant prepared from liver cell cultures 24 hr after plating of cells, and administered 12 and 24 hr after the galactosamine injection (n=10)

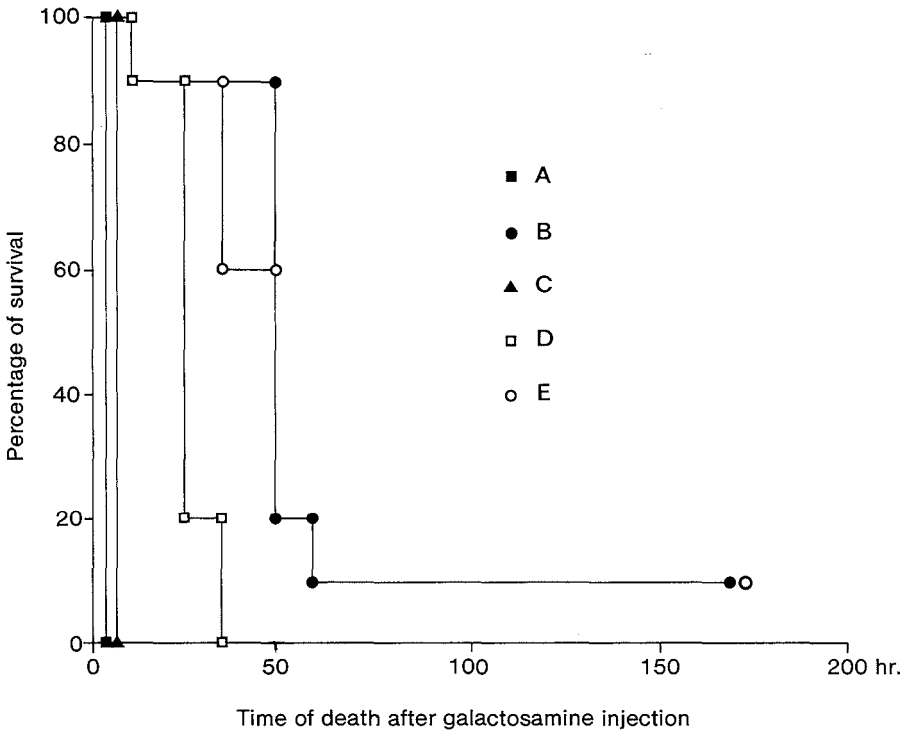

Fig. 4.5 A-E.
Results of implantation of hepatocytes in the spleen, expressed as the distribution of the survival times of rats given 1000 mg/kg galactosamine.

A: Rats given 20-30x10^6 hepatocytes injected into the spleen 24 hr after galactosamine (n=10)
B: Rats given saline injected into the spleen 24 hr after galactosamine (n=10)
C: Rats given 20-30x10^6 hepatocytes injected into the spleen 12 hr after galactosamine (n=10)
D: Rats given 20-30x10^6 hepatocytes injected into the spleen 6 hr after galactosamine (n=10)
E: Rats given saline injected into the spleen 6 hr after galactosamine (n=10)

4.5. Conclusions

Treatment with syngenically isolated hepatocytes did not improve the survival rate of rats suffering from acute liver failure induced by galactosamine. The same holds for the rats treated with hepatocyte fractions and cell-free supernatant. In the rats treated with hepatocytes implanted in the spleen, the surgical and implantation procedures had a lethal effect on the animals. The bleeding problems encountered after implantation of the hepatocytes were severe. Another problem was that some of the hepatocytes entered through the lienal vein and reached the liver, where they caused a severe portal hypertension as noticed during autopsy.

The distribution of the survival times of the control rats in the different treatment groups did not differ significantly. Rats given galactosamine only were compared with the rats treated with one of the modalities. Of the 42 control rats, 62% died during the second night after the galactosamine injection; 74% of the 42 rats given isolated hepatocytes died during the second day after galactosamine; 78% of the 40 rats given homogenate, 60% of the 45 rats treated with cytosol, and 90% of the 10 rats treated with cell-free supernatant also died on the second day.

Transplantation of viable hepatocytes led to a mean survival time of about 48 hr after the administration of galactosamine, which is rather short. This interval must be sufficiently long to allow expression of the influence of the treatment on the process of galactosamine-induced liver degeneration. However, it must be kept in mind here that hepatocytes transplantated between 6 and 12 hr after galactosamine administration increase the risk of damage due to galactosamine or its metabolic consequences, as will be discussed in the next chapter.

CHAPTER 5

Metabolite Deficit Period

Galactosamine has a patho-physiological effect caused by its trapping of the essential co-factor uridine triphosphate (UTP)(32). A marked decrease in UTP is accompanied by an inhibition of RNA and protein synthesis and other uracil dependant biosynthesis (76,77), which leads to organelle injury and cell necrosis.

Decker and Keppler (14) introduced the term metabolite deficit period to denote a well-defined interval of uridine deficiency, in the present case the interval between galactosamine and uridine administration. Farber and co-workers (18) reported that injection of uridine at the same time as galactosamine prevented the hepatic failure caused by galactosamine. Uridine given up to 3 hr after galactosamine reversed the signs of hepatic failure, but was unable to do so when given as late as 6 hr.

In connection with the short mean survival time (48 hr) of rats given galactosamine, which made it difficult to assess the effects of the therapy on survival, we wanted to lengthen the period after galactosamine injection. The interval between transplantation of hepatocytes and the time of death must sufficiently long to permit observation of the recovery process involved. Prolongation of the effective period of the injected hepatocytes must be obtained by transplantating the cells sooner, but that might expose them to damage arising from the galactosamine-induced UTP deficiency. As reported (34,15) the time when uridine should be given is 6 hr after galactosamine reversal of the liver damage is not obtained (14) but the negative effects on the transplanted hepatocytes are avoided as reported. This chapter deals with the studies performed to determine the dosage of uridine capable of preventing the death of the injected hepatocytes, as well as the most favorable time to apply it, in order not to influence the liver damage.

5.1. Determination of an effective metabolite deficit period

Administration of galactosamine in a dose of 1000 mg/kg, followed by injection of saline, results in a survival rate of 10 percent (Table 5.1. united controle groups: 2 out of 20 rats survived). This is in accordance with the results in previous experiments, in order to determine the LD_{90}. Administration of uridine in various doses together with galactosamine reduced the mortality in a dose-dependent fashion (18,32). The lowest dose of uridine that improved the survival rate was 500 mg/kg (on a molecular basis 11% of the dose of galactosamine (see Table 5.1). The other doses of uridine used in the study were 125, 250, 1000, and 2000 mg/kg uridine. Uridine improved the survival rate significantly when given up to and including 12 hr after galactosamine, but not when administrated after an interval of 18 hr. The duration of the interval between administration of the two compounds did not influence the minimal effective dose of uridine; for all dosages, 500 mg/kg uridine was the lowest dose to give significant enhancement of the survival rate. Figure 5.1 shows the percentage of rats which survived on each of the days after galactosamine injection. The percentage of rats given galactosamine and saline or galactosamine and uridine are presented separately. The majority of the rats in both groups died within 48 hr, which is in accordance with the earlier findings. No differences were found in the survival rate for successive periods between the saline and the uridine groups after galactosamine. Thus, the administration of uridine increased the survival rate but did not create a longer available therapeutic period.

Uridine was administered in doses up to 2000 mg/kg. The highest dose did not influence the SGOT level (Table 3.1: 52 $\pm$ 7 U/l, n=7) in the untreated control rats, where it did not differ from the levels (59 $\pm$ 6 U/l, n=8) measured in the rats given galactosamine and 2000 mg/kg uridine. Administration of uridine led to a dose- and interval-dependent depression of the SGOT levels after galactosamine

administration (Table 5.2). Uridine given 12 hr after galactosamine significantly increased the survival but did not normalize the SGOT levels. Comparison of the SGOT values in all of the rats which survived, with all of the rats which death occured within 48 hr after galactosamine injection and with all rats which died later than 48 hr after galactosamine administration showed for the first group a significantly lower value. (4179 $\pm$ 3055 U/l, n=35; 11378 $\pm$ 3357 U/l, n=18; 11453 $\pm$ 3139 U/l, n=11 (resp.; mean, $\pm$ S.D.)).

The effect of uridine on the serum bilirubin level was comparable to that observed for SGOT (Table 5.3). For the individual rats a significant correlation ($p<0.001$) was found between the SGOT and bilirubin values. Comparison of the bilirubin values of all surviving rats with those of all the rats which died before or after 48 hr also showed for the first group a significant difference (22 $\pm$ 18 umol/l; 58 $\pm$ 17 umol/l; 50 $\pm$ 16 umol/l (resp.; mean $\pm$ S.D.)).

Table 5.1.
Survival rate of Wag/Rij rats after intraperitoneal administration of 1000 mg/kg galactosamine and various doses of uridine, expressed as the number of survivors/total number of rats.

Uridine (mg/kg)	Interval between d-GL and uridine administration 0	6	12	18	24	(hours)
Saline	1/4	0/4	0/4	1/4	0/4	2/20
125	1/4	2/4	–	–	–	
250	0/4	1/4	–	–	–	
500	3/4	4/4	3/4	1/4	0/4	
1000	3/4	4/4	2/4	0/4	2/4	
2000	4/4	4/4	3/4	1/4	0/4	
	10/12	12/12	8/12	2/12	2/12	
	<0.01	<0.01	<0.01	N.S.	N.S.	← P

At various intervals after galactosamine injection, uridine or saline was injected intraperitoneally (0, 6, 12, 18, and 24 hr). Uridine was given in various doses (125 up to and including 2000 mg/kg).

p: level of significance between the pooled data of the control (saline) groups and the pooled data of the groups, which received the three highest doses of uridine (500, 1000, 2000 mg/kg) for the successive intervals.

Table 5.2.
Serum glutamic-oxalocetic transaminase (SGOT) levels (mean, U/l) determined after the injection of 1000 mg/kg galactosamine and various doses of uridine.

Uridine	Interval between d-GL and uridine administration			
(mg/kg)	0	6	12	(hours)
Saline	8350	9780	10693	9608
125	9194	8034	-	
250	12384	9235	-	
500	5618	6439	6641	
1000	4101	5481	10950	
2000	377	1330	9889	
	3365	4417	9160	
	<0.001	<0.001	N.S.	← P

At various intervals after galactosamine injection, uridine or saline was injected intraperitoneally (0, 6, and 12 hr). Uridine was given in various doses (125 up to and including 2000 mg/kg). Each group comprised 4 rats.

p: level of significance between the pooled data of the control (saline) groups and the pooled data of the groups, which received the three highest doses of uridine (500, 1000, 2000 mg/kg) for the successive intervals.

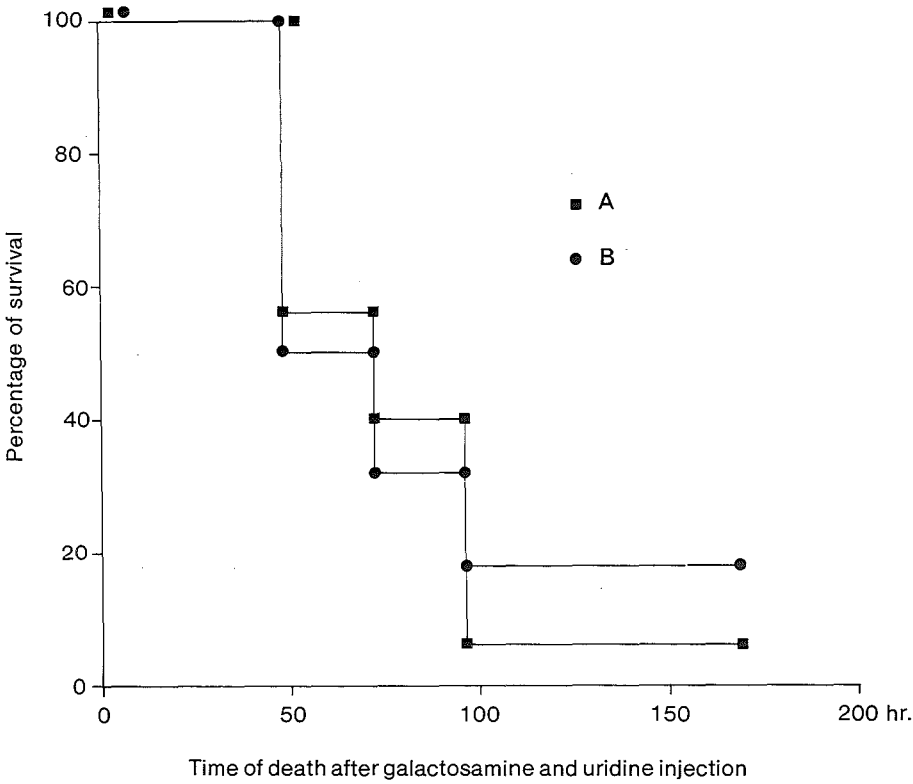

Fig. 5.1.
Distribution of the survival times of the rats which died after an intraperitoneal injection of galactosamine.

Group A: rats given saline at the indicated intervals after galactosamine (see Table 3.1: 18 non-survivors out of 20 rats).
Group B: rats given uridine at the indicated intervals after galactosamine (see Table 3.1: 38 non-survivors out of 76 rats).

Table 5.3.
Serum bilirubin levels (mean, umol/l) determined after the injection of 1000 mg/kg galactosamine and various doses of uridine.

Uridine (mg/kg)	Interval between d-GL uridine administration 0	6	12 (hours)
Saline	41	32	70
125	59	37	–
250	63	32	–
500	26	32	37
1000	14	40	54
2000	<2	11	38
	14	27	43
	<0.01	<0.05	N.S. ← P

At various intervals after galactosamine injection, uridine or saline was injected intraperitoneally (0, 6, and 12 hr). Uridine was given in various doses (125 up to and including 2000 mg/kg). Each group comprised 4 rats.

p: level of significance between the pooled data of the control (saline) groups and the pooled data of the groups, which received the three highest doses of uridine (500, 1000, 2000 mg/kg) for the successive intervals.

5.2. Conclusions

Uridine can reverse the effects of galactosamine but using survival as criterion we were unable to select a dose of uridine that would prevent hepatocellular death when given at the same time as galactosamine but not when given 6 hr after galactosamine. The maximum interval between administration of the two compounds in which uridine still significantly enhanced the survival rate lay between 12 and 18 hr after galactosamine injection. This interval is much longer than those reported by other investigators (14,18,32,76,77). Those authors used biochemical and histological parameters as criterion for liver cell injury, in particular enzyme leakage (SGOT), whereas we used the survival rate, which proved to be a more suitable parameter than enzyme leakage to define the duration of the period in which the toxin induced hepatic lesions are potentially reversible. Use of the SGOT values gives a shorter interval before the stage of irreversibility is reached than use of the survival rate does.

It is evident that with the present model of lethal galactosamine hepatitis and the use of uridine, experimental therapy should not be started earlier than 18 to 24 hr after the injection of galactosamine. Otherwise it will not be clear whether enhanced survival is due to a stimulation of the regeneration or to a reduction of the degenerative action of the galactosamine.

CHAPTER 6

Effects of different treatment modalities on deteriorating liver function in Wag/Rij rats

Chapter 4 dealt with the effect of transplantation of isolated hepatocytes and hepatocyte fractions and cytosol on the survival rate rats suffering from galactosamine-induced acute liver failure. Transplantation of syngenic viable isolated hepatocytes and hepatocyte fractions failed to improve the survival rate. It is possible that the implanted hepatocytes improved the function of the affected liver, but that the interval between transplantation of the hepatocytes and the moment of the animal's death was too short to allow the transplanted hepatocytes to improve survival by restoration of the function of the damaged host liver. Administration of uridine failed to lenghten this period, as reported in Chapter 5 (31,92).

It is also possible that the dose of galactosamine we used (1000 mg/kg) induces irreversible hepatic injury that cannot be reversed by any treatment. The reversibilty of damage to liver cells, as in a fulminant hepatic failure, is still a matter of debate.

In the studies discussed in this chapter we investigated the effect of transplantation of hepatocytes and cytosol on regeneration of liver tissue after a sub-lethal dose of galactosamine. Here, values given by aspecific liver function tests were used as parameter instead of the of survival rate.

6.1. Treatment with hepatocytes injected into the abdominal cavity

Isolated hepatocytes ($40x10^6$) were injected into the abdominal cavity 24 hr after the administration of 400 mg/kg galactosamine. The cell suspension ($20x10^6$ per ml) was injected via a teflon catheter to prevent damage to the cells and decrease the risk of injuring the intestines. The BSP elimination test was performed in rats 1,2,3,4,6

and 8 days after galactosamine injection. The BSP elimination test was performed in the treated rats was performed 1, 2, and 3 days after implantation of the liver cells, i.e., 2, 3, and 4 days after galactosamine. Only one test was performed in each rat, including the control rats given saline instead of galactosamine. Sham-treated rats received only the same volume of medium 24 hr after administration of galactosamine.

Figure 6.1 shows the half-life values for BSP. The value found in sham rats given medium without hepatocytes was significantly ($p<0.01$) enhanced to the same extent (16.5 ± 4.3 and 16.4 ± 5.2, respectively) on days 1 and 2 after galactosamine injection relative to the control rats. On days 3 and 4 the half-life values declined in sham rats (from 13.6 ± 6.2 to 12.1 ± 5.4) but still differed significantly ($p<0.01$) from the values of the control rats. On days 6 and 8 (7.2 ± 0.6 and 5.7 ± 0.2, respectively) the half-life values had returned to normal. The elimination rate of BSP in rats, in which isolated hepatocytes were implanted in the abdominal cavity one day after galactosamine injection, did not differ significantly from the elimination rate found for sham rats treated with medium only (day 2: 16.4 ± 1.4; day 3: 12.3 ± 1.1; day 4: 9.2 ± 2.4). It is noteworthy that the standard deviation of the half-life values within each group increased substantially after the injection of galactosamine.

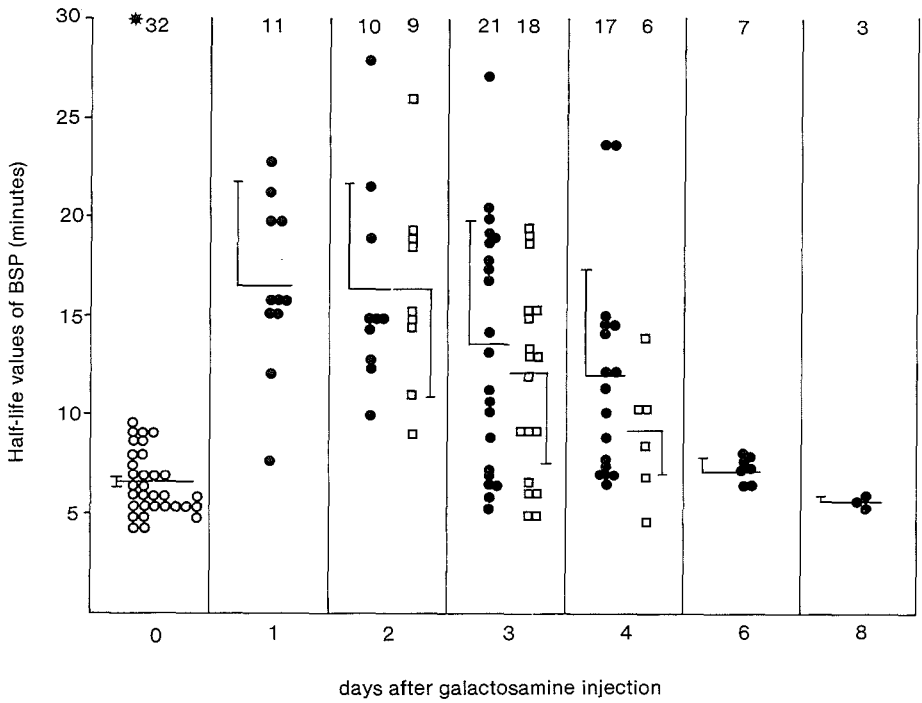

Fig. 6.1.
Half-life values for serum plasma bromosulfophthalein (BSP) in rats treated with isolated hepatocytes after receiving 400 mg/kg galactosamine intraperitoneally.

Isolated syngenic hepatocytes were injected into the abdominal cavity 24 hr after the administration of galactosamine. The half-life values of BSP for the hepatocyte-treated rats were determined 2, 3, and 4 days after galactosamine. The half-life values for the control rats were determined 1, 2, 3, 4, 6, and 8 days after galactosamine.

o: saline medium BSP clearance (control rats)
●: galactosamine medium BSP clearance (sham rats)
□: galactosamine hepatocytes BSP clearance
*: number of rats used
—: mean value ± S.D.

6.2. Treatment with cytosol

Cytosol was injected intraperitoneally 24 hr after the injection of 400 mg/kg galactosamine. The same volume of medium was injected intraperitoneally into the sham-treatment rats. The antipyrine elimination test was performed 24 and 48 hr after the injection of cytosol or injection medium (48 and 72 hr after galactosamine). Only one test was performed in each rat. The antipyrine elimination test was also performed in control rats given saline instead of galactosamine.

Figure 6.2 shows the half-life values for antipyrine. In sham rats, given the medium alone instead of cytosol, these values were significantly elevated 48 hr after injection of galactosamine (from 1.45 ± 0.29 to 2.93 ± 1.38) ($p<0.01$). The antipyrine elimination rate in rats given cytosol intraperitoneally 24 hr after galactosamine did not differ significantly from the values of the sham rats.

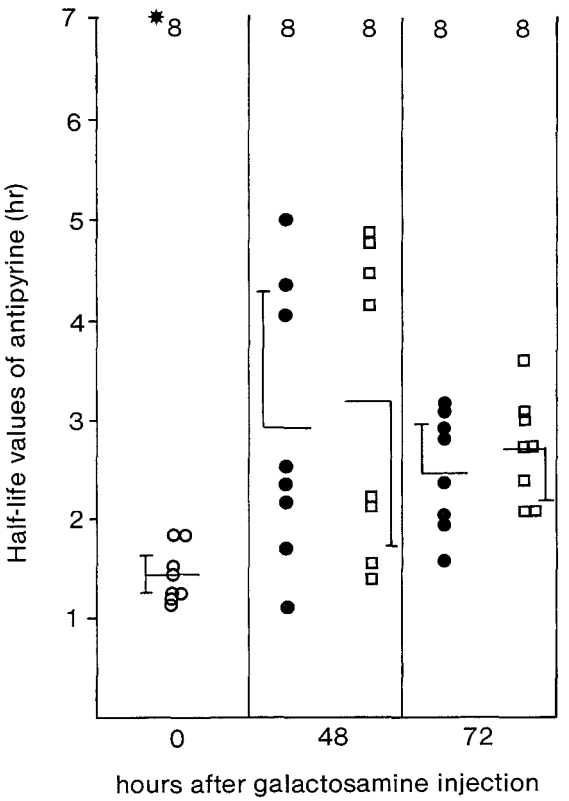

Fig. 6.2.
Half-life values for serum antipyrine in rats treated with cytosol after receiving 400 mg/kg galactosamine intraperitoneally.

The cytosol was injected intraperitoneally 24 hr after the administration of galactosamine. The half-life values for antipyrine for the cytosol-treated rats and control rats were determined 48 and 72 hr after galactosamine injection.

o: saline	medium	antipyrine (control rats)
●: galactosamine	medium	antipyrine (sham rats)
□: galactosamine	cytosol	antipyrine
*: number of rats		
—: mean value ± S.D.		

6.3. The fate of hepatocytes injected into the peritoneal cavity or the spleen

Earlier experiments having shown that improvement of the survival rate would not be obtained with any of the treatment modalities under study and even no improvement of the deteriorating liver function was accomplished. This might been explained by the early death of the injected hepatocytes.

Rats given hepatocytes in the abdominal cavity were killed 6 hr after the injection of the cells. At autopsy, a few hard white clumps of necrotic hepatocytes were found in the abdominal cavity (Fig.6.3). Samples of the same hepatocyte cell suspension were cultured, and the resulting colonies showed good growth (Fig.6.4).

Rats in which hepatocytes were implanted in the spleen were killed after various intervals up to 16 months. Light-microscopical examination of the spleen tissue showed hepatocytes with a normal appearance scattered throughout the organ (Figs.6.5 and 6.6) (56-58,94). After the implantation procedure we noticed that some of the hepatocytes injected into the spleen found their way through the splenic vein to the liver. Histologically, the liver tissue showed small islands of healthy aggregated hepatocytes (Fig.6.7).

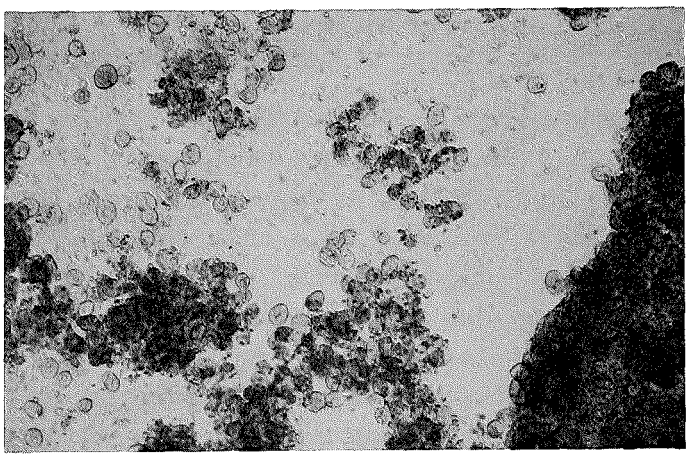

Fig. 6.3.
Clumps of necrotic hepatocytes found in the abdominal cavity 6 hr after intraperitoneal injection of the hepatocytes. The trypan blue exclusion test was applied to assess viability.

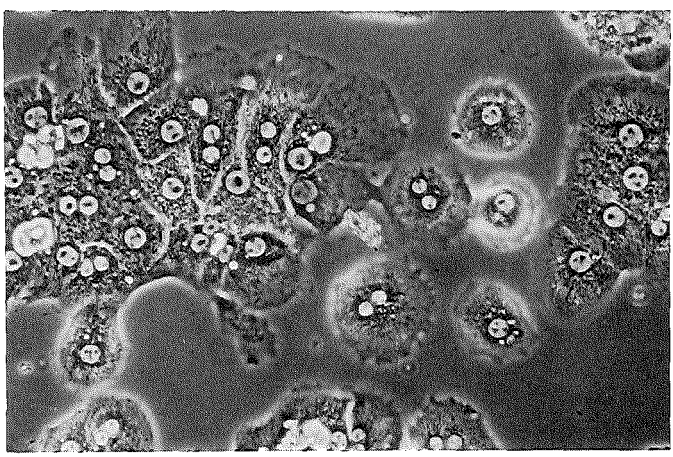

Fig. 6.4.
Phase contrast micrograph of a 24-hr-old cell culture of an isolated hepatocyte sample. x 400.

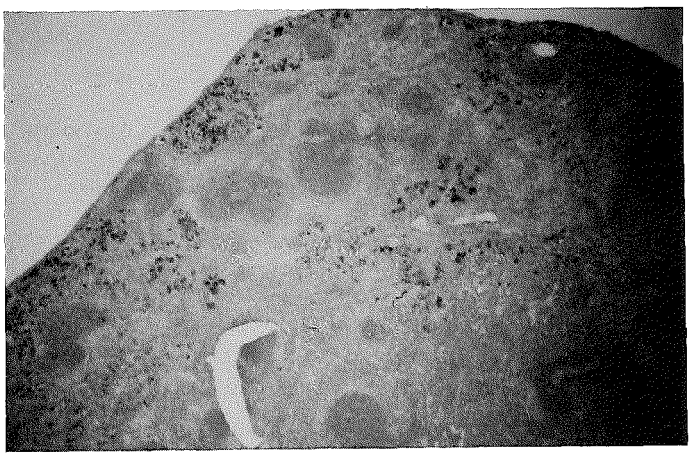

Fig. 6.5.
Light-microscopical section of the spleen with healthy hepatocytes in the spleen 16 months after the implantation. PAS. x 150.

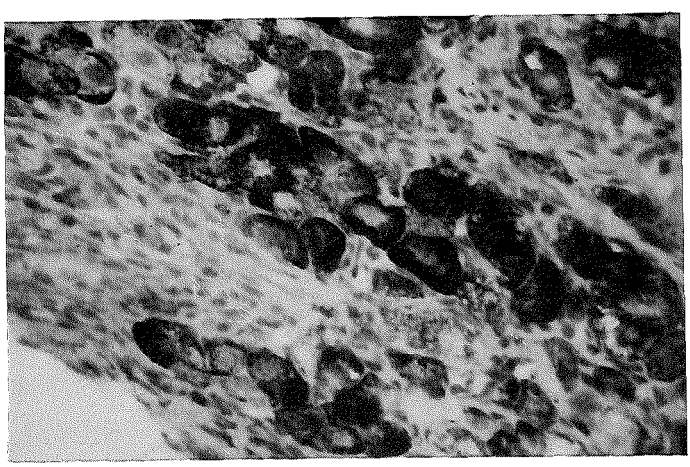

Fig. 6.6.
Light-microscopical section of the spleen with glycogen granules in the cytoplasm of hepatocytes 16 months after implantation.
PAS. x 600.

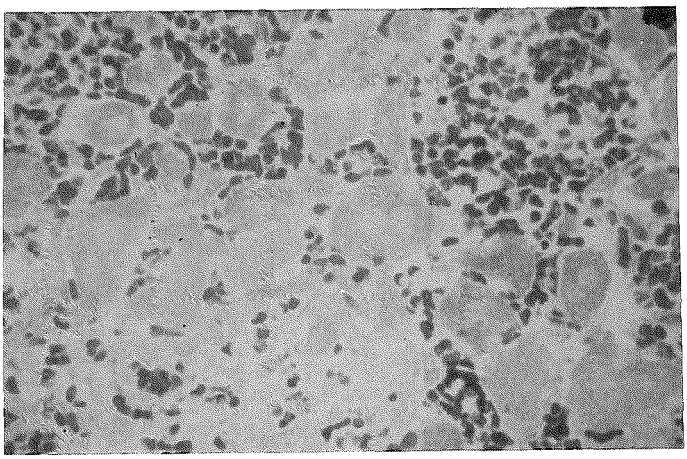

Fig. 6.7.
Light-microscopical section of the liver with hepatocyte island of aggregated hepatocytes 48 hr after implantation of hepatocytes in the spleen. HE. x 150.

6.4. Treatment by implantation of hepatocytes in the spleen

Isolated hepatocytes ($20-30x10^6$) were implantated in the spleen in a cell suspension ($40x10^6$ cells per ml) which was injected 24 hr after the intraperitoneal injection of 400 mg/kg galactosamine. The same volume of medium alone was injected into the spleen of sham rats 24 hr after galactosamine injection. The antipyrine clearance test was performed 24 and 48 hr after the implantation of the cells or injection of the medium, i.e., 48 and 72 hr after the injection of galactosamine. Only one test was performed in each rat. Figure 6.8 shows the antipyrine half-life values in sham rats given medium alone and rats treated with isolated hepatocytes in the spleen. The half-life values of the sham rats were significantly higher at 48 hr (1.65 $\pm$ 0.19 to 4.49 $\pm$ 2.32) than those of the control rats given

galactosamine alone ($p<0.01$). At 72 hr (2.29 $\pm$ 0.59) the half-life values declined, but still differed significantly from those of the control rats ($p<0.05$). The elimination rate of antipyrine in rats given isolated hepatocytes injected into the spleen 24 hr after galactosamine did not differ significantly from the values obtained in sham rats given only medium.

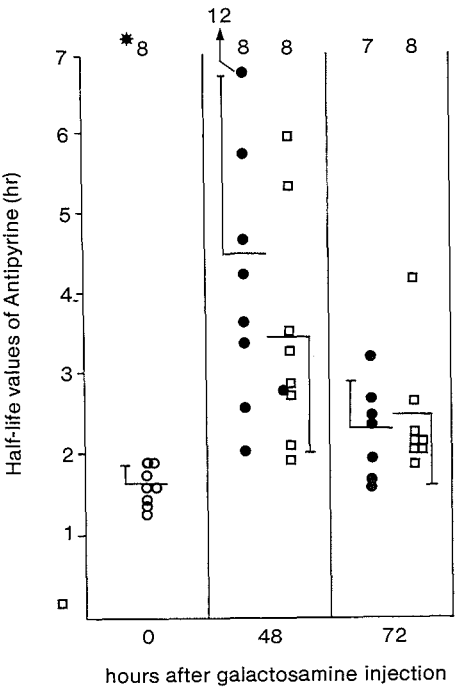

Fig. 6.8.
Half-life values for serum antipyrine in rats given isolated hepatocytes in the spleen after 400 mg/kg galactosamine injected intraperitoneally.

Isolated syngenic hepatocytes were implanted in the spleen 24 hr after the administration of galactosamine. The half-life values for antipyrine in the hepatocyte-treated rats and control rats were determined 48 and 72 hr after galactosamine injection.

○: saline	medium	antipyrine (control rats)
●: galactosamine	medium	antipyrine (sham rats)
□: galactosamine	hepatocytes	antipyrine
*: number of rats		
—: mean value $\pm$ S.D.		

Here again the half-life values fluctuated considerably after the injection of galactosamine, as had been observed for BSP clearance and antipyrine clearance.

6.5. Conclusions

For the studies reported in this chapter a spontaneously reversible degree of hepatic failure was induced. Implantation of syngenic isolated hepatocytes and cytosol did not accelerate the recovery of the deteriorating liver function, as measured by the BSP and antipyrine tests. Determination of the viability of hepatocytes implanted in different regions showed that those in the abdominal cavity died very soon after implantation, unlike those in the spleen, which proliferated up to 16 months' (19,21,35,36,55-58,94). In the view of the present findings it seems unlikely that hepatocytes can be expected to have a stimulating effect within the short period of survival after abdominal injection.

The results of these studies indicate that irreversibility of the injury does not explain the poor survival, because a sub-lethal dose was used. Furthermore, we found comparable and substantial variation of the degree of deterioration of the liver function after the injection of galactosamine. The increase of the half-life values was the same in the BSP and the antipyrine clearance tests. The variation of the clearance-test values within each group was not significantly greater than the variation of the values within each session ($p>0.05$). Marked variability of galactosamine-induced liver injury was also found by Tabata (87).

The results reported in this chapter concerning the influence of implanted hepatocytes on the process of recovery of liver function are similar to those obtained for the survival rate after treatment of the rats with hepatocytes or hepatocyte fractions (Chapter 4). We were unable in either case to reverse the hepatocellular damage induced by galactosamine.

CHAPTER 7

Effects of transplantation of hepatocytes and cytosol on deteriorating liver function in Lewis and Cpb:Wu rats

As discussed in the preceding chapters, we were unable to reproduce the favorable effect of hepatocyte transplantation in the presence of acute hepatic failure on animal survival reported by others (46-52,62,84-86). To be certain that our negative results were not due to a difference in strain sensitivity, we repeated the studies in other strains as used by other authors (24,25,46-52, 84-86). The effects of the same treatment modalities on deteriorating liver function were investigated in an inbred strain (Lewis rats) and an outbred strain (Cpb:Wu rats) given a sub-lethal dose galactosamine.

7.1. Lewis rats treated by implantation of hepatocytes in the spleen

Isolated hepatocytes ($20-30x10^6$) were implanted in the spleen 24 hr after intraperitoneal injection of 1500 mg/kg galactosamine, which is a sub-lethal dose, as reported in Chapter 3. The same volume of medium was injected into the spleen of the control rats as sham treatment. The antipyrine clearance test was performed 24 and 48 hr after implantation of the cells or injection of medium, i.e., 48 and 72 hr after the injection of galactosamine. Only one test was done in each rat. The antipyrine elimination test was also performed in the control rats given saline instead of galactosamine.

Figure 7.1 shows the half-life values of antipyrine. In the rats given medium instead of hepatocytes, the half-life values improved significantly (1.82 ± 0.24 to 4.23 ± 1.18) 48 hr after galactosamine injection ($p<0.01$). The half-life values of antypirine

in rats which received isolated hepatocytes implanted in the spleen 24 hr after galactosamine injection, increased compared with the values of the rats given medium (4.44 ± 0.7 compared with 4.03 ± 1.3). Eight animals died within 3 hr after implantation of hepatocytes in the spleen. The autopsy results showed severe bleeding in the intestine, possibly due to severe portal hypertension.

7.2. Lewis rats treated with cytosol

Cytosol was injected intraperitoneally 24 hr after injection of 1500 mg/kg galactosamine. The same volume of medium was injected into sham rats 24 hr after galactosamine. The antipyrine clearance test was performed 24 and 48 hr after the injection of cytosol or medium, i.e., 48 and 72 hr after galactosamine. Only one test was done in each rats. The antipyrine elimination test was also performed in the control rats.

Figure 7.2 shows the half-life values of antipyrine. The values determined in sham rats given medium instead of cytosol improved significantly (1.83 ± 0.3 to 3.41 ± 0.8) 48 hr after galactosamine injection ($p<0.01$). The antipyrine elimination rate in rats given cytosol intraperitoneally 24 hr after galactosamine did not differ significantly from the values obtained in the sham rats given medium.

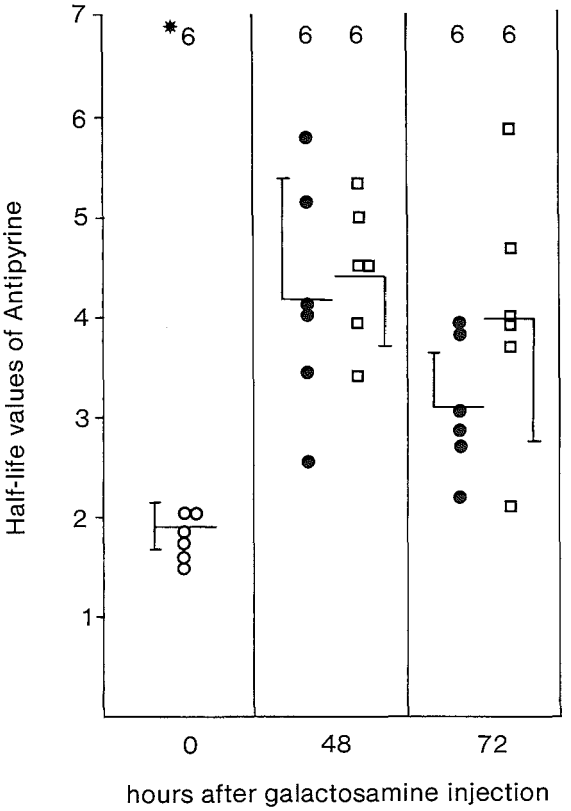

Fig. 7.1.
Half-life values for serum antipyrine in Lewis rats treated by implantation of isolated hepatocytes in the spleen, after 1500 mg/kg galactosamine intraperitoneally.

Isolated syngenic hepatocytes were implanted in the spleen 24 hr after the administration of galactosamine. The values for antipyrine in the hepatocyte-treated rats and control rats were determined 48 and 72 hr after galactosamine injection.

o :	saline	medium	antipyrine (control rats)
● :	galactosamine	medium	antipyrine (sham rats)
□ :	galactosamine	hepatocytes	antipyrine
* :	number of rats		
—— :	mean value ± S.D.		

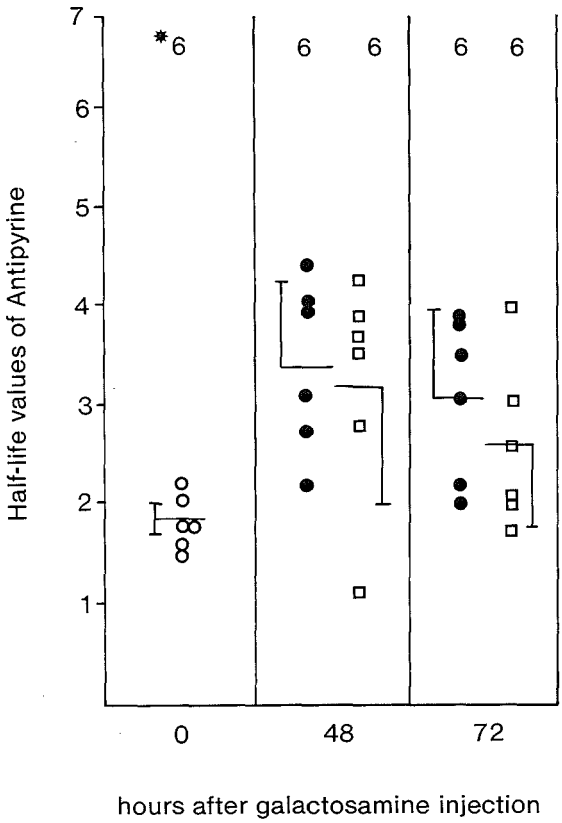

Fig. 7.2.
Half-life values for serum antipyrine in Lewis rats treated with cytosol, after receiving 1500 mg/kg galactosamine intraperitoneally.

Cytosol was injected intraperitoneally 24 hr after the administration of galactosamine. The values for antipyrine in the cytosol treated-rats and control rats were determined 48 and 72 hr after galactosamine injection.

o : saline	medium	antipyrine (control rats)
● : galactosamine	medium	antipyrine (sham rats)
□ : galactosamine	cytosol	antipyrine
* : number of rats		
—— : mean value ± S.D.		

7.3. Cpb:Wu rats treated with cytosol

Cytosol was intraperitoneally injected 24 hr after injection of a sub-lethal dose (1000 mg/kg) of galactosamine. The same volume of medium alone was injected into rats as sham treatment 24 hr after galactosamine administration. The antipyrine elimination test was performed 48 and 72 hr after the injection of cytosol or injection of medium, i.e., 72 and 96 hr after galactosamine. This time interval was chosen based on the course of antipyrine half-life values in the Cpb:Wu rat, as reported in Chapter 3. Only one test was done in each rat. The same test was performed in the control rats given saline instead of galactosamine.

Figure 7.3 shows the half-life values of antipyrine. In the sham rats given medium alone instead of cytosol the values decreased significantly (1.45 ± 0.2 to 6.18 ± 1.8) 72 hr after galactosamine ($p<0.01$). The elimination rate of antypirine in rats given cytosol intraperitoneally 24 hr after galactosamine did not differ significantly from the values obtained in the sham rats. Three rats died before the antipyrine clearance test could be done.

7.4. Conclusions

The conclusions drawn in Chapter 6 hold equally for Lewis and Cpb:Wu strains. The results of the same treatment modalities applied to these rat strains did not reflect causal differences that could explain the negative results obtained in the Wag/Rij strain. In this model of acute hepatic failure, treatment with hepatocytes and hepatocyte fractions failed to stimulate the liver sufficiently for adequate recovery.

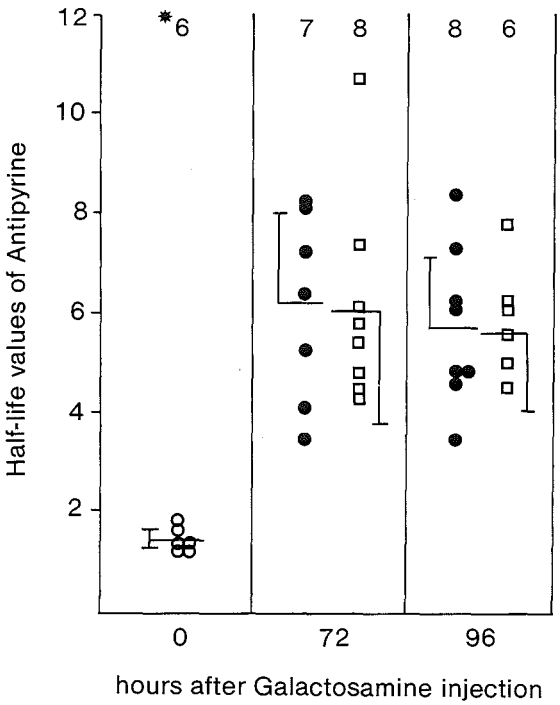

Fig. 7.3.
Half-life values for serum antipyrine in Cpb:Wu rats treated with cytosol, after receiving 1000 mg/kg galactosamine intraperitoneally.

Cytosol was injected 24 hr after the administration of galactosamine. The half-life values for antipyrine in the cytosol-treated rats and the control rats were determined 48 and 72 hr after galactosamine injection.

o : saline	medium	antipyrine (control rats)
● : galactosamine	medium	antipyrine (sham rats)
□ : galactosamine	cytosol	antipyrine
* : number of rats		
—— : mean value ± S.D.		

CHAPTER 8

Auxiliary partial liver transplantation

All treatment modalities investigated in this study failed so far to increase the survival rate or to have a restorative effect on the deteriorated liver function. With respect to auxiliary partial liver transplantation (APLT) the question as to whether the liver cell damage is reversible, is of equal importance. If the injury is irreversible and will terminate in necrosis, replacement of the liver function by transplantation of a donor liver is the only solution. If the damage is still reversible but not spontaneously, the hepatocytes of the donor liver can be expected to promote recovery. A graft offers two main advantages. First, the hepatocytes will adapt and start to function normally much sooner than isolated hepatocytes would, and second, damage to the hepatocytes is more likely to be imflicted by the isolation procedure than by the transplantation. Both of these factors should have a favorable influence on the process of regeneration.

For the presented study we started by transplanting 30% of a normal donor liver into rats with a normal liver and following the graft for eight days. After that we transplanted 30% of a normal liver into rats given various doses of galactosamine.

8.1. Transplantation of normal liver tissue into normal rats

Twelve normal rats received a graft consisting of 30% of a normal liver. All of the animals survived the operation. Rats were killed 1, 2, 3, and 8 days after the transplantation. Autopsy showed no sign of pathology in the contents of the abdomen or the graft. Histological examination of liver biopts showed normal liver tissue up to three days after transplantation. From the third to the eighth day the graft decreased in size to 30% and became pale.

Histologically, an abundance of connective tissue was seen.

Immediately after circulation of the blood was restored, the graft became swollen. About ten minutes later, the graft had adapted to the arterial pressure (27) through the portal vein and the swelling decreased, which persisted until the third day after the transplantation. The graft started to produce bile within 10 minutes after the commencement of blood flow.

8.2. Transplantation of normal liver tissue into rats treated with galactosamine

The effect of transplantation of 30% of a normal liver on the survival rate after intraperitoneal administration of various doses of galactosamine was investigated. Table 8.1 shows the survival rate of the rats given normal liver tissue.

The group of rats which received 1000 mg/kg galactosamine developed the most severe clotting problems, and therefore the operation was technically the most difficult to perform in these animals. All of the grafted rats survived the operation. Those which received only galactosamine (controls) lived 5 hr longer than the rats given a graft (APLT). The sham-operation rats died 4 hr earlier than the APLT rats. Samples of liver from the control rats, the sham-operation rats, and both the grafted and the recipient's liver were studied. All samples showed diffusely distributed, severely necrotic liver tissue whose architecture had disappeared.

The group of rats with 600 mg/kg galactosamine gave fewer surgical problems. The sham-operation rats died even earlier than the controls and the APLT rats, but significantly ($p<0.05$) more control rats survived than sham or APLT rats. Histologically, the sham rats showed severely necrotic liver tissue. The liver samples of the APLT rats and the controls (all of which died) was also necrotic but the livers of the APLT rats showed less damage than those of the control rats.

The rats of the groups which were given 1000 and 600 mg/kg

galactosamine and died after receiving a graft or sham operation showed the symptoms of acute hepatic coma.

In the group given 300 mg/kg galactosamine, survival differed significantly ($p<0.001$), between the sham-operation and the grafted animals. The divergence was most striking in this group. Besides the significant difference in survival after transplantation, there were histological differences (Fig.8.2 - 8.5) between the host liver and the graft. The samples of the host and graft liver tissue showed a normal architecture and normal healthy hepatocytes, whereas those of the rats which received a sham operation showed a severe loss of liver architecture and a diffusely necrotic parenchyme.

The group given 150 mg/kg galactosamine showed histologically normal liver tissue with the same quality as that of the transplantated normal rats.

Figure 8.1 shows a liver graft 48 hr after transplantation. The acceptor rat had received an injection of 300 mg/kg galactosamine 20 hr before the transplantation.

Figure 8.6 shows a section liver tissue from a normal healthy rat biopsied 24 hr after 70% hepatectomy. The striking difference here is that, unlike the liver tissue of all grafts, hosts, and sham-operation rats, the remaining liver tissue of the partially hepatectomized rats showed a high frequency of mitotic figures. None of the sections of the liver tissue of rats treated with galactosamine showed signs of mitosis, regardless of the dosage.

Table 8.1.
Survival rate of Wag/Rij rats given various doses of galactosamine before grafting of 30% of a normal rat liver (APLT).

mg/kg Galactosamine	A APLT	B Sham	C Controls
1000	* 0/6	0/6	0/10
600	0/6 [c]	0/6 [c]	7/10
300	10/12 [a,b]	0/12 [d]	10/10
150	5/5	5/5	10/10

* = Number of survivors/Total number of rats
A = Transplantation of 30% of a donor liver 24 hr after galactosamine injection
B = Sham transplantation 24 hr after galactosamine injection
C = Rats given only galactosamine
a $p < 0.001$ compared with sham-transplantation rats
b n.s. compared with control rats
c $p < 0.05$ compared with control rats
d $p < 0.001$ compared with control rats

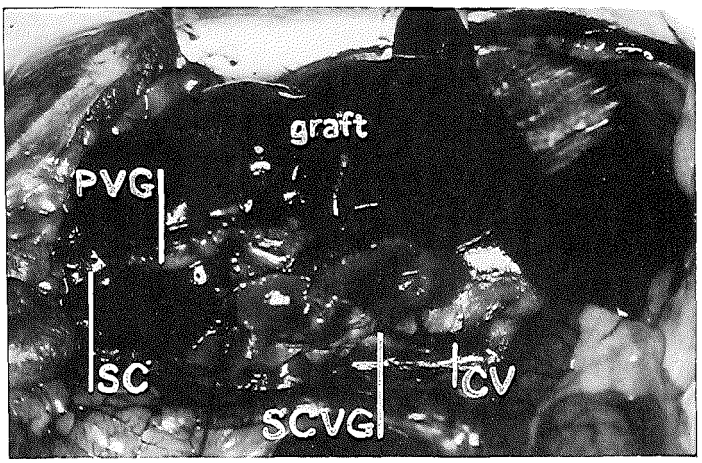

Fig. 8.1.
Liver graft 48 hr after transplantation into a rat given 300 mg/kg galactosamine.

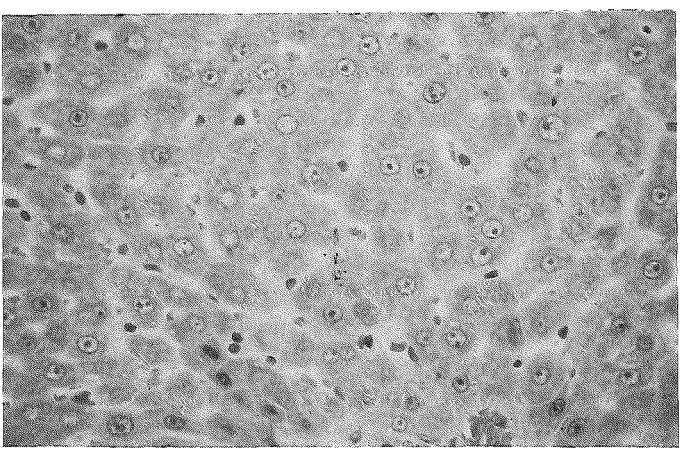

Fig. 8.2.
Light-microscopical section of liver tissue of a host liver 48 hr after administration of 300 mg/kg galactosamine. HE. x 250.

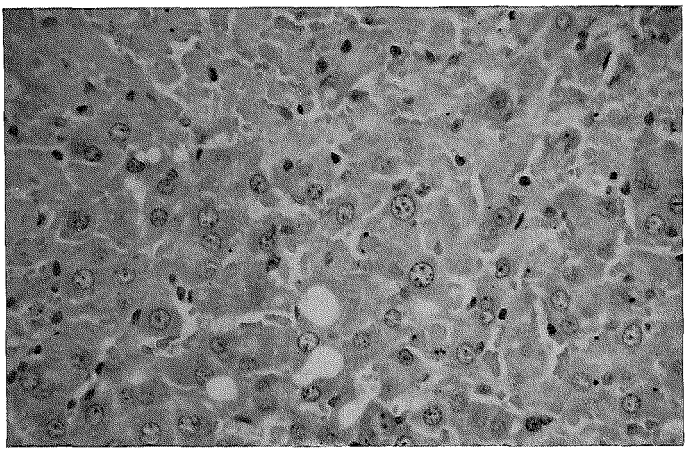

Fig. 8.3.
Light-microscopical section of liver tissue of a host liver given 300 mg/kg galactosamine and 20 hr after sham operation. HE. x 250.

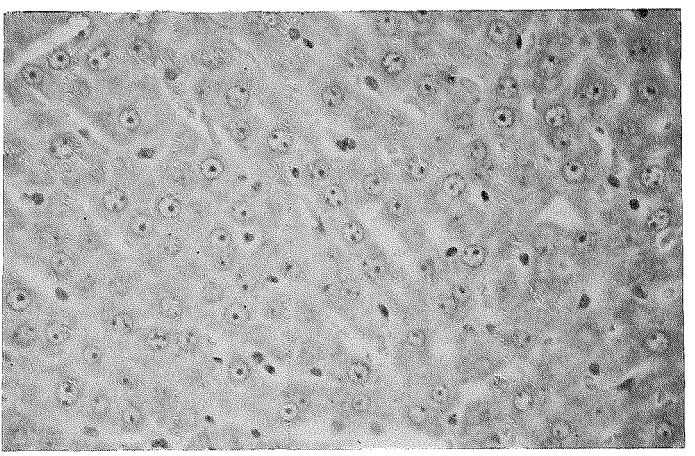

Fig. 8.4.
Light-microscopical section of host liver tissue 48 hr after administration of 300 mg/kg galactosamine and 24 hr after transplantation of 30% of normal liver tissue. HE. x 250.

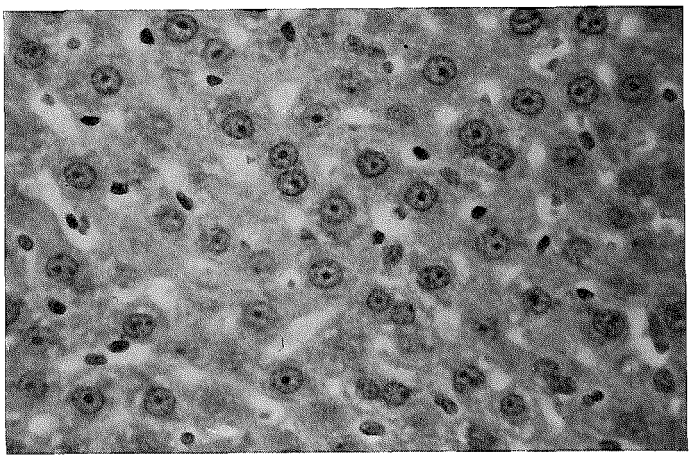

Fig. 8.5.
Light-microscopical section of liver tissue from a graft transplanted 24 hr after the donor had received of 300 mg/kg galactosamine. HE. x 600.

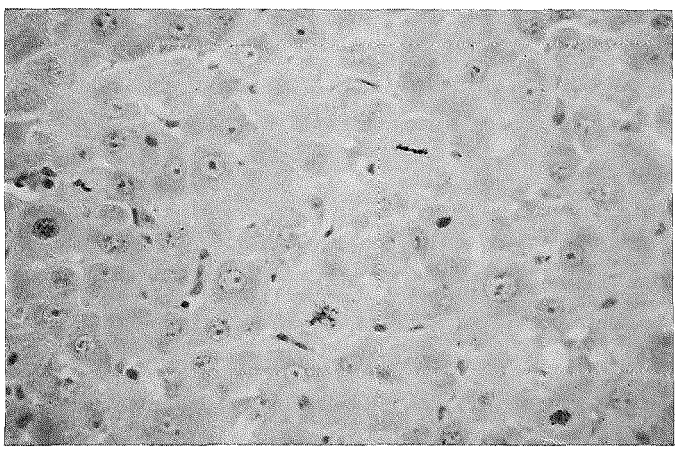

Fig. 8.6.
Light-microscopical section of normal liver tissue 24 hr after 70% hepatectomy. HE. x 250.

8.3. Conclusions

We were able to transplant 30% of a normal isogenic liver in rats which were in bad condition and had severe blood-clotting problems. All recipient rats survived the critical six hours after both the transplantation and the sham operation. Death was due to acute hepatic failure and not to effects of the operation. The finding that significantly more rats survived after receiving galactosamine only than after the sham operation (Table 8.1,^{d}) indicates that the operation inflicted further damage on the livers already injured by galactosamine. This suggests that sub-lethal damage might be made lethal by the operation.

Transplantation of 30% of a normal liver improved the survival rate significantly in rats which had received 300 mg/kg galactosamine as distinct from those given 300 mg/kg galactosamine and a sham operation (Table 8.1,^{a}). Furthermore, survival was significantly higher in rats given galactosamine alone instead of galactosamine and a sham operation (Table 8.1).

Group C in Table 8.1 received only galactosamine. Comparison of this group with the groups of grafted (A) and sham-operation rats (B) shows that the best survival rate was achieved after administration of 300 and 600 mg/kg galactosamine and no further treatment.

The effect of the donor liver seems to have been due to temporary detoxification rather than to stimulation of the host liver to regenerate. If we compare the histological findings in the graft and the host liver at the four galactosamine dosages it is evident that the cellular damage increased with increasing dose in both the graft and the host liver. The liver biopts of the sham-operation rats showed more damage at all four doses of galactosamine than the host livers and the grafts did. Histologically the same severe damage was seen in the biopts of livers of the sham-operated rats and the rats given 300 mg/kg galactosamine and a graft liver but which died (2/12) after the operation (Table 8.1). This suggests that the graft's capacity for detoxification was inadequate in the transplanted

animals. Histological examination also showed that there was a strong increase of synthesis by the hepatocytes of rats which had received 300 mg/kg galactosamine and survived (10/12). This increased activity was accompanied by an enlargement of the nucleolus which also shifted to the center of the nucleus (13). These prominent nucleoli occured frequently in the section of both the graft and the host liver, but were not seen in the rats which did not survive (Table 8.1).

An elevated mitotic rate was observed in liver tissue of normal healthy rats biopsied 24 hr after 70% hepatectomy. None of the liver biopts of APLT rats showed mitotic figures in either the graft or the host liver.

CHAPTER 9

GENERAL DISCUSSION

The results of several investigations indicate that acute liver failure induced by a toxic substance responds to treatment with viable isolated hepatocytes (15,48-52,62,78,80,84,90), a homogenate or cytosol (60,88) of normal or regenerating (20,63,64,88) liver tissue, and cell-free supernatant (2,65,66). The existence of a trophic factor that stimulates regeneration in the liver was postulated (10,37,38,61,81-83). Significantly more rats were expected to survive poisoning with a lethal dose of galactosamine after these forms of treatment.

The discrepancy between the results reported in the literature and our observations concerning the effects of implantation of hepatocytes on the survival rate (Chapter 5) might be explained by the difference in the duration of the interval between the time of hepatocyte implantation and the death of the animals. In the design of this type of experiment two periods are of paramount importance. The first is the interval between the injection of galactosamine and the implantation of the hepatocytes, and the second is the interval between the implantation of the hepatocytes and death, taken as the mean survival time after galactosamine in the absence of treatment.

Figure 9.1 includes the survival-time intervals reported by several investigators. A mean survival time of 96 hr without treatment was reported by Makowka (46,48-52), who concluded that transplantation of $40x10^6$ liver cells into the abdomen could improve the survival rate in rats suffering from galactosamine-induced acute liver failure if the cells were transplantated 24 or 48 hr after galactosamine injection. However, transplantation of hepatocytes was ineffective if it occurred 60 hr or longer after galactosamine injection.

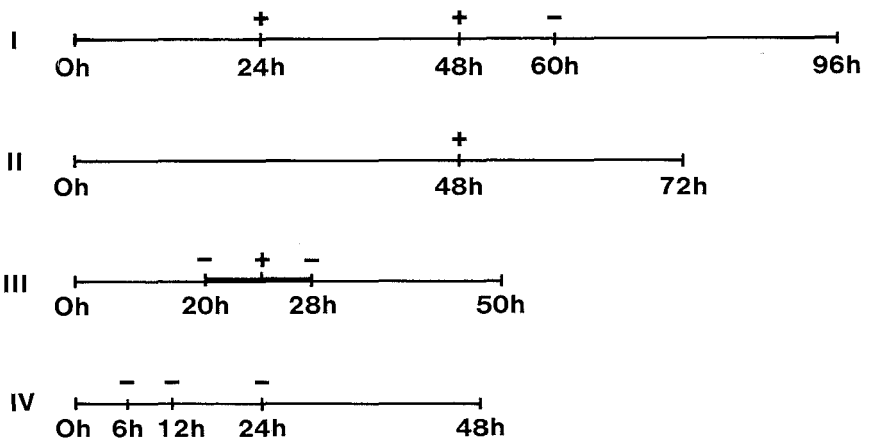

Fig. 9.1.
Intervals between galactosamine poisoning, treatment with isolated liver cells, and maximum mortality, in the published studies and the present investigation.

I. Makowka (52): Lewis rat; galactosamine 2600 mg/kg; hepatocytes implanted in the abdomen.

II. Sommer et al.(80): Lewis rats; galactosamine 1100 mg/kg; hepatocytes implanted in the abdomen, spleen, and liver.

III. Baumgartner et al. (2): Fisher rat; galactosamine 500 mg/kg; hepatocytes implanted in the spleen.

IV. Present study: Wag/rij rat; galactosamine 1000 mg/kg; hepatocytes implanted in the abdomen.

Numbers under the horizontal lines give the time (in hours) after administration of galactosamine at which the hepatocytes were injected in the different compartments. The last numbers on the right give the time of maximum mortality in the absence of treatment. Different groups of rats were used for each interval after galactosamine.
+ = treatment which improved the survival rate
- = treatment which did not improve the survival rate

In the experiments done by Sommer et al.(80) the mean survival time was about 72 hr and transplantation of $20x10^6$ liver cells into the liver or spleen 48 hr after the injection of galactosamine enhanced the survival rate of the rats. Baumgartner et al.(2) found a mean survival time of 50 hr for rats given galactosamine. Intrasplenic transplantation of $10-20x10^6$ liver cells improved the survival if given 20-28 hr after galactosamine, whereas earlier or later implantation did not.The published data indicate that the interval between galactosamine injection and the transplantation of hepatocytes must be at least 20 hr. Hepatocytes implanted after a shorter interval can be damaged by the effects of galactosamine. According to these reported findings the interval between transplantation and the mean survival time should be at least 22 or 36 hr, to give the implanted hepatocytes sufficient time to influence the process of liver cell necrosis induced by galactosamine. In the present study the maximum mortality occurred during the second night, about 48 hr after the administration of galactosamine. Comparison of the experimental schedule used in the present study with the protocols of the other three studies showed that for the rats given hepatocytes 6 or 12 hr after galactosamine injection, the interval between injection of the compound and transplantation of the cells was less than 20 hr. For the rats rats given the hepatocytes 24 hr after galactosamine injection, the interval between transplantation and the mean survival time was less than 22 hr. Thus, it seems possible that the implanted hepatocytes and hepatocyte fractions failed to improve the survival rate either because of the late effects of galactosamine or because the period in which they could contribute to the reversal of liver degeneration by stimulating regeneration was too short.

One critical factor in the present animal model of acute liver failure is that although the death of the animals from acute liver failure must occur within a limited interval, they must survive long enough to allow time for treatment to take effect. As already mentioned, the interval between administration of a lethal dose of

galactosamine and the mean survival time in our study was rather short (48 hr), and the implanted hepatoytes might not have had sufficient time to influence the processes of degeneration and/or regeneration. To lengthen this interval we wanted to use uridine in a dose that would prevent cell death if given at the same time as galactosamine but not if given as late as 6 hr after galactosamine. This would also eliminate the possibly deleterious effect of uridine deficiency on the transplanted cells. According to Farber and co-workers (18), uridine prevents liver cell necrosis if given up to 3 hr after galactosamine injection but has no effect if given as late as 6 hr after galactosamine injection. These authors described an irreversible lesion developing within 3 to 6 hr after galactosamine injection and ultimately resulting in the death of the animals. In a more recent paper Rasenack and co-workers reported that the time-dependent effect of uridine on galactosamine-pretreated isolated rat livers was exactly the same as that found in the liver of normal rats (68). Isolated rat livers were initially perfused for 3 hr with a medium containing galactosamine. The addition of uridine to the perfusion medium up to 3 hr after galactosamine prevented or reversed the galactosamine-induced damage. Uridine added to the perfusion medium as late as 6 hr after galactosamine was ineffective. In our study the longest interval between the administration of galactosamine and uridine, during which uridine significantly enhanced the survival rate of Wag/Rij rats, lay between 12 and 18 hr. This interval is much longer than those reported in the literature. On the basis of the survival rate, we were unable to find an appropriate dose of uridine meeting our conditions.

The second critical factor with respect to this model is that the hepatic lesion must be potentially reversible. It is possible that under the conditions we applied for the survival experiments the affected liver cells passed "the point of no return". For that reason we induced a spontaneously reversible grade of hepatic injury to be certain that the lesion is potentially reversible. The fact that implantation of hepatocytes failed to enhance the recovery of the

liver function indicates that the length of the interval and the irreversibility are not sufficient to explain the negative results in the survival experiments. The liver function tests were performed up to 48 hr after implantation of the hepatocytes, i.e., 72 hr after the injection of galactosamine, allowing enough time for the implanted hepatocytes to affect the regeneration process.

In a view of the substantial inter-strain differences (3,7,24,25) in the reaction to galactosamine, it is conceivable that the choice of the animal strain used for such studies on regeneration is of paramount importance. We induced a spontaneously reversible degree of hepatic injury in several rat strains (Chapter 7), and the failure of implanted hepatocytes to improve the recovery of the liver function indicates that the strain variation did not explain the negative results.

As discussed, rats within different groups showed substantial variation of the degree of deterioration of the liver function following galactosamine administration. Tabata reported (87) that the LD_{50} of rats for galactosamine varied widely between different batches of male WU rats with negligible differences in age and weight, which were supplied weekly by the same breeder. In the kind of experiments we performed, the number of control and treated rats must of course always be in the same range.

A parameter which is equivalent to the survival time is not yet available, because the process underlying the death of liver cells is still poorly understood. When all of the normal structures and functions of dying cells are disturbed, it is difficult or impossible to determine whether a change in a particular parameter has to do with cause, or effect, of the dying cell.

Liver transplantation seems to be the only remaining form of treatment to be evaluated with respect to the feasibility of improvement of the survival rate. But in the present study transplantation of 30% of a normal liver did not reverse lethal liver damage. Only rats given a sub-lethal dose of galactosamine survived, those given a sham operation as well, did not. It seems unlikely that

this is to be ascribed to the fact that the graft was only arterialized (27,93) and was not provided with portal blood.

Consensus has not been reached on the question as to whether portal blood is essential for regeneration in the transplant or the host's liver. Studies done by Rozga (69,70) showed that the presence of factors originating from the portal blood is mandatory for regeneration. However, the presence of these factors in the systemic circulation is sufficient to stimulate regeneration (69). For our liver transplantation model it may be assumed that the poor functioning of the host's liver guarantees the presence of adequate levels of the regenerative factors in the circulation to make regeneration possible. It is relevant that Holmin (27), Saric (71), and Castaing (12) reported that arterialization of the liver through the portal vein improves the clearance capacity and they found no ultrastructural lesions.

An important histological criterion for regeneration is an increase in the rate of mitosis, which we assessed after a 70% hepatectomy in normal rats. The mitotic rate increased within 24 hr, and one mitosis was found per field of vision at a magnification of 400x. Histological sections of tissue from recipients surviving after 300 mg/kg galactosamine showed no mitotic figures in either the graft or the host liver. We performed the transplantation 20 hr after the administration of galactosamine, because we had found that after 18 hr the liver injury caused by galactosamine could not be reversed by uridine and spontaneous reversal was not expected 18 hr or longer after injection of galactosamine (Chapter 5). The positive effect of transplantation of 30% of a normal liver seen after administration of 300 mg/kg galactosamine might mean that the graft's liver had undergone less than the critical amount of damage by secondary metabolites and had more time to regenerate/recover spontaneously, although we found no histological signs of regeneration. It seems probable that when higher doses of galactosamine were used the detoxification capacity of the graft was not adequate.

If we compare the three groups of rats - the group given a

graft, the group which underwent a sham operation, and the group given only galactosamine - it is evident that one must be certain that spontaneous recovery is impossible before one undertakes a liver transplantation intended to aim at stimulation of recovery of the sick liver.

The findings made in the presented study suggest that one or more still unknown factors inherent in the experimental models currently in use are of critical importance and that only a certain limited type of model of acute hepatic failure is suitable for the evaluation of the effectiveness of methods intended to promote regeneration of diseased livers.

It does not seem unreasonable to conclude that 40 x 10^6 liver cells injected into the abdominal cavity or the spleen would not improve survival, since ca. 300x10^6 (representing 30% of a normal liver) did not do so. It is conceivable that in a given model the use of hepatocytes and/or hepatocyte fractions triggers a non-specific reaction leading to survival. It is then also conceivable that when the hepatocytes or hepatocyte fractions are injected, the ongoing damage caused by galactosamine is arrested before it becomes lethal. This means that in cases of real fulminant hepatic failure the only chance of survival lies in the ability of the graft either to induce regeneration in the diseased liver or to take over all of the functions of the damaged liver. Because of the excessive risk associated with waiting to see whether the organ will recover is too great, it is preferable to aim at a transplantation enabling the graft to take over functions of the host's liver completely.

SUMMARY

The mortality rate of patients suffering from acute hepatic failure remains very high. The basic therapeutic clinical approach is intensive-care monitoring aimed at preventing accumulation of the liver damage, the prevention of complications due to liver failure, and attempts to give the sick liver enough time to regenerate. Investigations concerning regeneration factors pointed to the existence of a hepatotrophic factor that might stimulate regeneration in a damaged liver at the moment when most of the hepatocytes have reached the point of no return. This regenerative capacity of the liver could be triggered experimentally by transplantation of viable isolated hepatocytes, hepatocyte fractions of normal and regenerating donor liver tissue, and supernatant of liver-cell cultures. In the hands of some investigators, these forms of treatment led to improvement of the survival rate of rats suffering from acute hepatic failure. This reduction of the mortality rate associated with acute hepatic failure led to the present investigations on the effect of the transplantation of viable isolated hepatocytes and hepatocyte fractions on the survival of rats suffering from acute liver failure induced by a toxic agent, in particular as a step toward evaluation of the method for clinical use in patients with acute hepatic failure.

Chapter 2 deals with the materials and methods used in this research. For the evaluation of the therapeutic effect a model was needed, as discussed in Chapter 3 dealing with the dose-response relation of galactosamine, a substance which induces a liver damage in normal rats. The survival rate in two rat strains, BN and Wag/Rij, after injection of galactosamine was determined and the LD_{90} was estimated. The sensitivity of the Lewis, Wag/Cpb, and Cpb:Wu strains to galactosamine was also assessed.

The effects of transplantation of viable hepatocytes and hepatocyte fractions on the survival rate of rats suffering from acute hepatic failure are described in Chapter 4. The Wag/Rij rats

were injected with a lethal dose of galactosamine, and the different treatment modalities were administered 24 hr later. An improvement of the survival rate was not established for any of the forms of treatment. A possible cause of this failure might be that the period, the interval between administration of the different treatment modalities and the mean survival time, in which the process of liver degeneration would be influenced was too short. We attempted to find a way to lenghten this interval by giving the treatment sooner after galactosamine.

Uridine is a substance which can prevent or reverse liver damage, depending on the dose and time of injection after administration of galactosamine. Chapter 5 describes the studies performed to determine the uridine dosage and schedule of administration such that the galactosamine-induced liver damage would not be reversed but the transplanted hepatocytes would be sufficiently protected against the effects of galactosamine.

The negative results concerning improvement of the survival rate of rats suffering from acute hepatic failure might be explained by irreversibility of the induced liver damage. In Chapter 6 the induction of sub-lethal liver damage is described. We used specific liver function tests to determine whether any of the treatment modalities were able to stimulate recovery of a deteriorating liver function. These tests gave the same results as were obtained for improvement of the survival rate. We then induced sub-lethal liver damage in rats of two strains, Lewis and Cpb:Wu, as discussed in Chapter 7. These two strains were used to assess the effect of the treatment modalities on deteriorating liver function in relation to differences in sensitivity to galactosamine. None of the forms of treatment improved either the survival rate or the deteriorating liver function after a lethal or sub-lethal dose of galactosamine, respectively.

Since the liver has been reported to possess a hepatotrophic factor, we transplanted 30% of a normal liver into rats given galactosamine in various dosages, as described in Chapter 8. Only

transplantation of 30% of a normal liver after given a sub-lethal dose of galactosamine gave improvement of the survival rate compared with the sham-operated rats. Rats given the same dosage galactosamine alone survived spontaneously.

SAMENVATTING

Het sterftepercentage van patienten lijdend aan een acuut leverfalen is erg hoog. De basale klinische benadering is momenteel intensive-care begeleiding, gericht op het voorkomen van een toenemende leverschade, het voorkomen van complicaties ten gevolge van het leverfalen en pogingen om de zieke lever voldoende tijd te geven te regenereren. Onderzoekingen betreffende regeneratieve factoren wijzen op het bestaan van een hepatotrofe factor. Deze factor zou de leverregeneratie kunnen stimuleren op een moment dat de meeste hepatocyten zodanig beschadigd zijn, dat spontaan herstel niet meer mogelijk is. Het regeneratievermogen van de lever kan experimenteel in werking gezet worden door transplantatie van levensvatbare geisoleerde hepatocyten, of hepatocytenfracties van normaal en regenererend donor leverweefsel of supernatant van levercelkweken. Enkele onderzoekers konden een verbetering van het overlevingspercentage van ratten, lijdend aan een acuut hepatisch falen, bewerkstelligen door toediening van deze geisoleerde hepatocyten en hepatocyten fracties. De uitkomsten van die onderzoekingen dienden als basis voor dit onderzoek. Zowel het effect van transplantatie van geisoleerde hepatocyten, hepatocyten fracties, cytosol en supernatant op de overleving van ratten lijdend aan een acuut lever falen is onderzocht en beschreven en de eventuele klinische toepassing daarvan bekeken.

Hoofdstuk 2 behandelt de materialen en methoden gebruikt in dit onderzoek. Om een therapeutisch effect te kunnen evalueren is een model nodig. In hoofdstuk 3 wordt de dosis-effect relatie van galactosamine beschreven, een stof welke een leverschade induceert in gewone ratten. Het overlevings percentage van twee ratten stammen, de BN en de Wag/Rij, na het injecteren van galactosamine is bepaald en de LD_{90} is geschat. Tevens is de gevoeligheid voor galactosamine van de Lewis, Wag/Cpb en Cpb:Wu stammen beoordeeld. De effecten van transplantatie van vitale geisoleerde hepatocyten, hepatocyten-

fracties, cytosol en supernatant op het overlevingspercentage van ratten, lijdend aan een acuut leverfalèn, zijn beschreven in hoofdstuk 4. De Wag/Rij ratten zijn geinjecteerd met een lethale dosis galactosamine, en de verschillende behandelingsvormen zijn 24 uur later toegediend. Een verbetering van het overlevingspercentage voor alle vormen van behandeling werd niet verkregen. Een mogelijke oorzaak van dit falen kan zijn dat de gemiddelde overlevingstijd na de toediening van de behandeling te kort is om het proces van leverdegeneratie te beinvloeden. Een manier om dit interval te verlengen is door de behandeling sneller toe te dienen na de injectie met galactosamine.

Uridine is een stof die de aan te brengen leverschade kan voorkomen of doen herstellen, afhankelijk van hoeveelheid en moment van injectie na toediening van galactosamine. Hoofdstuk 5 beschrijft de onderzoekingen welke zijn verricht om een dosering uridine te bepalen en het tijdschema van toediening, zodanig dat de galactosamine-geinduceerde leverschade niet hersteld wordt maar de getransplanteerde hepatocyten voldoende beschermd worden tegen de effecten van galactosamine.

De negatieve resultaten met betrekking tot de verbetering van het overlevingspercentage van ratten lijdend aan een acuut leverfalen zou verklaard kunnen worden door de irreversibiliteit van de geinduceerde leverschade. In hoofdstuk 6 is het toebrengen van een sublethale leverschade beschreven. Specifieke leverfunctietesten zijn gebruikt om te bepalen of één van de behandelingsvormen in staat zou zijn het herstel van een verslechterende leverfunctie te stimuleren. Deze testen tonen dezelfde resultaten als welke zijn verkregen bij de onderzoekingen naar de verbetering van het overlevingspercentage. Vervolgens is een sublethale leverschade in 2 rattenstammen, te weten de Lewis en de Cpb:Wu, geinduceerd hetgeen is besproken in hoofdstuk 7. Deze twee rattenstammen zijn gebruikt om het effect van de behandelingsvormen te toetsen bij een afnemende leverfunctie in relatie met een verschil in gevoeligheid voor galactosamine. Geen van de vormen van behandeling verbeterden het overlevingspercentage of de

afnemende leverfunctie na een lethale resp. sublethale dosis galactosamine.

Aangezien wordt aangenomen dat de lever na partiële hepatectomie een hepatocyt-prolifererende factor uitscheidt, transplanteerden wij 30% van een normale lever in ratten behandeld met verschillende doses galactosamine, zoals beschreven in hoofdstuk 8. Alleen bij de groep ratten die een sublethale dosis galactosamine en 30% normaal donor-leverweefsel kregen is een verbetering van het overlevingspercentage geconstateerd in vergelijking met de "sham" geopereerde ratten. Ratten die alleen de sublethale dosering galactosamine kregen, overleefden spontaan.

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List of abbreviations

APLT	Auxiliary partial liver transplantation
ATP	Adenosine 5'-triphosphate
CTP	Cytidine 5'-triphosphate
CV	Caval vein
d-Gl	Galactosamine
GTP	Guanine 5'-triphosphate
PVG	Portal vein graft
RIA	Right iliac artery
RK/LK	Right kidney/Left kidney
SC	Silastic cannula
SCVG	Superior caval vein graft
UDP	Uridine 5'-diphosphate
UDP-galactose	Uridine diphosphate galactose
UDP-GalN	Uridine-galactosamine
UDP-GalNac	Uridine diphosphate N-acetyl-galactosamine
UDP-GlcN	Uridine-glucosamine
UDP-GlcNac	Uridine diphosphate N-acetyl-glucosamine
UDP-glucose	Uridine diphosphate glucose
UDP-glucuronate	Uridine diphosphate glucuronate
UMP	Uridine 5'-monophosphate
UTP	Uridine 5'-triphosphate

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CURRICULUM VITAE AUCTORIS

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In 1979 is het eindexamen Atheneum B behaald aan de Rotterdamse Avondschool. Zijn medische studie aan de Erasmus Universiteit in Rotterdam begon hij in 1980. Tijdens deze opleiding werd in januari 1982 het onderzoek naar het effect van verschillende behandelings methoden bij het experimenteel geinduceerde acuut leverfalen gestart. Het onderzoek is verricht onder leiding van Prof.Dr D.L.Westbroek en na zijn overlijden onder leiding van Prof.Dr O.T.Terpstra. Het doctoraal examen is in maart 1987 behaald.