LIVER TRANSPLANTATION IN THE RAT

PROEFSCHRIFT

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TO MARCELLE,
ROGIER, VINCENT AND MENNO

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Chapter I

INTRODUCTION

1 Indications for liver transplantation

During the past ten years progress in the field of vascular surgery and immunology has been such, that a steady improvement in the results of clinical organ transplantation can be observed. Also when a life threatening disease of the liver is present, liver transplantation may be considered.

In general these diseases may be described as follows: *Necrosis of the liver*, acute or sub-acute, can give rise to a progressive loss of the liver function. The causal factors are very often unknown. Sometimes certain hepatotoxins can be indicated as the cause. Halothane, for instance, may give rise to acute hepatic necrosis of the liver (Silverman 1975).

Viral hepatitis can also produce such serious damage to the liver, that it endangers life.

The largest group of patients for whom a liver transplant may be considered, consists of those with *cirrhosis*: appearing for example after liver necrosis, prolonged biliary obstruction, metabolic disorders such as Wilson's disease or from unknown causes. Cirrhosis, in the long run produces a loss of liver function to such an extent, that too small an amount of endogenous poisons are removed, and liver coma ensues. At the same time a shortage of protein production gives rise to oedema and/or ascites, while a serious lack of coagulation factors may lead to a pronounced haemorrhagic diathesis. If cirrhosis is the result of excessive alcohol consumption, then, as a rule, a liver transplant will not be considered (Starzl 1969, Starzl et al. 1975, Williams 1970, Rybak 1974); in the first place because continued alcohol consumption would also endanger the new liver and secondly, because in many cases, concurrent disease such as pneumonia or chronic bronchitis hamper transplantation of the liver.

Biliary atresia is the most common life threatening liver disease in childhood, usually bringing about a fatal biliary cirrhosis, leading to death within a few years of birth. In a series of 39 untreated patients the average life span was 19 months. 16 patients (41%) died within 1 year, 15 died between 1 and 2 years (in total 77% deaths within 2 years) and 8 died at an age varying from 27 months to 7½ years (Hays and Snyder 1963). Wilkinson (1973) reported an incidence of congenital biliary atresia of

1 : 13,000 to 15,000. According to Silverman (1975) the incidence is 1 : 8,000 to 1 :10,000 but 4 to 5 times higher among "orientals". Liver transplantation could be considered for these patients, when biliary cirrhosis is well advanced. Kasai (1974) however, reported that after a modified hepatoporto-enterostomy operation a cure was observed in 80% of the cases, provided the operation was performed within 10 weeks of birth. This statement, however, is based solely on a series of 4 patients: in all cases the jaundice disappeared and the faeces acquired a normal colour whilst only 3 of the patients were kept under observation for more than 1 year. Without giving any figures, Wilkinson (1973) and Silverman (1975) appear to be much more pessimistic. They state that a mortality of almost 100% occurs even if the hepatoporto-enterostomy operation is performed before the third month. Experience in this type of operation, probably plays an important role.

In conclusion one must state that patients with biliary atresia who suffer from biliary cirrhosis are to be considered as candidates for a liver transplant. However, since favourable results have been achieved in Japan after a modified hepatoporto-enterostomy, a more detailed evaluation of this alternative treatment should take place first. For this purpose it is necessary that the diagnosis of biliary atresia is made within ten weeks of birth. It seems of great importance that these patients are sent to one centre in the Netherlands so that one team of paediatricians and paediatric surgeons can obtained the necessary experience in treating such children.

Although the removal of a malignant process in the liver can hardly be expected to be curative, one does find in the literature than primary carcinoma or sarcoma or even localized metastases in the liver have formed the motive to remove the liver and replace it by a transplant. No recurrence could be found after liver transplantation by the Cambridge-London group in 2 out of 12 patients, who originally had hepatic malignancy (Williams et al. 1973). In view of these individual successes they consider malignant growth, confined to the liver, as an indication for orthotopic liver transplantation.

Starzl found metastases shortly after the operation in every one of a series of 6 patients, in whom malignancy was the indication for the transplantation. Only one patient, in whom quite incidentally a small hepatoma was found in the resected liver, was still alive 32 months after the operation (v. Wyk et al. 1972). The motive for the transplant in this patient however was biliary cirrhosis. Consequently the Denver liver transplantation group no longer considers malignancy as a motive for liver transplantation (Groth and Starzl 1973).

2. Incidence of life-threatening liver diseases in the Netherlands

In order to obtain some impression of the number of patients concerned, some data collected by the (Dutch) Central Bureau of Statistics are given below, in table I.1.

Diagnosis	*Age group*	
	0-15 yrs.	*15-50 yrs.*
Necrosis of the liver, acute and sub-acute	2	20
Hepatitis infectiosa	3	17
Congenital anomalies of gall bladder, biliary tract or liver	56	----
Cirrhosis, excluding that associated with alcohol consumption	13	146

Table I.1. Number of patients dying in the Netherlands during a period of 3 years: 1970, 1971 and 1972

35 of the children dying as a result of congenital biliary tract defects died before the age of 1 year, the others before they were 5. Thus, the greater part of this group of 56 patients (15 to 20 per year) probably suffered from biliary atreasia. This tallies with the number of patients to be expected in the Netherlands according to Wilkinson (1973) and Silverman (1975): birth rate 150 to 200,000 per annum, incidence of ± 1: 10,000 → 15 to 20 patients with biliary atresia per year. In total, according to the C.B.S., 25 patients under 15 and 58 patients from 15 to 50 years, die each year as a result of the above stated liver diseases. In principle these could be considered for liver transplantation, save those with additional problems such as obstinate local and/or generalized infection. Also patients with biliary atreasia together with severe vascular anomalies such as absent inferior vena cava, preduodenal portal vein and anomalous hepatic artery, seem to be unsuitable candidates for liver transplantation (Lilly and Starzl 1974). It is quite clear that the number of patients expected to be in need of liver transplantation depends upon the indications used. Williams (1970) made such an estimate for Britain. According to his view, roughly one half of the patients dying per year from diseases such as primary hepatoma (145 patients), primary biliary

tract carcinoma (195), cirrhosis (490) and acute hepatitits (160) would have been potential recipients of a liver transplant. Thus, while including malignant diseases of the liver (in contrast to Starzl and co-workers), he estimates that in Britain approximately 500 patients could be in need of liver transplantation per year. Knowing the number of patients who actually receive a liver transplant in America (± 15 patients per year) and in England (± 8 patients per year) (Bergan 1975, Williams et al. 1973), it is obvious that this number will not be achieved in the near future.

3. Clinical liver transplantation compared with transplantation of other organs.

How do the results of kidney, heart, lung and pancreas transplantation compare with those of liver transplantation?

As regards kidney transplantation, it may be noted in table I.2 that one year after the operation on average 50% of the cadaver kidneys still function. After 2, 3 and 4 years the percentage of functioning cadaver kidneys is still 42.6, 41.8 and 40.6% respectively (ACS/NIH, 12th Report of the Renal Registry Committee, 1975).

By comparison, 80, 69 and 60% of the cadaver kidneys transplanted in Rotterdam still functioned after 1, 2 and 3 years L.D.F. Lameyer 1976, personal communication).

Heart transplantation was reported 257 times to the ACS/NIH from 1967 to 1975 (Bergan 1975). The percentage of surviving patients was 21, 8.5, 3.5 and 2 after respectively 1, 3, 5 and 6 years. These patients, however, were treated in many (59) different centres. Experience, as regards heart transplantation, was sometimes at a minimum and the operations were not performed any more after the first disappointing results.

Experience, as regards heart transplantation, is much wider at Stanford University, where 90 of the operations were performed. Up until August 1975 the percentage of surviving patients was: 47, 37, 27, 24 and 20 after 1, 2, 3, 4, and 5 years respectively (Schroeder et al. 1976).

Transplantation of the pancreas was reported 36 times. One year's survival with a functioning transplant was only found twice.

Lung transplantation was also carried out 36 times up until 1975. The 3 patients who survived the operation longest lived 2, 6 and 10 months.

Since 1963, 181 patients have undergone orthotopic liver transplantation. The number of survivors was 24, 12 and 6 after 1, 2 and 3

Table I.2. Number of patients with solid organ transplants and the percentage surviving with a functioning transplant up to 6 years.

organ	number of patients	% surviving patients with a functioning transplant					
		1st yr.	2nd yr.	3rd yr.	4th yr.	5th yr.	6th yr.
kidney (1)	14806	50.6	42.6	41.8	40.6	35.2	24.0
kidney, Rotterdam (2)	91	80	69	60	–	–	–
heart (3)	257	21	–	8.5	–	3.5	2
heart, Stanford (4)	90	47	37	27	24	20	–
liver, orthotopic (3)	181	13	6.6	3.3	2.2	1.1	–
liver, orthotopic (5)							
Denver, 1963-1973 (excluding patients with carcinoma)	43	29.3	9.3	4.7		–	
Denver, 1971-1973 (excluding patients with carcinoma)	16	38					
Denver, biliary atreasia	20	30					
liver, heterotopic (3)	39	2.5	2.5	–			
pancreas (3)	36	5.5	0				
lung (3)	36	0					

References: (1) ACS/NIH 1975. (2) Lameyer 1976. (3) Bergan 1975. (4) Schroeder et al. 1976 (5) Groth and Starzl 1973.

years. Four patients are still living varying from 3½ to 5½ years after the operation. In Denver, where experience in liver transplantation is largest, only 4.7% of the patients survived the operation 3 years, excluding those who were operated upon because of a malignancy of the liver. Patients operated upon from 1971-1973 however, survived in 38% of the cases more than one year. The results in comparison with heart transplantats are poor. Heterotopic liver transplantations were performed 39 times. Only one patient lived longer than 1 year and this one was still alive with a functioning transplant 2 years after the operation (Groth 1975). What circumstances have contributed to the fact that the results of liver transplantations compared with those of heart and kidney transplantations are so poor? Several possible factors, namely donor selection, sensitivity to anoxia, bacterial and viral injections, rejection and immunosuppressive therapy, will be discussed on the following pages.

4. Problems concerning liver transplantation

a. Donor selection

It is possible to achieve a prolonged average survival time in dogs matched for the major histocompatibility complex (DL-A), compared to "mismatched" dogs as is shown by Chavez-Peon and Malt (1971), Dausset et al. (1971), Chandler et al. (1972), Ranson et al. (1974) and Lambotte and Westbroek (1975). Prolonged survival after orthotopic liver transplantation in dogs was also obtained by Schalm et al. (1975) (8 of the 9 dogs lived more than 1 year), using DL-A "matched" dogs and mild immunosuppressive therapy (azathioprine 2 mg/kg/day). A good donor-recipient matching on the basis of HL-A tissue antigens is however as yet impossible in the practice of clinical liver transplantation since there is not a large "recipient pool". The same problem of small recipient pools occurs however with heart and lung transplantation. Only blood group compatibility plays a part and usually a clear histo-incompatibility exists between donor and recipient. In cadaver kidney transplantation, a large recipient group is available, kept alive by haemodialysis, so that HL-A matching of the kidney donor and recipient becomes possible. European results indeed indicate that better results are obtained, when donor and recipient are matched, according to Hl-A tissue antigens (Koch et al. 1971, Parsons et al., 1975). But in America differences in survival between patients with good or bad matching tissue antigens

(serologically determined) could only recently be demonstrated, thanks to the analysis of very large groups of kidney transplant patients. With good matching (2, 3 or 4 identical tissue antigens, n = 2,025) the percentage of functioning kidney transplants after 1 year was 8.9% higher than when the matching was poor (0 or 1 antigen the same, n = 1,535): respectively 53.2% and 44.3% survival (ACS/NIH 12th report Renal Registry Committee). It is, however, unlikely that better tissue typing and matching would have had much influence on the results of clinical liver transplantation, since until now, rejection of the liver graft was not the major complication causing death of the liver transplant recipients (Williams et al. 1973, Starzl et al. 1975).

b. Sensitivity to anoxia

From experiments with dogs, Martin (1972) concluded that both heart and liver are more sensitive to anoxia than the kidney. At a temperature of ± 37 °C the liver, after 30 minutes anoxia, is so damaged that successful transplantation is impossible (Schalm 1968). Irreversible liver damage, already existing before the donor liver was inserted, has certainly contributed to the death of a limited number (6) of patients (Starzl 1969). In Denver, therefore, only livers are used which come from brain dead donors with a good heart, lung and liver function. In England unlike in America, the heart of the donor must have ceased to beat before the patient is certified dead (Williams et al. 1973, Calne et al. 1974). This means that the liver has to be preserved quickly, preferably in a simple way. (Schalm 1969, Calne 1972, Calne et al. 1972, Calne et al. 1973, Calne et al. 1974, Otte et al. 1973 a,b). The duration of the preservation will then be 3 to 6 hours, namely until the hepatectomy in the recipient is completed. Simple, clinically applicable, preservation systems are indeed available (Schalm et al. 1969, Calne et al. 1972, Calne et al. 1974, Lie et al. 1974) so that poor preservation does not actually contribute to the poor prognosis of liver transplanted patients (Starzl 1969, ACS/NIH report I 1971, Williams et al. 1973, Groth and Starzl 1973).

c. Bacterial and viral infections

Bacterial and viral infections form a pronounced risk for the liver transplant recipient. The cause of death in 83 patients with orthotopic liver transplants was in 35 of them (42%) associated with bacterial infections (for the most part Gram-negative), and thus infection implies a considerable risk for patients with liver transplants (Starzl 1969, Starzl et al. 1975, Murray-Lyon et al. 1970, ACS/NIH 1971, 1972 and 1973, Williams et al.

1973). In cadaver kidney- and heart transplant recipients only 30% (n = 25,000) and 24% (n = 137) of the causes of graft failure or death were associated with infections (ACS/NIH 1971, E.D.T.A. combined report V 1974). The intimate contact of the donor liver via portal vein and biliary anastomosis with the continuous reservoir of bacteria and viruses present in the intestines, apparently forms an essential threat to the liver transplant recipient. The problems of bile duct obstruction, immunosuppression and graft rejection are obviously associated with these infectious complications.

C. 1 Problems of bile duct obstruction

Problems of bile duct obstruction are seen by some (Williams et al. 1973) primarily as a mechanical problem. The various forms of bile duct anastomosis (choledocho-enterostomy, choledocho-choledochostomy, cholecysto-choledochostomy and cholecysto-enterostomy with or without "Roux Y Loop" or gastro-enterostomy) have, however, not yet produced any clear solution (Starzl 1969, Starzl et al. 1973, Calne 1969, Williams et al. 1973). It is possible that choledocho-choledochostomy with the use of a lumen widening plasty has theoretical and practical advantages. The lumen widening technique offers less chance of mechanical obstruction and the advantage of an autogenous bile duct - intestines transition is maintained. Consequently, there is at this transition place no chance of rejection with obstruction and invasion of bacteria (Schalm et al. 1975). Others (Martineau et al. 1972, Coleman et al. 1973) emphasize the viral genesis of bile duct obstruction appearing late after liver transplantation. Whatever the causes of the obstruction may be, they are all followed by ascending bacterial infection. Local bile duct inflammation with abscess formation and generalized sepsis contribute considerably to the mortality seen after liver transplantation.

C. 2 Immunosuppression and graft rejection

General suppression of resistance against viral and bacterial infections by means of the immunosuppressive agents used and a predisposition to infections on account of the bile duct anastomosis are possibly not the only factors which contribute to the high risk of infection after a liver transplantation. The liver has an extensive reticulo-endothelial system, that eliminates bacteria which penetrate the portal system from the intestines (Starzl 1969). This system of phagocytic cells, residing within the donor liver, also has its function suppressed by the immunosuppressive agents.

In our opinion, thought should be given to the possibility that these phagocytic cells are also impeded in their activity by the "Host versus Graft" or rejection reaction. This hypothesis is supported by the experiences of Starzl and co-workers (Starzl 1969 p. 327), who found that with insufficient immunosuppression septic hepatic infarctions occurred in five consecutive cases. After the immunosuppressive therapy was intensified, no other cases of regional hepatic gangrene with septicaemia were seen.

Multiplication of bacteria to a great extent occurs during the operation and directly afterwards due to the paralytic ileus, caused by the abdominal operation. Penetration into the bloodstream (portal vein) and biliary system will be seen in many instances (Brettschneider et al. 1968).

If, during the time of the transplantation and directly afterwards, the bacterial density in the intestines is decreased, then this should theoretically lead to less infections after transplantation of the liver. Schalm et al. (1975) indeed found few infectious complications after liver transplantation in dogs and pigs, in comparison with earlier experiments, when the quantity of intestinal bacteria was reduced for a period of 2 days at the time of the liver transplantation by means of selective bowel decontamination. Adequate control experiments, carried out at the same time are however, still required. In order to reduce the chance of bacterial invasion from the intestines, a reduction of the number of bacteria might also be of importance during surgical re-explorations or at the time of an acute rejection of the transplanted liver.

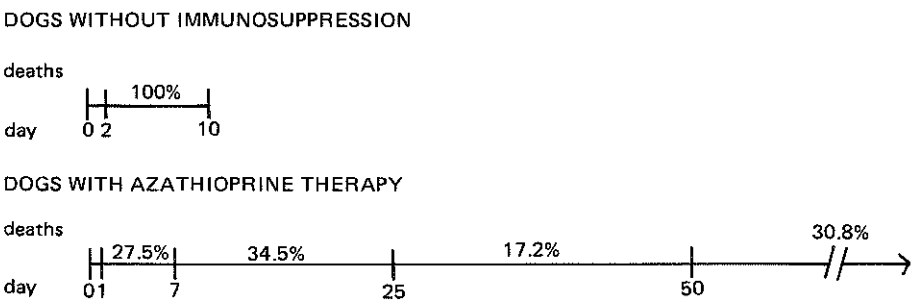
d. Rejection and immunosuppressive therapy — rationale for the experiments

Originally clinical liver transplantation was carried out, after which the recipients were given very little or no immunosuppressive therapy (Starzl 1969, Williams et al. 1973, Aune 1973). This usually led to irreversible rejection of the liver. Just as with heart transplantation a strong immunosuppression will, however, be necessary in order to combat rejection of the liver. A real problem is that there are so few certain indications for determining an imminent rejection process (Starzl 1969, Williams et al. 1973, Groth and Starzl 1973). Neither the leucocyte migration test (an aspecific cellular reactivity test) nor successive liver biopsies, provide unequivocal information concerning the rejection process (Eddleston et al. 1971, Williams et al. 1973). Both the side effects of azathioprine (hepatoxicity and intrahepatic cholestasis, Starzl et al. 1965) and bile duct obstruction and hepatitis may give those liver function disorders

which are also found in rejection of the liver: decrease in liver function (production ↓ and poison-removing effect ↓), liver cell loss, cholestasis, hyperbilirubinaemia, rise in temperature, enlargement of the liver, general discomfort etc.

All transplantation centres use corticosteroids to prevent or treat rejection of the transplanted liver. Opinions on the nature and dosage of the additional immunosuppressive agents used, such as azathioprine, cyclophosphamide or Antilymphocyte Globulin (ALG) do, however, differ. (Starzl 1969, Lie et al. 1971, Aune et al. 1972, Fortner et al. 1973, Williams et al. 1969, 1973, Groth and Starzl 1973). Because of the supposed hepatotoxicity of azathioprine, Starzl and co-workers have replaced this agent by cyclophosphamide, during the first three months after liver transplantation. However, bone marrow depression during therapy with cyclophosphamide (2–4 mg/kg/day) is more likely to occur and azathioprine may still be preferable for chronic treatment (Groth and Starzl 1973). Rejection episodes occur mostly within 3 months after transplantation (Starzl 1969, Williams et al. 1973). It is because of this reason that, just as with heart transplantation, ALG is used during that period in order to suppress rejection of the liver transplant most vigorously. (Groth and Starzl 1973, Schroeder et al. 1976, Starzl et al. 1975).

The immunosuppressive treatment used in liver transplantation in fact is based on the experiences obtained through clinical and experimental kidney transplantation. Systematic research concerning immunosuppressive therapy after liver transplantation is scarce. The rejection of a pig liver transplant is slow and not very aggressive, in contrast to human liver transplant rejection. The pig species is therefore not suitable for research concerning prevention or treatment of acute liver rejection (Calne et al. 1967(a), 1967(b), Battersby et al. 1974, Terblanche et al. 1968). The dog species and subhuman primate, in whom acute rejection of the transplanted liver occurs, have strong individual disparity of histocompatibility antigens, possibly also of "immune responsiveness" (Vriezendorp 1973). Thus, Starzl et al. (1965) observed survival times of unmodified liver transplanted dogs varying from 2 to 10 days ($n = 22$, mean survival time ± s.d.: 7.1 ± 2.2 days). After azathioprine therapy (8 mg/kg/day tapered to 2 mg/kg/day after 1 week, thereafter 2–4 mg/kg/day) they found a mean survival time of 27.9 ± 16.5 days. However, in order to compute the mean survival time, the maximal survival time was arbitrarily limited to 50 days post transplantation. In fact survival differed from 1 day to more than one year, 30.8% of the animals living for 50 days or more (see scheme below).



Consequently very large series of dogs would be needed in order to demonstrate differences in effectiveness between immunosuppressive regimens.

Chandler et al. (1972) also investigated the influence of immunosuppression on survival of dogs after orthotopic liver transplantation. They used multispecific cytotoxic allo-antisera to determine if histocompatibility matching would improve the survival rate. The "mistmatched" Beagles lived for 8.7 ± 1.2 days after operation (mean ± s.e.m.). "Matched" animals lived without treatment for 43.9 ± 13.7 days (arbitrary limit of 100 days). However, additional treatment with azathioprine (2 mg/kg tapered to 1 mg/kg/day) or Antilymphocyte Globulin (ALG) (40 mg/kg/day tapered to 20 mg/kg/2 days) failed to demonstrate a significant prolongation of mean survival time compared to untreated matched animals.

Myburgh and co-workers have chosen the monkey (Baboon) as their experimental animal for orthotopic liver transplantation. They first demonstrated the acute course of the liver rejection in these animals. Of 12 monkeys with an orthotopic liver transplant, 75% died within two weeks, the other animals lived up until 36 days (mean ± s.e.m.: 12.9 ± 2.5 days). Prolongation of survival time of several animals could be observed, when treated with Antilymphocyte Globulin (ALG), azathioprine, prednisolone or combinations of these agents. However, no significant difference in survival time could be demonstrated after 50 days between treated and untreated animals (Myburgh et al. 1971). Further experiments were directed towards obtaining "tolerance and enhancement" effects, using for instance donor bone marrow cells and alloimmune antisera. Thus, using sera obtained from 10 baboons after their immunisation with allogeneic lymphoid tissue, together with bone marrow cells, a significant prolongation could be obtained. (Mean survival time ± s.e.m.: 50.9 ± 7.6 days, 6 out of 10 animals being alive

after 50 days) (Myburgh and Smith 1972). Subsequent experiments demonstrated, just as with dogs a pronounced variation of survival time in the treated animals (Smit and Myburgh 1974, Little et al. 1975 and Myburgh and Smith 1975).

Rats, like dogs and monkeys, also have acute rejection of allogeneic liver transplants. Their use for liver transplantation experiments, would have several advantages compared to those with larger experimental animals.

The financial aspects plays a significant role, since the total costs of breeding, care and accommodation before, during and after the operation are much less in rodents. One problem however is, the microsurgical operative technique needed to transplant the liver in these small animals.

A second important factor is that inbred strains can be obtained easily. Since antigenic properties within one inbred rat species are equal in all members of that species, the differences between two inbred rat species are constant. It is therefore likely that less individual variation between the rejection patterns will occur. The anatomic differences within one inbred strain of rats are less pronounced than in dogs or monkeys, consequently there will be less individual differences in the operative procedure compared to larger animals.

A suitable auxiliary technique for liver transplantation, in which the recipient liver still sustains the life of the animal, did not exist, let alone a non-auxiliary liver transplantation technique, in which no functional recipient liver tissue would be left at the time of transplantation. This last technique in fact would be needed when different immunosuppressive regimens were to be compared and results of liver function tests were to be used as parameters for the function of the transplanted liver.

The purpose of the investigations described below, were:

- to develop an auxiliary technique of liver transplantation in the rat, in order to obtain better survival figures;
- to develop a non-auxiliary technique of liver transplantation in the rat;
- to investigate the acute unmodified rejection pattern after allogeneic livertransplantation, through serial liver function tests and histology;
- to evaluate the efficacy of some short term immunosuppressive regimens upon the modification of acute liver transplant rejection, while the histocompatibility differences between donor and host were controlled through the use of inbred rat strains.

Chapter II

HETEROTOPIC AUXILIARY LIVER TRANSPLANTATION IN RATS

The following chapter is reproduced by permission of the Williams and Wilkins Company, Baltimore. It was published before in Transplantation Vol. 12, pages 415-420 in 1971. Authors: Kort, W.J., Wolff, E.D. and Eastham, W.N.

SUMMARY

Heterotopic auxiliary liver transplantation in rats was performed with a portal afferent blood supply, together with either the infra- or suprahepatic part of the inferior vena cava (IVC) as the efferent vessel. Animals in which the suprahepatic part of the IVC was used had a high postoperative mortality which was associated with moderate or severe congestion of the graft in over one-half of the cases. Substitution of the infrahepatic part of the IVC prevented the congestion and increased the postoperative survival rate.

1. Introduction

Experimental liver transplantation has been performed in different animal species. Among the animals which have been used are monkeys (*1*), pigs (*2*), dogs (*11*), and rats (*4, 5*). Lee's original technique of auxiliary liver transplantation in rats consists of a 30% liver graft receiving its afferent blood supply from the coeliac axis, with the suprahepatic part of the IVC serving as the efferent vessel. The common bile duct, portal vein, and infrahepatic IVC are ligated.

We used this method in a pilot experiment in which 22 rats were given liver transplants. Extreme congestion of the grafted liver occurred in 12 of the 16 animals which survived the operation but which died within 24 hr. Thrombotic occlusion of the coeliac axis was present in the remaining four. These findings sggest that the principal factor responsible for the failure of this technique in our hands was an impaired hepatic outflow. For this reason, we modified the technique by substituting the infrahepatic part of the IVC for the suprahepatic part. Although an inadequate blood flow through the coeliac artery probably contributed to only one-third of the deaths, we also decided to change to a portal blood supply, as this source is known to contribute approximately 80%

of the blood flow through the liver and to provide, on the average, 70% of the available oxygen (*12*).

In order to evaluate the effectiveness of the modified operative procedure, comparative studies were performed in which either the infra- or suprahepatic parts of the IVC were used in combination with a portal blood supply.

2. Material and methods

Animals

Forty inbred, specific pathogen-free female rats of either the Wag/Rij or BN/Bi strain, weighing 150-200 g, were used as donors. Forty males of the same strains, weighing 300-350 g, served as recipients.

Experimental Procedure

Nineteen animals received liver transplants with an infrahepatic outflow, and 21 received liver transplants with a suprahepatic outflow. All those animals which were operated upon but which failed to recover from the anaesthetic were designated as having had an operative death. The different operative procedures were performed on donor-recipient combinations, Wag/Rij → Wag/Rij, BN/Bi → Wag/Rij, BN/Bi → BN/Bi, and were repeated until five animals from each of the combinations survived the operation. The different operative methods were performed in the sequence indicated by the operation numbers listed in Tables 1-3.

Infrahepatic IVC Method

Isolation and perfusion of donor livers. To reduce respiratory tract secretions, donor as well as recipient animals were given 0.01 mg of atropine s.c. 20 min before being anaesthetised with ether. Following exposure of the donor's IVC, 0.5 mg/kg of heparin (Leo) in 0.5 ml of physiological saline was injected into that vessel. A 70% hepatectomy was performed according to the method used by Higgins and Anderson (*3*), and the common bile duct was ligated close to the liver hilus. Heparin (2.5 mg/kg in 1.0 ml of saline) was then injected directly into the portal vein before it was cannulated with a Braun i.v. catheter (1.0 x 1.5 mm ϕ). The hepatic artery was isolated and a loose tie was placed around it near the liver hilus. The thorax was then opened, the suprahepatic part of the IVC was ligated above the diaphragm, and the infrahepatic IVC was cut transversely and proximal to the right renal vein. The hepatic artery was ligated and divided. The liver was then freed from surrounding tissue and

was removed with a large piece of attached diaphragm. After isolating the graft, we perfused it through the portal vein with cold saline (4 °C) at a rate of 20 drops/min and at a pressure of 20 cm H_2O until transferring it to the host. During this interval, which lasted about 10 min, the liver was immersed in cold saline at 4 °C and was perfused by a total of ± 10 ml of perfusate (Fig. 1).

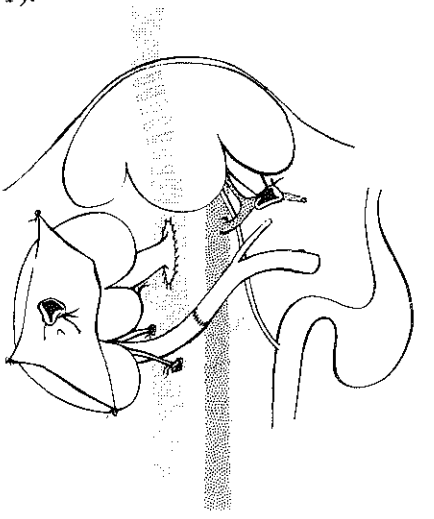


Figure 1. Heterotopic auxiliary rat liver transplantation using the infrahepatic vena cava as the efferent vessel. The donor's suprahepatic IVC, hepatic artery, and common bile duct and the host's right renal vein all are ligated.

Transplantation technique. After receiving ether anaesthesia, recipient animals were given 0.5 mg/kg of heparin into a lateral tail vein. The operative procedures were performed under clean but non-sterile conditions, and the contents of the peritoneal cavity were intermittently moistened with saline. Hepatectomy (removing only 70% of the liver), together with right nephrectomy, was performed through a midline abdominal incision. The vena cava was freed from perivascular tissue proximal to the ligated right renal vein. The portal vein was separated from the hepatic artery by blunt dissection, and a loose ligature (4/0 silk) was placed around it. A section of the wall of the vena cava, proximal to the ligated right renal vein, was then isolated in a Satinsky-like curved arterial clamp, and an oval aperture was cut in it with iris scissors. The graft was removed from the cold saline and placed in the right side of the abdomen, and an end to side anastomosis of the infrahepatic IVC was made with the clamped part of the host vena cava (continuous sutures; 7/0 silk; Ethicon).

The loose ligature, previously placed around the recipients portal vein, was then tightened, prior to cutting of the vessel. The two portal veins were joined by end to end anastomosis (continuous sutures; 7/0 silk; Ethicon) during which time (± 10 min) the host vessel was occluded by a bulldog vascular clamp. The donor liver was lifted toward the recipient's portal vein by placing a saline-soaked dental pack beneath it. We restored the circulation through the graft by releasing the caval and portal vascular clamps, in that order. In order to compensate for blood loss, each animal received 1 ml of saline i.v. We stabilised the position of the graft by stitching the attached diaphragm to the lateral abdominal wall and retroperitoneal fat with three separate sutures of 4/0 silk. The intestines were replaced, the abdominal wound was sutured with continuous catgut, and the skin was closed with autoclips.

Suprahepatic IVC Method

Isolation and perfusion of donor livers. This procedure was essentially the same as that previously described, with the following exceptions. Instead of ligating the suprahepatic part of the IVC, we resected this vessel, together with a rim of right atrial tissue, after which we ligated the infrahepatic IVC (Fig. 2).

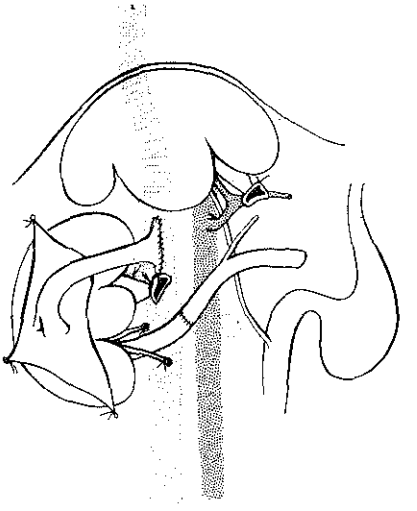


Figure 2. Heterotopic auxiliary rat liver transplantation using the suprahepatic vena cava as the efferent vessel. The donor's infrahepatic IVC, hepatic artery, and common bile duct and the host's right renal vein all are ligated.

Transplantation technique. The operative procedure varied only in that the suprahepatic part of the IVC was anastomosed to the host's vena cava. The methods of anastomosis were the same as those employed in the infrahepatic IVC method.

Postoperative Care

Immediately after operation, the animals were placed under an infrared light for 2–3 hr, and 50,000 international units of penicillin G/kg and 50 mg of streptomycin/kg were injected i.m. During this time and for the following 24 hr, the rats were given a drinking bottle containing 5% glucose, after which they were given their preoperative diet of acidified water and food pellets. The antibiotic therapy was repeated on the next day.

Evaluation of the Transplanted Liver

Animals dying on day 1 died within 24 hr of the operation. Subsequently, the designated day of death corresponds to death within the equivalent number of 24-hr periods. All animals in the pilot series died within 3 days and, therefore, survival for 4 days in the present series was taken as an indication of successful vascularisation of the graft. Thereafter, animals were examined under ether anaesthesia, and the organs were removed for histological evaluation. Animals with allogeneic grafts were killed on day 5, so that developing changes attributable to rejection would not obscure the appearance of the transplanted liver. Isogeneic grafted animals were killed on days 7, 8 and 9. Necropsy was performed on those animals which died spontaneously. The tissues were fixed in 4% buffered formalin and 5-μ paraffin-embedded sections were stained with hematoxylin, azophloxin, and saffron.

Congestion, when observed in the donated livers, was graded as being of a mild (+), moderate (++), or severe (+++) degree. Donor livers without congestion were graded as (–).

3. Results

In addition to the observations listed in Tables 1-3, bile duct proliferation was noted in both isogeneic and allogeneic transplanted livers of animals surviving for 4 days or more. Changes attributed to rejection of the graft (periportal mononuclear cellular infiltration) were observed in allogeneic transplanted livers.

Table 1. Survival and pathological findings of BN/Bi → BN/Bi liver-transplanted rats with a supra- or infrahepatic outflow

Operation No.	Day and mode of death[a]	Type of operation[b]	Degree of congestion in donated liver[c]	Relevant pathological data and/or cause of death
1	1; †	SUP	+++	Severe congestion with necrosis of donor liver; congestion of spleen and intestines
2	*	SUP		Unsuccessful portal anastomosis
3	*	SUP		Respiratory failure
4	*	SUP		Unsuccessful portal anastomosis
5	*	SUP		Severe congestion of donor liver, portal vein, and intestines; circulatory failure
6	*	SUP		Torn suprahepatic IVC
7	*	INF		Unsuccessful portal anastomosis
8	*	INF		Unsuccessful portal anastomosis
21	2; †	SUP	+++	Severe congestion with necrosis of donor liver; congestion of spleen and intestines
22	7; †	SUP	+	Icterus attributable to biliary obstruction; multiple haemorrhages
23	1; †	SUP	+	Severe congestion and necrosis of the butterfly lobe; centrizonal necrosis of host liver
24	7; k	INF	–	Icterus attributable to biliary obstruction
29	1; †	SUP	++	Congestion and early necrotic changes in donor liver; centrizonal necrosis of host liver
30	7; k	INF	–	No significant pathological findings
32	7; k	INF	–	No significant pathological findings
33	7; k	INF	–	Icterus with intestinal haemorrhages; centrizonal necrosis of host liver
36	5; †	INF	–	Diffuse fatty change in both donor and host livers

[a] *, operative death; †, spontaneous death; k, killed under anaesthesia.
[b] INF, infrahepatic part of the IVC used as the efferent vessel; SUP, suprahepatic part of the IVC used as the efferent vessel.
[c] See Materials and Methods for explanation.

Table 2. Survival and pathological findings of BN/Bi → Wag/Rij liver-transplanted rats with a supra- or infrahepatic outflow

Operation No.	Day and mode of death[a]	Type of operation[b]	Degree of congestion in donated liver[c]	Relevant pathological data and/or cause of death
16	3;†	INF	–	Peritonitis
17	3;†	INF	+	Hydrothorax and (?) septicaemia
18	2;†	SUP	++	Centrizonal necrosis of host liver; solitary, small pulmonary thromboembolus
19	1;†	SUP	++	Congestion of donor liver and intestines with blood in the peritoneal cavity
20	1;†	SUP	+	Congestion of intestines with intestinal haemorrhage
25	5;k	INF	–	No significant pathological findings
26	5;k	INF	–	No significant pathological findings
27	5;k	SUP	–	Centrizonal necrosis of host liver
28	1;†	SUP	++	Thrombosis in and possible occlussion of the thoracic part of the VIC with haemorrhage into the peritoneal cavity; centrizonal necrosis of host liver
31	5;†	INF	–	No significant pathological findings

[a] *, operative death; †, spontaneous death; k, killed under anaesthesia.
[b] INF, infrahepatic part of the IVC used as the efferent vessel; SUP, suprahepatic part of the IVC used as the efferent vessel.
[c] See Materials and Methods for explanation.

4. Discussion

Examination of the tabulated results reveals an increased 4-day postoperative mortality in those animals in which the suprahepatic part of the IVC was used as the efferent vessel (11 of a total of 13 animals dying within this period). Although the grafted livers in these 11 animals

Table 3. Survival and pathological findings of Wag/Rij → Wag/Rij liver-transplanted rats with a supra- or infrahepatic outflow

Operation No.	Day and mode of death[a]	Type of operation[b]	Degree of congestion in donated liver[c]	Relevant pathological data and/or cause of death
9	9; k	INF	–	No significant pathological findings
10	9; k	INF	–	No significant pathological findings
11	*	INF		Unsuccessful portal anastomosis
12	*	INF		Unsuccessful portal anastomosis
13	9; k	INF	–	No significant pathological findings
14	8; k	INF	–	No significant pathological findings
15	8; k	INF	–	No significant pathological findings
34	8; k	SUP	–	No significant pathological findings
35	1; †	SUP	+	Extreme congestion of host liver with early degenerative changes in donor liver
37	2; †	SUP	–	Peritonitis, ascites, hydrothorax, and pulmonary congestion
38	*	SUP		Unsuccessful portal anastomosis
39	2; †	SUP	+	Ascites, hydrothorax, and pulmonary congestion
40	5; †	SUP	–	Peritonitis

[a] *, operative death; †, spontaneous death; k, killed under anaesthesia.
[b] INF, infrahepatic part of the IVC used as the efferent vessel; SUP, suprahepatic part of the IVC used as the efferent vessel.
[c] See Materials and Methods for explanation.

acquired a normal colour after the vascular clamps had been released, they subsequently became swollen and progressively darker, despite a detectable outflow in the efferent vessel. At necropsy, 6 animals had moderate or severe congestion of the liver. This sequence of events was particularly evident in animal No. 5, in which circulation through the graft eventually stopped, resulting in an operative death.

It is doubtful that intrahepatic outflow block, of the type described by Starzl et al. (*10*) and Schalm et al. (*8*) in dogs, played a significant role in our series, as the congestive changes were never seen at operation

when the infrahepatic part of the IVC was used as the efferent vessel.

When the suprahepatic part of the IVC is used as the vascular outlet for the graft, the length and position of this vessel render it particularly susceptible to stretching and torsion, especially when the animal assumes its normal walking position. At such a time, the grafted liver is at least partly supported by the vessel which is then situated directly beneath it. Any compression suffered by the vessel in this position may also be compounded by the presence of mobile or distended intestinal loops coming to lie between the vessel and the abdominal wall. Kinking of the vein is also possible, this having been previously recorded in pig liver transplants (*6, 7*). The possibility that spasm of the vessel may be significant is unlikely, as the wall of the suprehepatic IVC is virtually devoid of muscle cells. The importance of the contractions of the atrial patch, which often continues to beat independently and for some time after the anastomosis is completed, is difficult to assess.

When the infrahepatic part of the IVC is used as the outlet, the opportunity for any of these factors to become operative is greatly lessened. In the first instance, the length of the outflow vessel is very much shorter (2 mm), so that kinking and torsion are virtually impossible, and blood leaving the liver enters the host's IVC almost immediately. Second, the site of the infrahepatic part of the IVC and its anastomosis is surrounded by the donor liver and, therefore, protected from any possible compression. Third, if vascular spasm does play a part in the suprahepatic method, it is less likely to occur in the infrahepatic IVC which is, to some extent, held open by its intimate associations with the liver parenchyma on the one hand and the suture line of the anastomosis on the other.

It is evident from Table 3 that, although the 4-day postoperative mortality rate was also high in Wag/Rij animals with suprahepatica IVC efferent vessels, significant congestion of these isogeneic liver grafts were not encountered. We do not have an adequate explanation for this observation, particularly when one considers that the diameters of the anastomosing vessels of the Wag/Rij female donors were much smaller (approximately one-half) than those of their BN/Bi counterparts. This also made the isogeneic Wag/Rij transplantations technically more difficult.

In two animals (Nos. 17 and 23), severe congestion occurred in the caudate lobe of the donated liver, while the remainder of the graft was only mildly congested. The particular anatomical features of this part of the liver have led us to refer to it as the "butterfly lobe". In the normal situation, the "wings" of this lobe embrance the stomach and are partly

supported by it and by the gastrohepatic ligaments. However, after transfer of the graft to the host, the butterfly lobe loses these points of support, and it is then sometimes difficult to position this part of the graft satisfactorily. When encountered, this problem appears to be unrelated to the method employed for efferent vessel anastomosis and is simply the result of obstruction of a lobular vascular pedicle.

A similar situation in a human subject was described by Starzl et al. (*9*) in which the arterial supply to a lobe was impaired. Should positioning of the butterfly lobe prove troublesome and should its vascularization appear to be jeopardised, it can be removed without adversely affecting the remainder of the graft.

Centrizonal necrosis of the remaining host liver was observed in 6 recipients. Three of these were of the BN/Bi strain and 3 were Wag/Rij animals. All had been given BN/Bi liver grafts and, with the exception of 1 animal, the suprahepatic IVC method had been employed. Of these animals, 3 died within 24 hr, 1 died within 2 days, and the 5th and 6th survived the 4-day postoperative period. Therefore, it appears that, when the suprehepatic part of the IVC is used, there is an increased risk, not only of inducing extrahepatic outflow obstruction but also of precipitating centrizonal necrosis in the remaining host liver.

If the operations are viewed chronologically, it is obvious that in the early stages of the series there was a very high operative mortality. This result is readily explained on the basis of inexperience with the end to end porta-portal anastomosis and, once this was overcome, the operative mortality dropped to almost zero. The average operation time was 1 hr, including the 15 min required for isolating the donor liver.

Portacaval shunted rats can survive 70% hepatectomy and, therefore, the auxiliary transplantation procedure does not completely eliminate the functional capability of the residual host liver. Nevertheless, an accelerated decrease in host liver function can be achieved by ligating the common bile duct. In these circumstances, and especially if a longer period of survival is desired, it becomes necessary to implant the donor's common duct into the duodenum. Our subsequent experience has shown that such a manoeuvre is in no way hindered when the described method of hepatic transplantation is adopted.

We concluded that, in this series of heterotopic auxiliary rat liver transplants in which small female rats were used as donors, the utilisation of the infrahepatic part of the IVC as the efferent vessel prevented congestion of the graft and resulted in an improved 4-day postoperative survival rate.

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Chapter III

NON-AUXILIARY LIVER TRANSPLANTATION IN THE RAT

1. Introduction

Immunological mechanisms of liver transplant rejection and its functional consequences have been studied in a number of animal species (Calne et al. 1967 (b), Calne et al. 1970, Lee and Edgington 1968, Myburgh et al. 1971, Slapak et al. 1970, Starzl et al. 1965). The use of rats is advantageous in two ways, first because genetic factors are controlled by the use of inbred strains and secondly, costs are limited compared to the use of dogs, pigs or monkeys.

Lee and Edgington (1968) first reported on histopathological changes of allogeneic liver transplants in rats. Fisher and Fisher (1970) further characterised these changes and in addition performed ultrastructural studies. Histochemical investigations have been performed by Jerusalem et al. (1971) and Jap (1972, Jap et al. 1972). However, all these studies were performed on auxiliary liver transplants, leaving a life-sustaining part of liver in the recipient, together with the transplanted liver. This leads to a complicated situation: for instance "functional competition" (Hess et al. 1972, v.d. Heyde 1966, Schalm 1966) between host and donor liver, causing atrophy of the graft, may obscure the immunological processes involved in the rejection of the graft (Marchioro et al. 1965). Another important non-immunological factor is the difficulty in interpretation of the biochemical results when two livers are present in the recipient.

We therefore decided to develop a non-auxiliary transplantation technique in the rat. The animals were allowed to live for 7 weeks since this observation period would permit the evaluation of the majority of the liverallograft rejection crises. Also when immunosuppression would subsequently be used (see chapter IV), it could be expected that as with kidney transplantation experiments, acute rejection of the liver was to occur within this period (de Bruin 1970, Tinbergen 1972).

2. Material and Methods

a Animals

Inbred, specific pathogen free* female BN/Bi rats weighing 150 - 200 g were used as donors. Male animals of the same strain or inbred Wag/Rij males both weighing 300 - 350 g served as recipients, for isogeneic and allogeneic transplants, respectively. The complete female donor livers were heterotopically transplanted into hepatectomised male recipients. Mean donor liver weight was 6.08 ± 0.50 g, while male BN livers weighed 8.70 ± 0.55 g and male Wag livers weighed 9.58 ± 0.62 g, 39 isogeneic and 38 allogeneic transplantations were performed.

b Surgical technique

Isolation and perfusion of donor livers is essentially as described before (chapter II) but instead of performing partial hepatectomy, the donor liver is kept intact. After ether anaesthesia atropine 0.01 mg together with heparin 50 I.U. (Leo) in 1.0 ml of saline is injected into the exposed I.V.C. The *whole* donor liver with its infrahepatic I.V.C., common bile duct, canulated portal vein, diaphragm and ligated suprahepatic I.V.C. is dissected from surrounding tissues and immersed in cold saline (4 °C). Portal vein perfusion is performed at a rate of 5 drops/3 minutes, pressure 10 cm H_2O, while a total volume of 3 ml perfusate is used.

Hepatectomy and insertion of the donor liver (see Fig. III 1). Under ether anaesthesia recipient animals receive 0.01 mg of atropine and 10 I.U. of heparin in 0.5 ml saline I.V. Through a midline abdominal incision the procedure is started with only a 70% hepatectomy. Right nephrectomy and separation of the portal vein is performed thereafter. Recipient hepatectomy is continued with removal of the posterior part of the right lateral lobe, after ligation of its pedicle with a single ligature (2/0 slik). The inferior vena cava is isolated in a curved arterial clamp, the graft is transferred to the right side of the recipient's abdomen and an end-to-side anastomosis of the donor's infrahepatic I.V.C. is made with the host's vena cava, proximal to the ligated right renal vein.

The administration of ether is interrupted while the end-to-end anastomosis between the portal veins is made. Both anastomoses are made with continuous sutures using 7/0 silk; following their completion the caval

*Free from Salmonella, Pasteurella and pleuro-pneumonia like organisms.

and portal clamps are released and ether is then given again. Saline (1 ml) is given I.V. to compensate for possible blood loss (usually not more than ½ ml). Total anoxia time of the liver transplant was ± 40 minutes. The host's hepatic artery and common bile duct are double ligated and cut. The caudate lobe is exposed after dissection of its ligaments, ligated around its pedicle with 2/0 silk and removed. Recipient hepatectomy is now completed by removal of the superior part of the right lateral lobe, in the same way, as near as possible to the I.V.C. Hemostasis occurs usually spontaneously due to the discontinuation of portal and arterial blood flow. The duodenum is approximated towards the donor liver and the smallest possible length of common bile duct implanted into the duodenum and fixed with 7/0 silk to the wall. The intestines are placed evenly within the abdominal cavity. The peritoneum and abdominal wall are closed with catgut, the skin with autoclips. A transfusion consisting of 1 ml isologous blood with 1.5 ml 5% glucose is given I.V. Recipient operation took approximately 60 minutes. Afterwards animals are warmed and provided with a drinking bottle with 5% glucose in the drinking water for 24 hours. Acidified water and commercial food pellets were provided ad libitum to the individually caged animals thereafter.

Antibiotic treatment. Penicillin, Streptomycin and Cephaloridin were given s.c., daily during three weeks after the operation, in doses of 10.000 I.U., 10 mg and 20 mg respectively.

c Laboratory techniques and experimental design

Body weight, hematocrit, clotting activity and biochemical determinations were performed twice a week during the two weeks following transplantation and once a week thereafter. Bromsulphtalein (B.S.P.) retention was determined once a week. Blood from animals under ether anaesthesia was obtained by retro-orbital sinus puncture using a non heparinised glass capillary, wetted with 5 µl sodium citrate. The first two drops were discarded to obviate possible clotting activation by tissue thromboplastin or overdosage of the sodium citrate. 0.8 ml of blood was collected in polyethelene micro reaction flasks (Eppendorf) containing 20 µl sodium citrate 0.55 mol and immediately mixed (Whirlmix Vortex). Blood samples were centrifuged for one minute (12000 g Eppendorf Microcentrifuge) and the supernatant plasma collected in polystirol crystal tubes with polyethene caps (Emnosa Belgium). All plasma samples and their dilutions were kept on melting ice until used. Diluant was barbital sodium acetate buffer pH 7.4, as described by

Michaelis (1930). One heparinised capillary (0.08 ml) was used to determine haematocrit values.

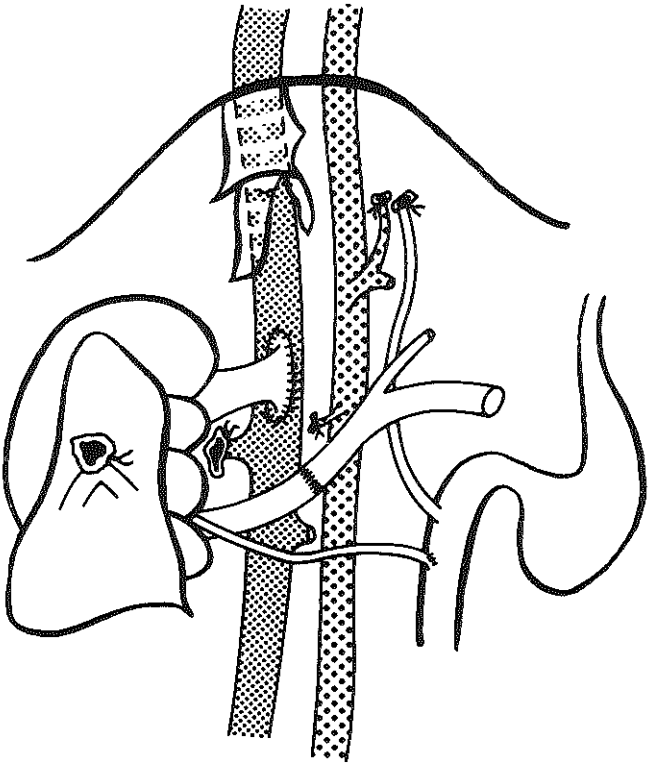


Fig. III 1. Schematic representation of liver transplantation in the rat.
The recipient is fully hepatectomized, the bile duct and hepatic artery are ligated. The portal vein is diverted to the heterotopically placed liver transplant. Efferent blood flow goes through the infrahepatic inferior vena cava. The donors hepatic artery and suprahepatic inferior vena cava are ligated.

Prothrombin activity was determined according to the one stage principle (Owren, 1949). Prothrombin deficient rat plasma was prepared according to Koller et al. (1951). Citrated rat plasma was absorbed with aluminium hydroxide to obtain rat plasma, free of Prothrombin, Factors VII, IX and X. This plasma was mixed with rat serum in a ratio of 2 : 1. The resulting prothrombin deficient rat plasma contained 1.4% Prothrombin (F II), 41 mg% Fibrinogen (F I), 71% F V and > 100% F VII. At intervals of 30 seconds, 0.050 ml of each of the following 4 specimen

were successively added to a glass tube, and maintained at 37 °C in a water bath:

1. the test sample (diluted 1 : 10)
2. rat plasma deficient in Prothrombin
3. rat Thromboplastin (37 °C)
4. calcium chloride (0.033 mol, 37 °C).

The constituents were vigorously mixed after each successive addition. Coagulation time (time from recalcifation until formation of a fine fibrin thread) was recorded by repeatedly drawing a "Kolle" hook through the mixture. Prothrombin activity was expressed as % of normal after comparison with a time-concentration curve made with pooled normal rat plasma (fig. III 2).

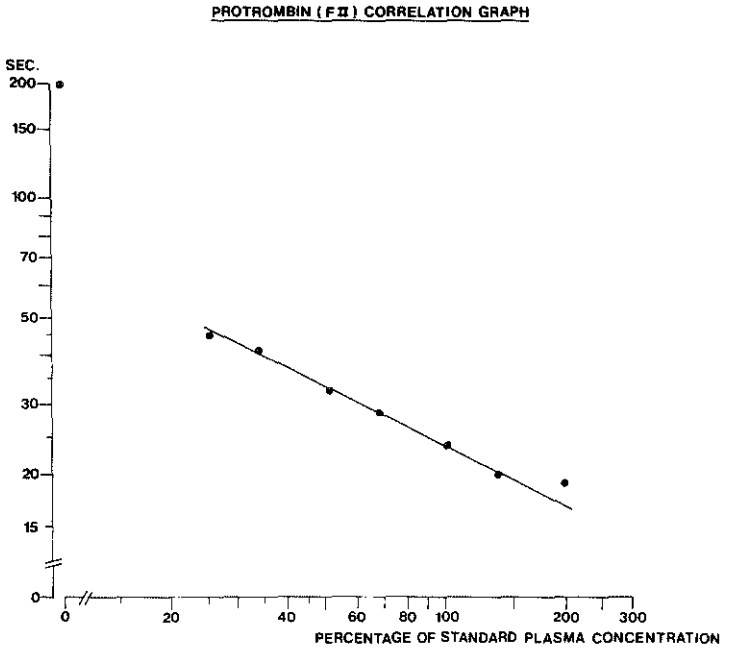


Fig. III 2. Rat prothrombin time-concentration graph.

Each dot represents the mean time of five clotting time measurements using a certain dilution of pooled rat plasma mixed with prothrombin deficient plasma, thromboplastin and calcium chloride (see text). The "0" point of plasma concentration is obtained by successively adding the herefore mentioned constituents to Michaelis buffer instead of pooled rat plasma.

Normotest® which can determine on a percentage ratio the presence or absence of the liver synthetised clotting factors II, VII and X were performed (v. Oosterom and Veltkamp 1974). Because of the sensitivity of Thrombotest® for the endogenous clotting antagonist P.I.V.K.A. (Protein Induced by Vitamin K Absence or Antagonist), this test was used in order to distinguish vitamin K deficiency (11, 34). Thrombotest and Normotest reagents (kindly provided by Nyegard, Oslo) were prepared with 3.2 mmol calcium chloride and distilled water respectively. Aliquots of 0.25 ml were stored (−25 °C) in small glass tubes, tightly closed with rubber caps. The test sample of plasma (0.050 ml diluted 1 : 10) was added to the reagent (37 °C), and vigorously mixed. Coagulation time was determined with a Kolle hook, and activities expressed as % of normal (compared to time-dilution curves made with pooled normal rat plasma).

Plasma albumin was determined with a dye binding method (Hydroxy-Benzeneazo-Benzoic-Acid: H.A.B.A.) according to De Leeuw-Israel et al. (1967).

S.G.P.T. was determined with a colorimeter according to Reitman and Frankel (1957), (Boehringer biochemical test combination).

Total- and conjugated-bilirubin were measured colorometrically (Hollander et al. 1968, Boehringer).

B.S.P. retention test was performed with animals under ether anaesthesia, after being warmed under an infrared light to stimulate peripheral circulation. The dye (40 mg/ml, 0.35 ml per rat) was injected into a lateral tail vein and blood collected from the tail tip in heparinised capillaries exactly 1, 10, 20, and 30 minutes thereafter. The supernatant plasma (20 µl) was added to 1.5 ml 0.5 mol NaOH in 0.9% NaCl and the extinction determined on a colorimeter at 579 nm.

Portal vein radiography was performed under ether anaesthesia at seven weeks; Urografin 76% (0.3 ml) was injected into the spleen and/or a canulated mesenteric vein.

Thereafter the relevant organs were removed for histological examination. Necropsy on the animals which died spontaneously was performed within 15 hours after their death. The tissues were fixed in 4% buffered formalin and 5µ Paraffin embedded sections were routinely stained with hematoxylin, azofloxin and saffron (HAS).

3. Results

Total time required for the transplantation was about 75 minutes. Recipient operation lasted 1 hour, the liver transplant was ischaemic for 40 minutes, including 20 minutes of saline perfusion. Death during the operation (animals not recovering from the anaesthetic) occurred in 16 cases (20.8%) of the 77 attempted transplantations. Death within 48 hours of the operation, occurred 30 times of the first 57 transplantations, equally devided over iso- and allo-transplanted animals. Thereafter some alterations in the transplantation procedure were effectuated, and death within 48 hours occurred only once among the following 20 transplantations (see discussion). Overdoses of ether, during the observation period, resulted in the death of 2 allo- and 1 isografted animals. In 4 isogeneic transplanted animals unsuccessful biliary anastomosis gave rise to abundant bile stained ascites, progressive hyperbilirubinaemia and death, between 2 and 4 weeks after transplantation. They were therefore considered to be technical failures and excluded from the experiment. The functional, histopathological and roentgenographic studies were performed on the remaining 13 animals with allogeneic transplants and 9 animals with isogeneic transplanted livers.

a Isogeneic transplantations

All 9 animals recovered remarkably well after the operation and continued to be clinically well during the 7 weeks observation period (fig. III. 3). The mean maximal weight loss was 9.6% of preoperative values but the animals gained weight until the mean value was 100% of the preoperative values (fig. III 3).

Haematocrit values (fig. III 3) rose steadily from 35.2% immediately after the transplantation to 40% at seven weeks, notwithstanding the weekly withdrawal of 1½ ml of blood.

Five animals were transiently jaundiced, maximal bilirubin being 2.6 mg/100 ml (fig. III 4). The mean BSP uptake by the transplanted liver as indicated by the 1-10 minute serum excretion was within normal limits and in some cases even higher than normal (60% 1-10 min. excretion) values were reached. Excretory capacity of the liver (BSP 20-30 min. excretion) was also normal (fig. III 4).

Four of the nine animals had elevated SGPT levels (> 50 mU/ml) 3 days after transplantation, but only two animals at maximum had elevated SGPT values thereafter (fig. III 4).

Albumin levels were decreased but tended to recover: 2.6 gr/100 ml

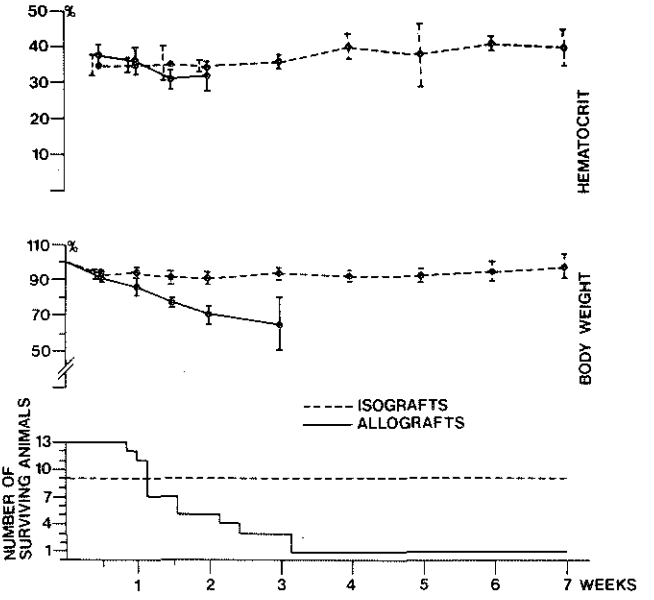


Fig. III 3. General parameters.

Survival of rats bearing liver isografts or liver allografts. Mean survival time after liver allotransplantation: 11.9 days. Mean body weight and hematocrit ± s.d. of rats bearing liver isografts or liver allografts.

after seven weeks (normal 3.0 gr/100 ml) (fig. III 5). All clotting tests: prothrombin, Thrombotest® and Normotest® activity normalised or values slightly above 100% were obtained (fig. III 5).

Evaluation of the radiographs, made just before the end of the experiment, showed normal splenoportograms. The portal veins were of normal caliber and the intrahepatic branches were undistorted (fig. III 6a). The sinusoidal filling in the hepatogram phase was normal (fig. III 6b). Portal-systemic venous shunts, indicative of portosplenic hypertension, through collateral veins like the coronary vein, left adrenal or renal veins were not observed.

At autopsy the macroscopic aspect of intestines, kidney, adrenal and testes was normal. The spleen and splanchnous veins were not enlarged or dilated. The autologous liver remnant could not be found or a necrotic, encapsulated remainder of 0.4 gr at the most was located near the subdiaphragmatic part of the caval vein. The donor livers were, compared to their mass at the time of transplantation, enlarged (mean 11.4

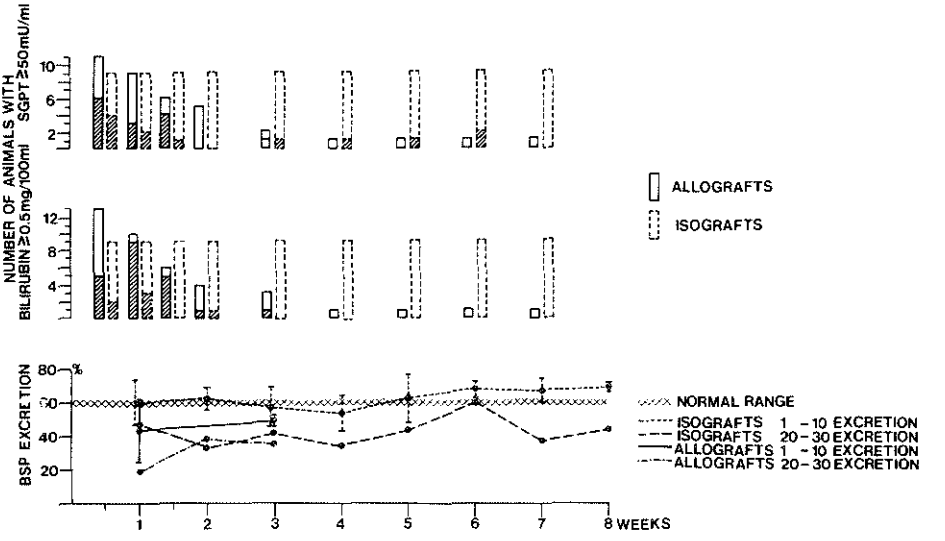


Fig. III 4. Excretory capacity and cell loss.

Mean B.S.P. excretion ± s.d. in rats bearing liver transplants. 1–10 min. excretion represents maximal capacity of the liver to take up B.S.P. from the blood into the livercell; while the 20–30 min. excretion represents the capacity of the liver to excrete B.S.P. from the livercell into the biliary system. The normal range of B.S.P. 1–10 min. excretion ± s.d. is indicated by the shaded area.

Number of animals, bearing liver transplants, with bilirubin ⩾ 0,5 mg/100 ml and with significantly raised S.G.P.T. values (⩾ 50 mU/l).

± 2.5 gr S.D.). Their surface had a normal smooth aspect, the colour being a normal red-brown. When cut, the consistency was normal and no congestion of blood was seen.

Histological examination showed normal architecture and liver cell appearance, although some proliferation of bile ducts was present (fig. III 7). In the liver hilus and along the intrahepatic branches of the portal vein, many extensively coiled arteries were noted.

b Allogeneic transplantations

Although recovery of the 13 animals, incorperated in the study, after the operation was very good, their clinical condition deteriorated progressively from the end of the first week. Survival time varied from 6 to 22 days disregarding one animal which survived until killed at the end of the observation period (fig. III 3). Mean and median survival time were 11.9 and 11 days, respectively.

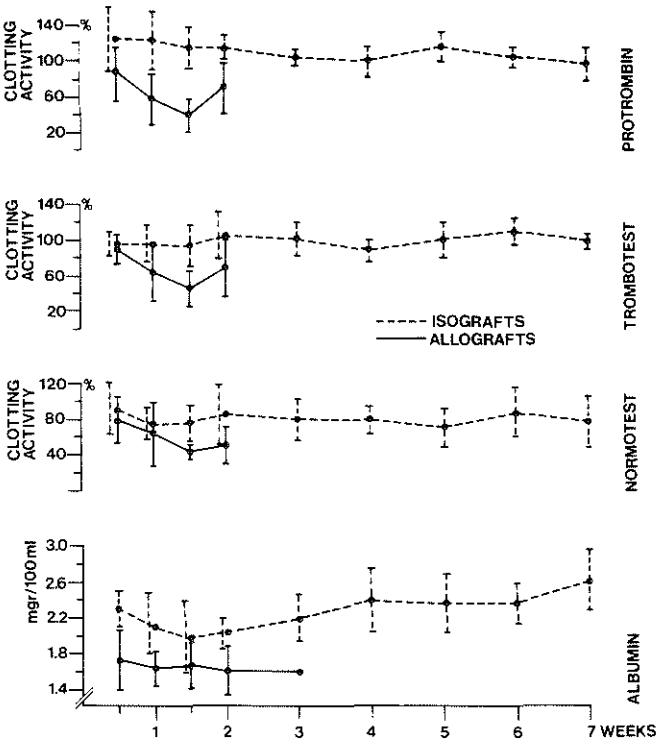


Fig. III 5. Productive capacity.

Mean ± s.d. of Prothrombin, Thrombotest and Normotest activity and albumin levels of rats bearing liver transplants. The prothrombin activity was measured with the one stage method, using rat constituents (see text).

Average body weight of the experimental animals (in % of preoperative values) diminished sharply to 65%, 3 weeks after operation (fig. III 3).

Hematocrit diminished from 48% (normal hematocrit) to 32% in two weeks (fig. III 3). In two animals hematocrit rose thereafter until they died.

The incidence of jaundice became about three times as high as in the isografted series. Three days postoperatively already five of the 13 animals (38.5%) were clearly jaundiced. At the 10th postoperative day and at 14 days 90% and 85% of the animals were jaundiced respectively. In contrast, only 30% and none of the nine isografted animals were jaundiced at that time (fig. III 4). The elevated serum bilirubin was

conjugated in the majority of the animals suggesting that the hyperbilirubinaemia was mainly of the obstructive type. Maximal bilirubin values were 4.2 mg% at 4 days, 1.8 mg% at 1 week and 2.2 mg% at 10 days.

Mean plasma albumin levels became very low (1.6 g/100 ml (fig. III 5).

Impaired synthetic capacity of the allo-transplanted livers, was also reflected in the results of the clotting tests: mean prothrombin activity rapidly fell from normal levels to 40% 10 days after transplantation (fig. III 5). Although the five remaining animals had an increased clotting activity 4 days later, four animals died the subsequent week. Parallel to the results of the prothrombin activity measurements, Thrombotest and Normotest values diminished to 45% of normal after 10 days. At 14 days postoperatively an increase in clotting activity was noted in the 5 remaining animals (fig. III 5).

One week after transplantation Bromsulphalein (BSP) excretion was low at 10 and also at 30 minutes after injection of the dye (fig. III 4)

SGPT did not rise to high levels. Significantly raised levels of 50 mU/ml or more were present in 6 of the 11 animals after 3 days. One week postoperatively, only 3 of the 9 remaining animals had raised SGPT-levels while 4 of the remaining 6 animals had high levels 3 days later (maximum 255 mU/ml) (fig. III 4).

At necropsy all allografted livers were very much enlarged (twice to six times their original weight), and showed a dark red-brown colour; sometimes the liver lobes were partly necrotic. Although not noticed clinically before the animals were moribund, 9 of the 12 animals had yellow stained ascites, which also contained some blood. They all had distinct amounts of bile within their intestinal lumen indicating that the biliary anastomosis was functioning well.

Histology of the liver grafts showed infiltration with mono-nuclear cells probably of lymphoid origin; this was especially prominant in the portal tracts. These cells extended into the liver parenchyma and were supposedly associated with haemorrhages from the liver sinusoids seen during the first week. Focal necrotic areas were increasingly seen during the second week (fig. III 8). The animals which died in the third week after transplantation had large areas of coagulative liver-infraction probably caused by intrahepatic thromboses of major portal branches (fig. III 9). During the first two weeks after transplantation one animal's clinical condition deteriorated, while loss of liver function was noted. However, it

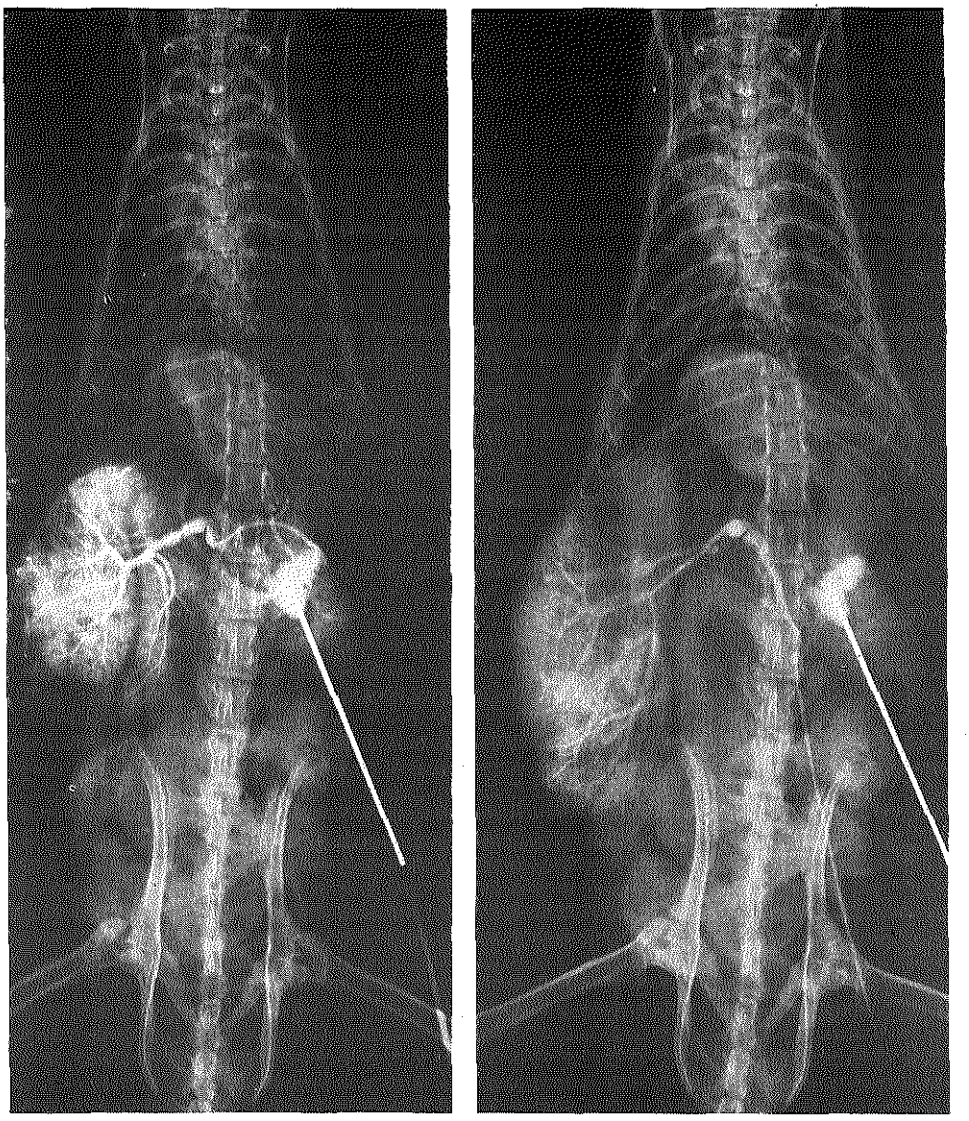
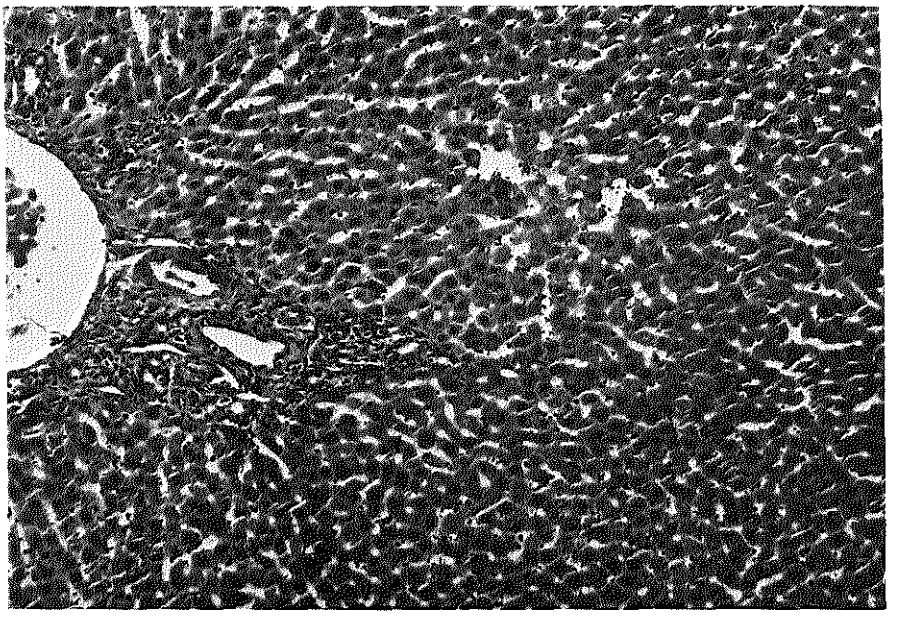


Fig. III 6a, 6b.
Spleno-portography of a rat after isologous liver transplantation.

6a: the radioopaque dye is injected into the spleen: the portal vein and its intrahepatic branches are intensely stained.
The heterotopic place of the liver is well recognisable, filling the right lateral side of the abdominal space.

6b: the radioopaque dye is injected into a mesenteric vein and intensely stains the major intrahepatic portal vein branches. The photograph is taken in a later phase than 6a showing an even distribution of the dye through the liver sinusoids.



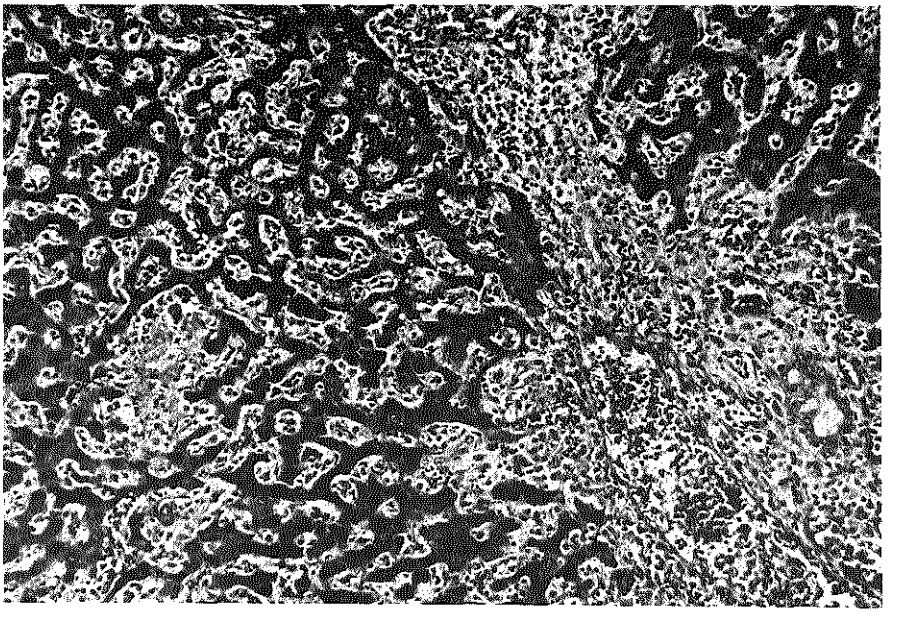
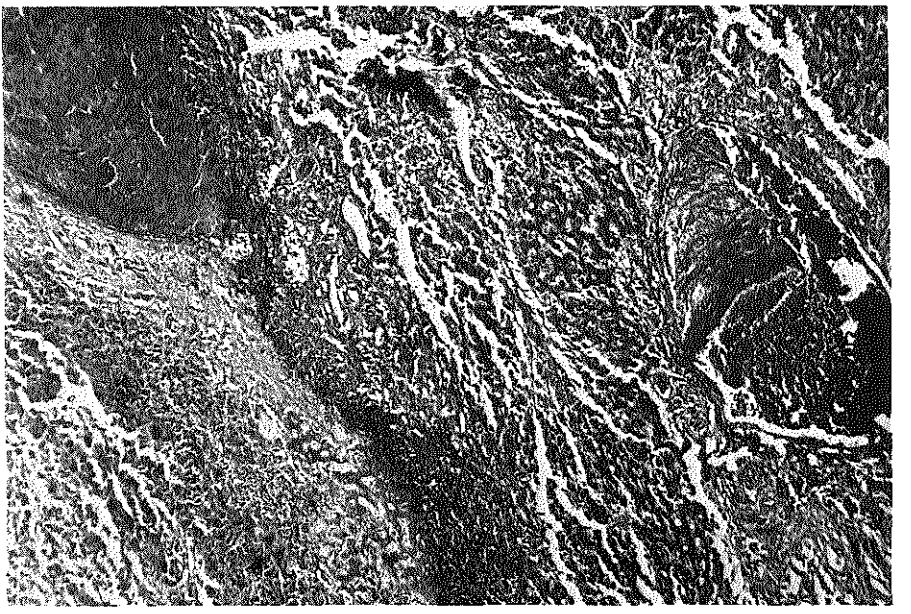


Fig. III 7.

Rat liver isograft. Untreated animal, 7 weeks after transplantation showing well preserved hepatocytes. There is some bile duct proliferation around the portal vein. Although the hepatic artery was not anastomosed at the time of transplantation several arteries are distinguishable. H.A.S. x 150.

Fig. III 8.

Rat liver allograft. Untreated animal, day 12 after transplantation. There is a strong mononuclear cell infiltration along the portal vein, extending into the liver parenchyma. Focal necrotic areas are present. H.A.S. x 150.



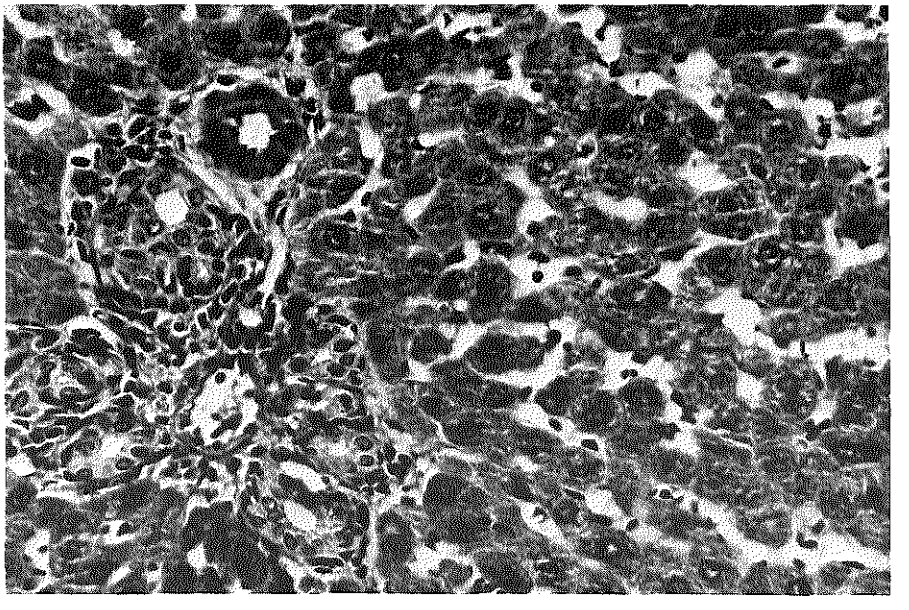


Fig. III 9.

Rat liver allograft. Untreated animal, day 12 after transplantation. Two major branches of the portal vein are seen with a thrombus. There is a large area of necrotic liver tissue and also a part in which the liver structure is partially preserved. H.A.S. x 60.

Fig. III 10.

Rat liver allograft. Untreated animal, day 49 after transplantation. Hepatocytes and liver architecture are normal, there is some bile duct proliferation, several arteries are seen along the portal vein. H.A.S x 380.

ameliorated to an excellent clinical condition, gaining weight until preoperative values were reached, while also its liver function restored to normal.

At autopsy no macroscopial abnormalities were noted, in particular no infracts of the liver were seen; nor were there signs of porto-splenic hypertension. Histology, at 50 days postoperatively revealed at that time normal liver tissue without any signs of rejection (fig. III 10).

Statistical evaluation using the test of Welch (Welch 1947, de Bruin 1970), on a 1% significance level, revealed significant differences between the iso- and allografted series of the following parameters: survival, weight, clotting tests, albumin and BSP-excretion.

4 Discussion

a Transplantation procedure

Initially, postoperative mortality (defined as death within 48 hours of successful operation) was high: 30 animals died in the first series of 57 transplanted animals (52%). Though the animals recovered from the anaesthetic and were clinically well for 1 - 6 hours after the operation, their condition thereafter deteriorated until death occurred, usually within 15 hours. The clinical pattern of these dying animals in fact resembled that seen after experimental temporary occlusion of the portal vein (v.d. Meer et al. 1971). This high postoperative mortality was initially attributed to the liver transplantation procedure as such. However, several other factors had to be considered as a possible contribution for this high mortality. Overdosage of ether was readily prevented by temporary discontinuation of the ether administration.

During the operation a *decrease of body temperature* to ± 26 °C was noted in the first series of transplanted animals. This may have been due to evaporation (and thus temperature loss) of fluid from the intestines which were until then lateralised outside the recipients abdomen during the transplantation procedure.

Through this lateralisation of the recipients intestines, direct *stimulation of splanchnic nerve endings* may also have led to a neurogenic fall in blood pressure. Thirdly, Aldrete (1969) recognised that preventive measures had to be taken in order to prevent diminuation of *blood glucose* levels during the transplantation procedure: 0.2 g glucose/kg/hour should be administered. Further, not only external *blood loss* should be considered when giving a blood-transfusion but an unknown amount of blood is removed with the extirpated liver and in addition

some blood probably remains sequestrated in the viscera, kidneys and hind limbs.

Three simple measures were taken in the subsequent series to prevent the four mentioned factors which could have contributed to the initial high postoperative mortality. First, the intestines were lateralized *within* the abdominal cavity, secondly not only the estimated extravascular blood loss of 0.5 ml was compensated for, but an additional blood transfusion of 1 ml isologous blood was given. Thirdly 1.5 ml glucose 5% was administered together with the blood to prevent hypoglycaemia.

As a result, the early mortality was reduced from 30 of the first 57 (52%) animals operated upon, to 1 out of 20, of the subsequently operated animals. This significant improvement could not be attributed to an increased dexterity of the operator since improvement was prompt and (intra) operative death remained ± 20% throughout this and subsequent series.

b Liver function and pathology

The biochemical analysis of liver function indicated that hepatic synthetic capacity as reflected by albumin levels, F II values and the results of the other clotting tests, was significantly reduced during the acute rejection process of liver allografts. Since Thrombo- and Normotests gave practically the same mean coagulative activity percentages, one may conclude that no sign of vitamin K deficiency was present at any time of the study (Hemker and Muller 1968, Owren 1969). As expected, the BSP excretion was decreased, also indicating hepatocellular damage.

In the isografted rats, BSP excretion during the first 10 minutes after its injection, was increased above normal. This indicates that the number of liver cells available for detoxication was increased (Cornelius et al. 1967, de Leeuw-Israel, 1971). Interestingly the mean liver mass of the isografted rats was 11.4 g after seven weeks, while the male recipients original liver weight at the time of transplantation was only 8.7 g and the female donor liver at the time of transplantation weighed only 6.8 g. Thus the increase in liver weight does not seem to be limited by intrinsic factors, that is genetically determined within the (female donor) liver cells, but considerable influence must have come from outside the liver. Since the productive capacity of the liver had become normal, as may be concluded from the normal clotting activities, or close to normal (see albumin levels), it suggests that the liver has a lower productive capacity per unit of cell. This may have been the consequence of lower oxygena-

tion (only portal blood was provided for the liver) or other hemodynamic factors. The data favour the concept that there exists a controlling mechanism based on the maintenance of a constant functioning hepatic cell mass: body weight ratio (Maki and Slapak, 1974). The fact that there was overcompensation of excretory function and of mass rather than of productive capacity suggests that the latter provides the stimulus for compensatory liver growth.

Survival time of the untreated non-auxiliary liver allografted animals was similar to that seen in untreated kidney allografted animals of the same strains (de Bruin 1970, Tinbergen 1972), the kidneys being transplanted across the same strong histocompatibility barrier (H-1 histocompatibility locus (Štark and Křen 1969, Stark et al. 1969). This is not the case when livers are transplanted using the auxiliary method of transplantation. Although the major Ag-B locus is not a strong locus in the Buffalo rat strain (Guttman et al. 1967, Murray 1969), total or 30% auxiliary liver allografts between Buffalo and Lewis rats appeared to be completely infarcted by day 14 (Fisher and Fisher 1970). In Lee and Edginton's studies all auxiliary liver grafts were destroyed by day 10 or 11. Our own experiments in which auxiliary grafts were used (chapter II) also indicated a shorter survival time of the allografted liver when compared to non-auxiliary liver grafts.

The present experiments indicate that liver cell proliferation is strongly stimulated in non-auxiliary liver allografts, leading to a somewhat prolonged survival and apparently an increase of functioning liver mass. In this respect it is interesting to note that one animal survived the acute rejection period, although functional analysis revealed severe impairment of liver function during that time. Even more interesting is the fact that histopathological examination of the grafter liver 7 weeks after transplantation showed no signs of rejection or cell damage whatsoever. Apparently the recipient had accepted the transplanted organ. We have not carried out liver biopsies in order to obtain histopatholocial proof of rejection in this animal. Hence, we can only point to the similar function pattern of this and other animals while we have no absolute proof that indeed rejection did occur at 10 days after transplantation. The same phenomenon was noted in respect to kidney transplantation in the rat by Dunn and Rendall (1974) and they suggested that auto-enhancement might be the reason for this prolongation of survival. It is not known if similar processes like tolerance or enhancement as supposed by Calne (1969) in respect to pig liver transplantation played a role.

Other workers (de Bruin 1970, Lubbe et al. 1972, Vuzevski, 1976) have described that in the rejection of organ transplants two different types of lesions are noticed:

1. a lesion of capillary endothelium and parenchymal cells associated with lymphoid cell infiltration, probably to be explained by a cell-mediated rejection, and
2. an inflammation and necrosis of the larger transplant arteries and veins associated with deposition of IgG and complement in the vascular wall. This so-called vascular rejection is possibly the result of an antibody-mediated reaction.

Histopathological examination revealed that unmodified rat liver rejection also had two main features: animals dying within 1½ weeks predominantly showed acute rejection which had the features of a cell medicated process. During the first week the portal tracts were increasingly invaded by mononuclear cells which also infiltrated between the liver cells apparently causing liver sinusoid rupture with haemorrhages and individual liver cell loss (fig. III 8). Animals dying after two weeks showed endothelial proliferation, necroses of the wall of the portal veins and portal vein thromboses, possibly associated with an antibody mediated process (fig. III 9). The acute rejection pattern of these liver allografted rats is in contrast to procine liver transplantation (Calne et al. 1967a) and is comparable to that seen in canine, monkey and human liver transplantation (Myburgh et al. 1971, Porter 1969).

In clinical and experimental liver transplantation, the biliary-intestinal anastomosis and the portal vein are the suspected sites for ascending infections (Starzl 1969, Starzl et al. 1975, Murray-Lyon et al. 1970, Schalm et al. 1972, Schalm et al. 1975, Williams et al. 1973). The susceptibility to this infection seems to be promoted by the rejection process. Histologically we have seen however, no cases of dissiminated focuses of bacterial growth. This may be explained by the fact that we used specific pathogen-free animals and treated them during three weeks with antibiotics.

In our series, many animals developed yellow-brown stained ascites during the final stage of rejection. Macroscopic examination and histology showed that at that stage the extrahepatic biliary tract was intact and unobstructed by the rejection process. Since many hepatocytes were still working at that time, the ascites must have been of transhepatic origin.

In the liver isografts a great number of arteries was observed than in the untransplanted liver. This was not found up to 3 weeks after

transplantation in a separate series of animals which were sequentially killed. Possibly, re-arterialisation occurred, the new arteries being formed along the portal tracts.

In 1972 we preliminary reported on the results of the non-auxiliary transplantation model as described above (Wolff 1972). It is relatively simple, can be performed by one person in one hour, and because of the sequence of operative actions, it circumvents the need for a blood shunt from the portal vein to the jugular vein, while the recipient is not anhepatic at any time of the operation. Rats bearing isogeneic liver transplants, placed heterotopically as described above were able to survive up to 1½ years without indications of diminuation of liver function. Recently another non-auxiliary way of transplantation was described (Lee et al. 1974) using the classical orthotopic liver transplantation technique in the rat. Although in that way the liver transplant is clearly in a more physiological position, the procedure is more difficult to perform and it necessitates a porto-jugular shunt.

Donor livers in our series, half the size of the original recipient livers, were able to function adequately and to maintain the 100% hepatectomized recipient in a healthy state. The strong proliferative activity of the livers as shown in our experiments may have clinical implications. It suggests that, livers from infants might be used for orthotopic transplantation in one to three year old children or children's livers for adult recipients (Calne 1969, Ranson et al. 1972).

The technique of non-auxiliary liver transplantation in rats as described above, may be useful in studying the patho-physiologic consequences of various immunosuppressive regimens.

Chapter IV

IMMUNOSUPPRESSIVE THERAPY OF LIVER ALLOGRAFTED RATS

1. Introduction

Experimental allo-transplantation of such organs as heart, kidney, skin, intestines, islets of Langerhans or bone marrow between inbred rat strains, have provided valuable information on rejection and function of the allograft. Different immunosuppressive regimens have been tested in rodents with such transplants (v. Bekkum et al. 1969, de Bruin 1970, Kort et al. 1973, Marquet and v. Bekkum 1973, Marquet and Heystek 1975, Tutscka and Santos 1975, Wustrack et al. 1975). However, in the case of liver transplantation so far only larger animals could be used (Chandler et al. 1972, Myburgh and Smit 1975, Starzl et al. 1965).

Immunosuppression of liver allografted rats could not be adequately investigated because a life-sustaining part of host liver had to be left within the recipient rat. Often the auxiliary liver allografts in rats were rejected within 14 days without significant loss of liver function or clear signs of liver cell damage, while the animals remained alive and in good condition (personal observation).

A liver transplantation technique in which no functional host liver tissue was left at the time of transplantation provided the possibility to study survival of the animals, as well as the function of the grafted liver (chapter III). "Functional competition" between the graft and the host liver would not influence the fate of the graft, and changes in liver function would in fact reflect the different aspects of rejection. In the experiments described here, a number of immunosuppressive regimens were used in rats, carrying such life-sustaining allotransplanted livers.

2. Material and methods

a. Animals.

Inbred specific pathogen free rats of the same strains as described in chapter III were used. Female BN/Bi rats were used as liver donors.

Male animals of the Wag/Rij strain served as recipients to be treated with immunosuppressants. The liver isografted group of BN/Bi rats described in chapter III served as control animals.

b. Surgical technique. Antibiotic treatment.

The surgical technique as well as antibiotic treatment were as described in chapter III. Whole female livers (weighing 6.08 ± 0.50 g) were heterotopically transplanted into hepatectomized recipients (male BN livers weighed 8.70 ± g and Wag livers weighed 9.58 ± 0.62 g). Antibiotic treatment consisted of daily doses of 10,000 I.U. penicillin, 10 mg streptomycin and 20 mg cephaloridin s.c. given during 3 weeks following the operation.

c. Laboratory techniques.

The techniques and frequency of determinations were as described in chapter III.

Body weight measurements and haematocrit determinations were performed bi-weekly. Synthetic capacity of the liver was evaluated by albumin measurements and clotting activity tests. Prothrombin (FII) was measured with a one stage method (Owren 1949) using rat thromboplastin and prothombin deficient rat plasma (chapter III). Excretory capacity of the livers: total and conjugated bilirubin measurements and sequential bromsulphtalein (B.S.P.) retion tests were performed. SGPT, indicating possible liver cell loss, was regularly measured in all animals. Portal vein radiography was performed at 7 weeks. The animals were then killed during the same anaesthesia and the relevant organs for histological examination removed. Paraffin-embedded sections of 5μ thickness were routinely stained with hematoxilin, azophloxin and saffron (H.A.S.).

d. Immunosuppression and experimental design.

One badge of horse Anti Rat Lymphocyte Globulin (ALG) (kindly provided by the Radiobiological Institute TNO, Rijswijk) was used in two of the experimental groups. It was produced and purified in the same way as described for horse anti monkey lymphocyte globulin (Balner et al. 1970). When tested in vivo, in the same weekly dose regimen as used in these liver allograft studies, it effectively prolonged skin allograft survival between inbred rat strains. Azathioprine (Imuran®, Burroughs Wellcome, London) and Prednisolone sodium succinate

(Organon, Oss, Holland) were administered for three weeks after the transplantation. A period of three weeks to treat the animals was chosen since it was known that unmodified rejection of the allografted liver occurred within this period in the majority of the animals (chapter III). It was also known that the majority of liver homograft rejections, in spite of immunosuppressive treatment, in dogs or monkeys, occur within 50 days (Myburgh et al. 1971, Starzl et al. 1965). Thus, an observation period of seven weeks was chosen since this seemed a reasonable period to study the acute rejection of the transplanted livers. It also allowed comparison with experiments concerning rat kidney grafts and immunosuppression (de Bruin 1970).

The experimental groups were:

I One group of nine allografted recipients was treated with ALG only. The ALG treatment consisted of 4 intra-peritoneal injections of 1 ml: day -7, day 0 (day of operation), day +7 and day +14.

II A second group of 10 animals received daily 4 mg/kg .azathioprine s.c. from day 0 to day 21.

III A third group of 10 animals received a combination of the treatment of group I and II, i.e. ALG and 4 mg/kg azathioprine.

IV A fourth group of 9 animals was treated with daily s.c. injections of prednisolone 4 mg/kg and azathioprine 2 mg/kg during three weeks after transplantation.

3. Results

Fiftythree allotransplantations were performed. Fourteen animals died during the operation or within 48 hours after transplantation. Since their death could be related to technical errors, they were excluded from the experiment.

a Liver function

Group I

The nine ALG treated animals survived in excellant clinical condition, comparable to nontransplanted or isografted animals, until they were killed. However, two of these animals were accidentally killed, by an overdosage of ether, 4 and 5 weeks respectively after transplantation (fig. IV 1). The results of the liver function tests were also good (the two animals, which were accidentally killed, were included in the results). Mean weight (fig. IV 2) was only slightly lower than that of the

isografted group. Haematocrit and albumin values (fig. IV 3, IV 4) were higher than those of the isografted group. Prothrombin (fig. IV 5) did not differ, but BSP excretion (fig. IV 6) became better than the results of the isografted group. The proportion of animals with an elevated serum bilirubin ($\geqslant$ 0.5 mg/ml) was somewhat greater than in the group of isografted animals (fig. IV 7).

Group II

Of this group, treated with azathioprine 4 mg/kg, 5 of the 10 animals died during the first two weeks after transplantation (fig. IV 1). During this time, the results of the weight, haematocrit, albumin, prothrombin and BSP excretion estimations were depressed in all ten animals (fig. IV 2 - 6). In the animals that died spontaneously, at autopsy a pale donor liver with haemorrhages was found. Haemorrhages were also found scattered over the body, for instance subcutaneously, in the lungs, intestinal wall and occasionally in the testes. In those animals which survived the first two weeks, the results of the function tests tended to normalise, which was closely correlated with an amelioration of the clinical condition of these animals.

Group III

Azathioprine 4 mg/kg added to the ALG treatment gave low values of weight, haematocrit and albumin determinations, especially so when compared to the group treated with ALG alone (fig. IV 2-4). All 10 animals survived for 3 weeks after the liver transplantation but 3 animals subsequently died within 3 days, an additional death occurring 2 weeks later, leaving 6 out of 10 animals to survive the observation period of 7 weeks (fig. IV 1). The animals which died at 3 and 5 weeks post operatively were found to have at autopsy dispersed haemorrhages and very pale bone marrow. Blood congestion as well as haemorrhagic areas were found in the donor livers. Macroscopically, also thrombotic processes within the caval vein, adherent to the ligated suprahepatic part of the donors caval vein and partly occluding the efferent traject of blood flow through the liver, were found.

Group IV

Although initially all nine animals treated with azathioprine 2 mg/kg and prednisolone 4 mg/kg clinically recovered quite well after the transplantation, their clinical condition deteriorated after approximately 3 days. Four deaths occurred within the first post operative week (fig.

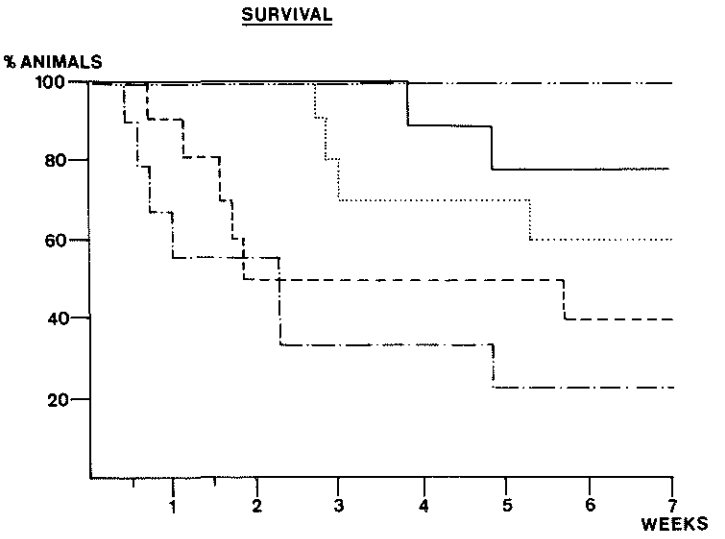


Fig. IV 1.

Survival time of recipients of a liver isograft, without immunosuppression and allografted rats with a different immunosuppressive regimen: see text.

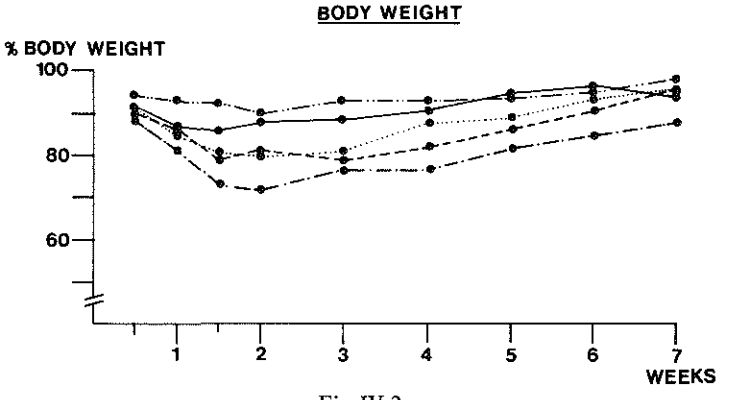


Fig. IV 2.

Mean bodyweight of liver isografted control rats and allografted rats, with a different immunosuppressive regimen.

ISOGRAFTS without immunosuppression

ALLOGRAFTS with immunosuppression

ALG
ALG+IM.4mg/kg
IM.4 mg/kg
IM.2 mg/kg+PRED.4mg/kg

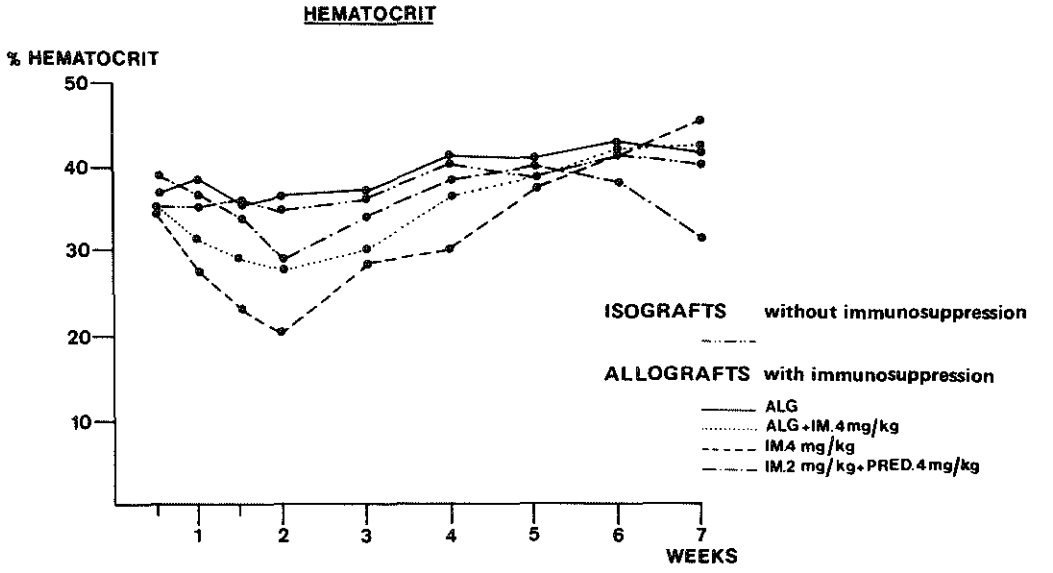


Fig. IV 3.

Mean haematocrit of liver isografted control rats and allografted rats, with a different immunosuppressive regimen.

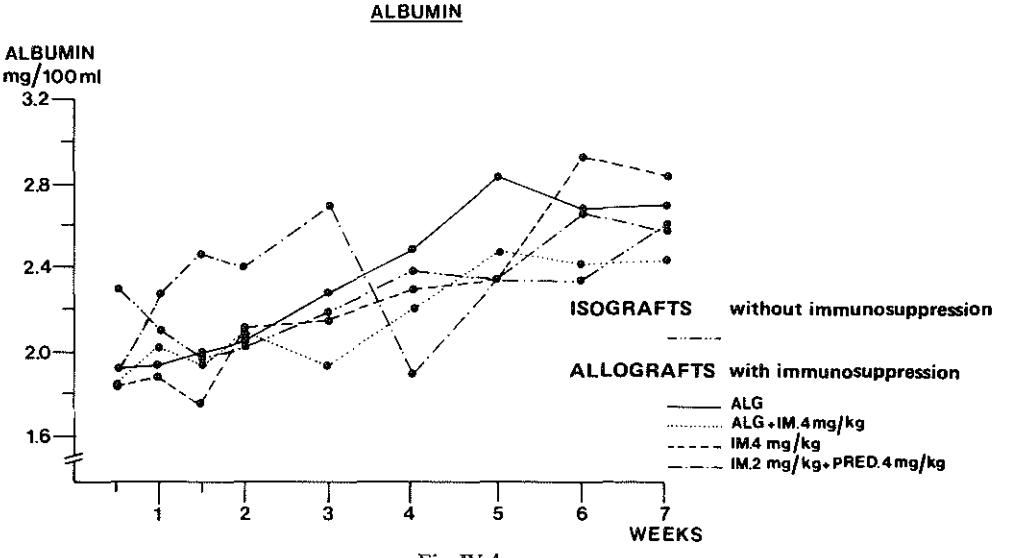


Fig. IV 4.

Mean albumin of liver isografted control rats and allografted rats, with a different immunosuppressive regimen.

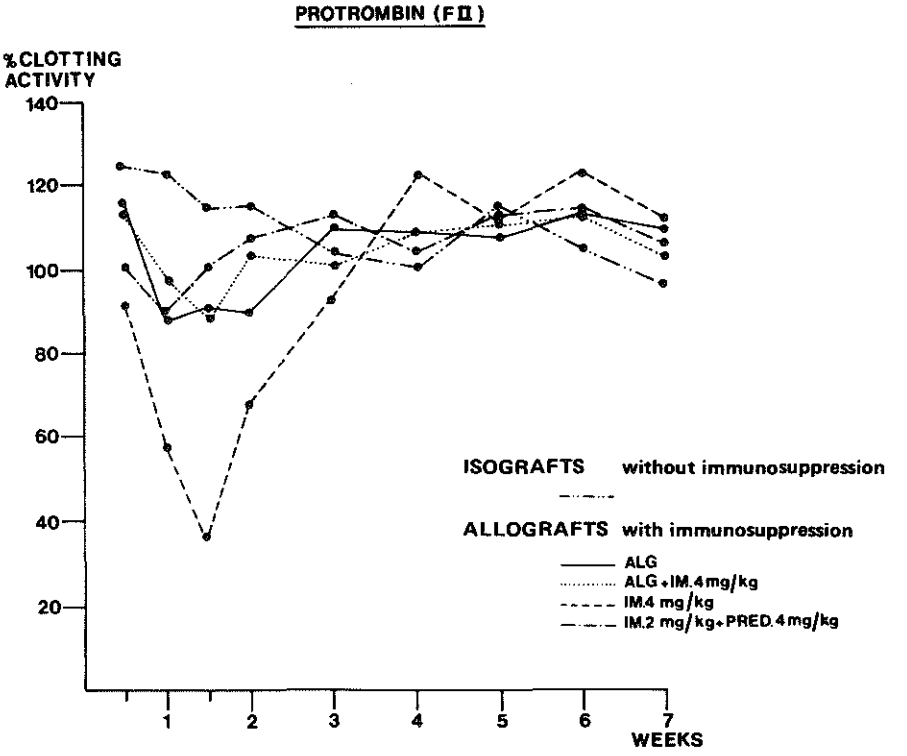


Fig. IV 5.

Mean Prothrombin (F II) of liver isografted control rats and allografted rats, with a different immunosuppressive regimen.

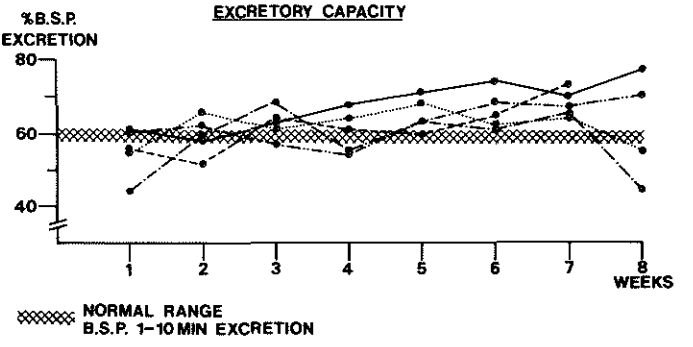


Fig. IV 6.

Mean B.S.P. excretion (expressed in % of 1 min. concentration, 10 min. after injection of the dye), of liver isografted control rats and allografted rats with a different immunosuppressive regimen.

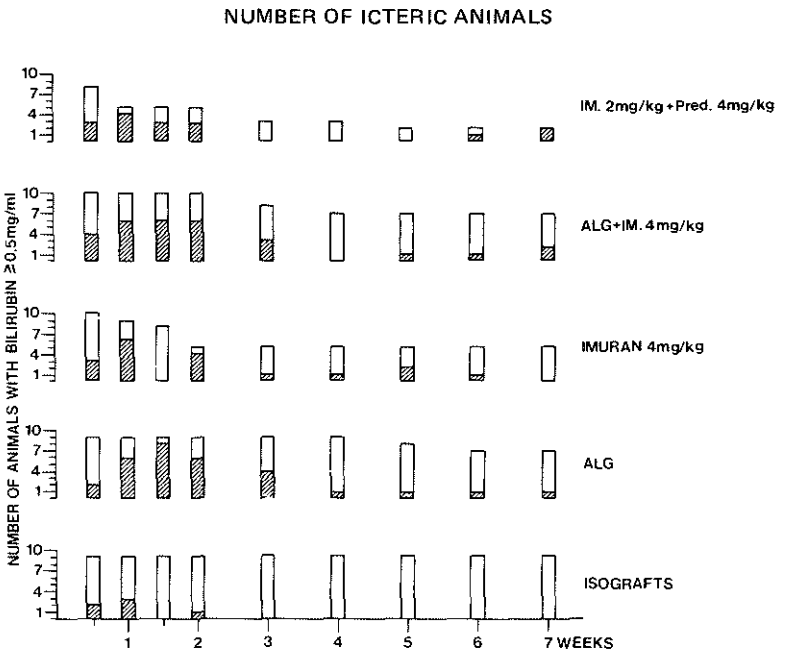


Fig. IV 7.

Number of animals with bilirubin ⩾ 5 mg/ml after receiving a liver isograft without immunosuppression, or liver allograft, with a different immunosuppressive regimen.

IV 1). Liver function, as reflected by the mean albumin values and prothrombin activities were however not significantly impaired (fig. IV 4, 5 table IV 1). Autopsy on the animals that died spontaneously showed that thrombotic processes partly occluding the intrahepatic caval vein but also wound dehiscention and peritonitis were present. In three animals significant intra-abdominal or external blood loss had obviously occurred.

Mean SGPT values of the allografted animals were elevated (⩾ 50 mU/ml) immediately after the operation and even more so 1½ to 2 weeks after the operation. The values normalised in the following 5 weeks. There were no significant differences noted between the differently treated groups.

B.S.P. excretion rates, during the first 10 minutes after injection of the dye, were significantly better in the ALG treated group than in the

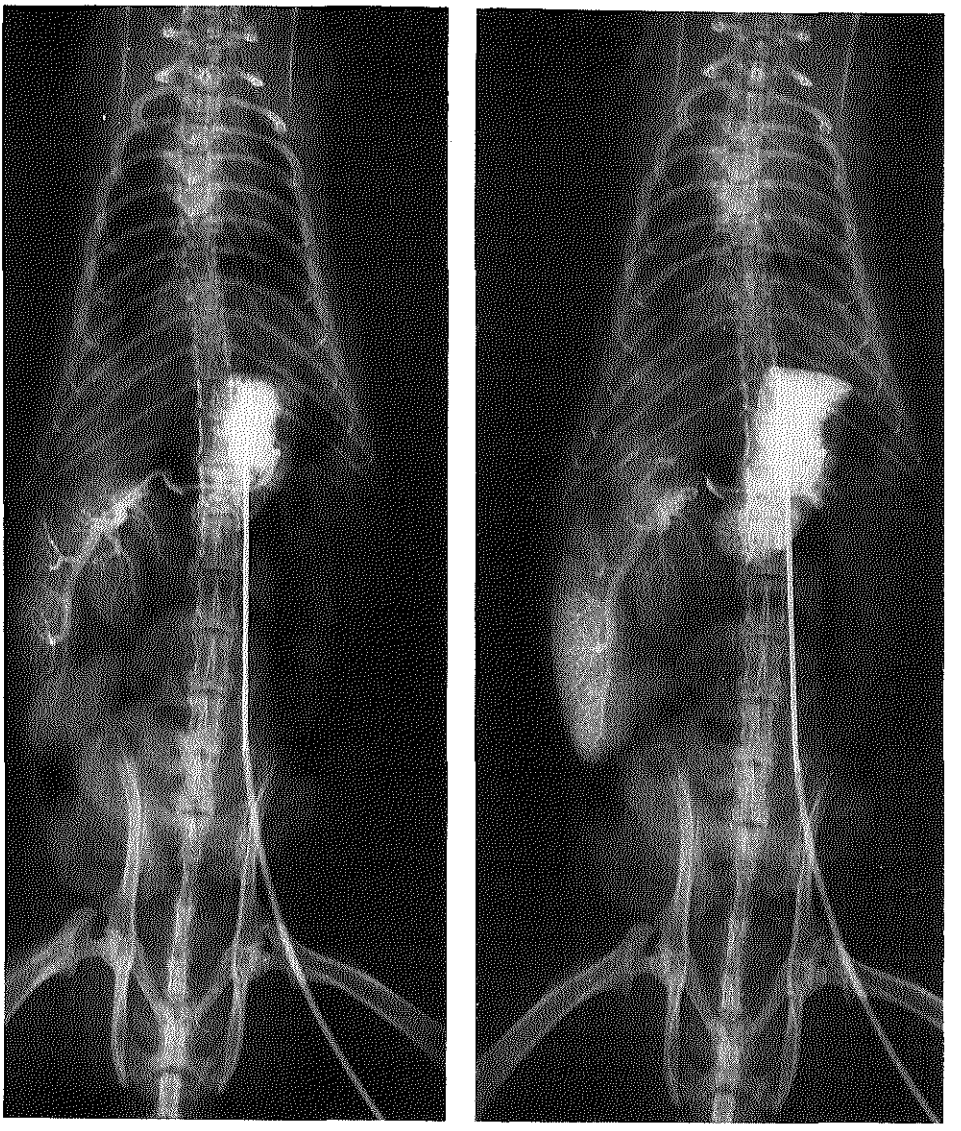
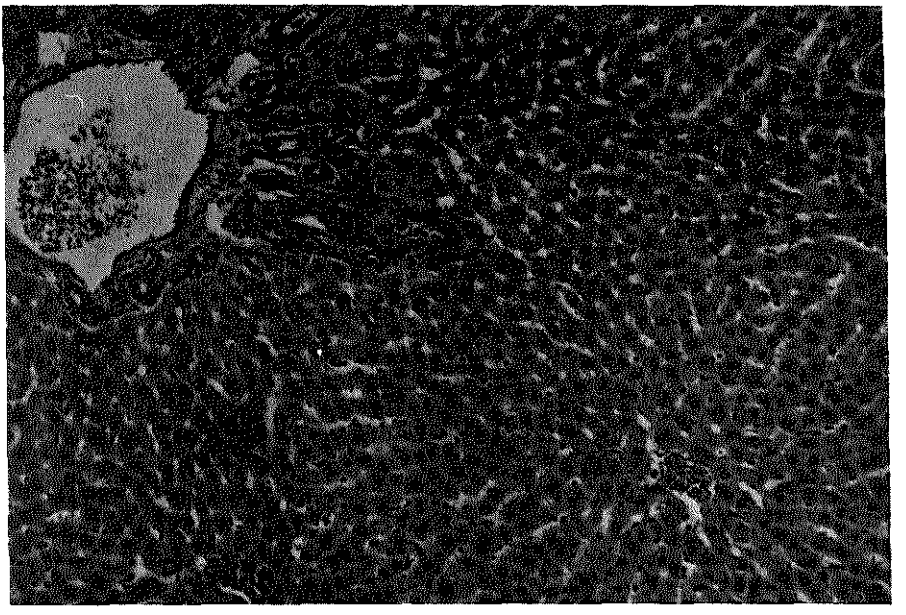


Fig. IV 8.

Spleno-portography of a rat, after allotransplantation of the liver and treatment with ALG.
A There is good visualisation of the portal vein and its intrahepatic branches.
B Picture taken in a later phase than A, there is an even distribution of the radio-opaque dye through the portal vein and its branches but it is also evenly distributed through the liver sinusoids.



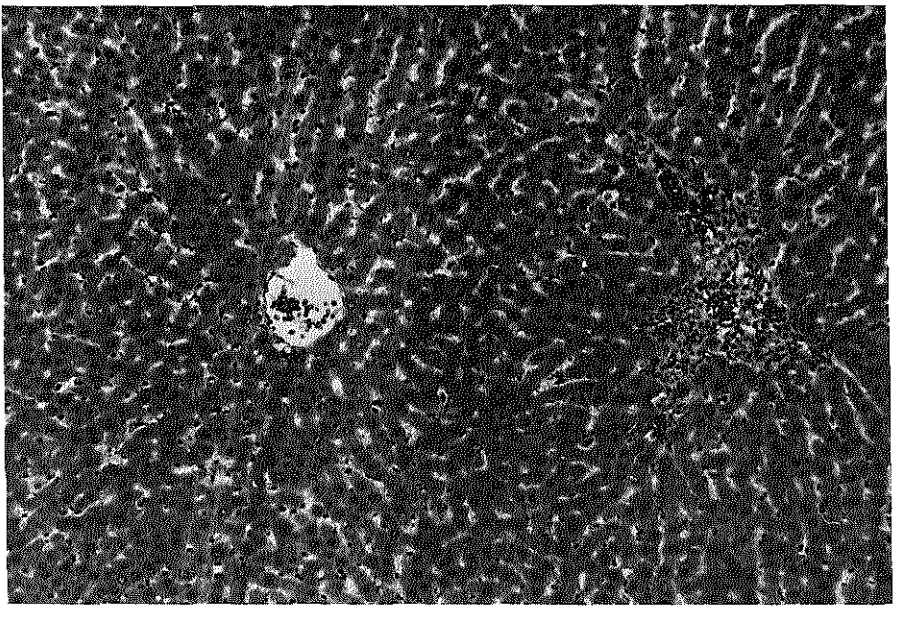
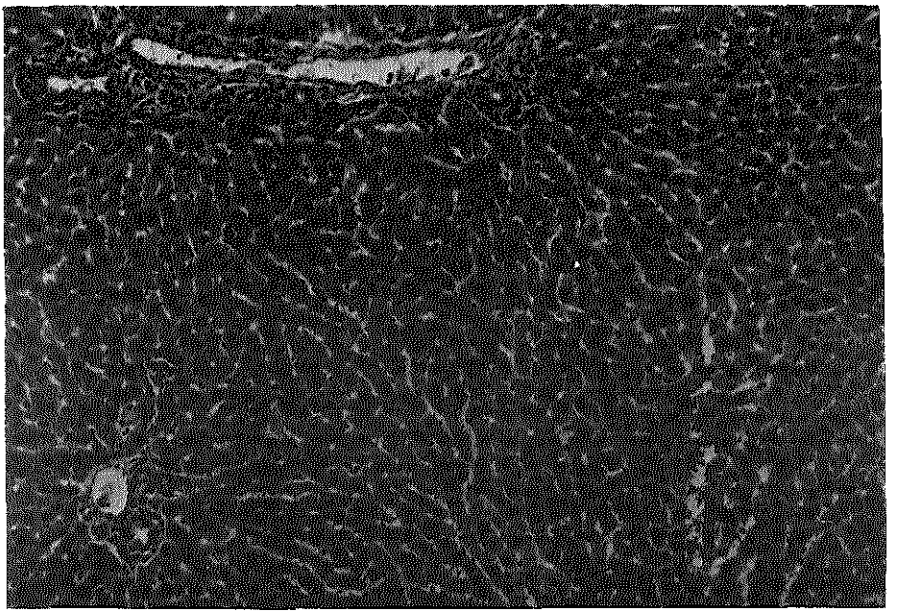


Fig. IV 9.

Rat liver allograft, 7 weeks after transplantation. Animal treated with ALG. The liver parenchyma is well preserved. There is little mononuclear infiltration. H.A.S. x 150.

Fig. IV 10.

Rat liver allograft, 7 weeks after transplantation. Animal treated with ALG and Imuran 4 mg/kg/day. Preservation of liver parenchyma is good. There is little mononuclear cell infiltration into the portal triangles. H.A.S. x 150.



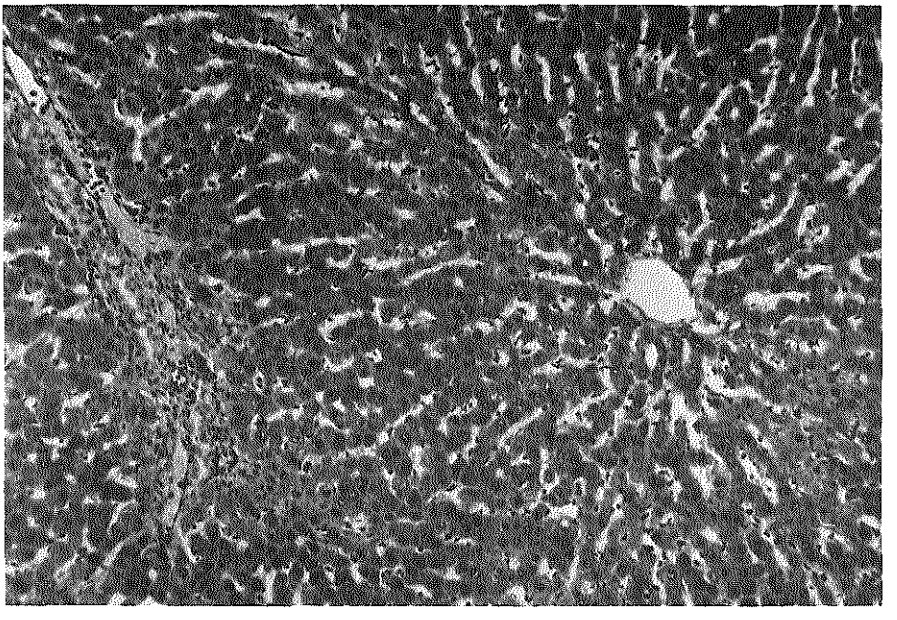


Fig. IV 11.

Rat liver allograft, 7 weeks after transplantation. Animal treated with Imuran 4 mg/kg/day. In general the liver parenchyma is well preserved, but there is evidence of cholanchitis and liver cell loss. H.A.S. x 150.

Fig. IV 12.

Rat liver allograft, 7 weeks after transplantation. Animal treated with Imuran 2 mg/kg/day and Prednisolone 4 mg/kg/day. The liver tissue is well preserved and there is only moderate mononuclear cell infiltration H.A.S. x 150.

isografted group (fig. IV 6, table IV 1). Those animals treated with azathioprine alone, or in combination with ALG or Prednisolone however, had initially a lower excretion rate than the isografted animals, later, they had good but variable excretion of the dye.

Statistical evaluation of the results using the test of Welch (1947) is summarised in table IV 1.

Spleno-portografy at 50 days showed that the portal veins of all animals were functioning well, their branches being distributed evenly into the periphery of the liver transplants. Also the hepatogram showed a regular sinusoidal distribution of the roentgen opaque dye (fig. IV 8 A, B).

b Histology (fig. IV 9-12)

Histology of the livers of all animals surviving for 50 days, showed that the general architecture of the livers was well preserved and that the liver cells appeared normal. Along the portal veins, many vessels with a muscular media and normal intima (arteries), were noted.

Group I

In the ALG treated animals histology showed that well preserved and regenerating liver tissue was present. There were moderate signs of bile duct proliferation, sometimes with evidence of cholanchitis, that is mononuclear and polynuclear cell infiltration around the bile ducts and the biliary structures in the liver hilus. There was hardly any mononuclear cell infiltration within the liver, associated with liver cell damage (fig. IV 9).

Group II

The animals of the group treated with azathioprine 4 mg/kg/day, which died spontaneously, had histologic signs of liver cell damage: fatty degeneration of the liver cells. Major ischaemic liver infarcts or infarcts congested with blood, due to thrombotic occlusion of the portal vein or caval vein of the donor liver, were also seen. In other animals bone marrow aplasia was diagnosed: myeloid and erythroid precurser cells together with a solitary megacaryocyte were only scarcely seen. There were histologic evidences of a herewith associated multifocal bacterial invasion of the animals. Histology at 50 days showed a more pronounced cholanchitis than in the ALG treated animals. Substantial mononuclear infiltration with lymphocytes and plasma cells, associated with multifocal, hepato-cellular necroses was noted (fig. IV 11).

Table IV 1. Statistical evaluation of the differences between liver isografted control animals and the differently treated allografted rats, tested on a 1% level of significance. + = significantly higher, – = significantly lower, 0 = no significant difference.

		isografted animals	allografted animals			
		untreated	ALG	Azathioprine 4 mg/kg	ALG+ Azathioprine 4 mg/kg	Azathioprine 2 mg/kg +Pred. 4 mg/kg
Isografted animals untreated	weight	X	+	+	+	+
	Hct	X	–	+	+	0
	Albumin	X	0	0	0	0
	Protrombin	X	0	+	0	0
	BSP	X	–	0	0	0
Allografted animals ALG	weight	–	X	+	+	+
	Hct	+	X	+	+	+
	Albumin	0	X	0	+	0
	Protrombin	0	X	+	0	0
	BSP	+	X	0	0	0
Allografted animals Azathioprine	weight	–	–	X	0	+
	Hct	–	–	X	0	–
	Albumin	0	0	X	0	–
	Protrombin	–	–	X	–	–
	BSP	0	0	X	0	0
Allografted animals ALG + Azathioprine	weight	–	–	0	X	+
	Hct	–	–	0	X	–
	Albumin	0	–	0	X	–
	Protrombin	0	0	+	X	0
	BSP	0	0	0	X	0
Allografted animals Azathioprine + Prednisolone	weight	–	–	–	–	X
	Hct	0	–	+	+	X
	Albumin	0	0	+	+	X
	Protrombin	0	0	+	0	X
	BSP	0	0	0	0	X

Group III

Microscopic evidence of the complications, leading to spontaneous death as noted in group II (azathioprine 4 mg/kg/day) was also seen in the group treated with ALG in combination with azathioprine 4 mg/kg/day. After 50 days virtually the same picture was seen as in recipients treated with ALG alone (fig. IV 10).

Group IV

7 Animals died spontaneously after treatment with prednisolone 4 mg/kg/day together with azathioprine 2 mg/kg/day. Mononuclear infiltration was increasingly seen with time not only in the portal triangles, but also in between the liver parenchyma. In the animals dying ± 1 week after transplantation, vacuolar degeneration, atrophy and liver cell death was noted. The two animals of this group which survived the 50 days after liver transplantation, had only moderate mononuclear infiltration in the donor livers (fig. IV 12).

4. Discussion

The advantages of using inbred rat strains were confirmed in this study of rats with life sustaining liver transplants. Intra-operative mortality and post-operative mortality (death within 48 hours after operation) together are ± 26%, showing that the transplantation technique is feasable and is highly reproducable. The technique is well-suited to investigate the effects of immunosuppressive therapy and histocompatibility differences. In respect to the immunosuppressive agents used, several observations can be made.

First, the complications caused by the use of azathioprine (Imuran) are considerable. It is known, that Imuran is hepatotoxic (Starzl 1969) and that it is metabolised in the intact liver while only the derivatives are responsible for its immunosuppressive activity (Bach, 1972). It is therefore not surprising that we observed signs of toxicity: strong diminution of liver function and histologic signs of liver cell damage. In other animals bone marrow aplasia, with consequent anaemia, haemorrhagic diathesis and susceptibility to fulminant infections was observed. In our series a 50% mortality was obtained within 2 weeks after transplantation in the azathioprine treated group, this is similar to results of rat kidney transplantation in the same inbred rat strains, in which, with identical immunosuppressive therapy 40% survival was obtained (Tinbergen 1968, de Bruin, 1970).

The side effects of Imuran i.e. bone marrow aplasia and hepatotoxicity are similar to those observed by Starzl (1969) in human liver transplantation. Further (experimental) evaluation of less hepatotoxic immunosuppressants (for instance cyclofosfamide) seems to be indicated.

Secondly, in the group in which Imuran 2 mg/kg was combined with prednisolone 4 mg/kg, only 22% survival after 50 days occurred. This is in contrast with kidney transplantation in which with the same rat strains and host-donor combination survival was 80% (de Bruin 1970).

The liver function tests clearly point to a progressive loss of liver function in the Imuran treated animals, while the liver function of the Imuran/prednisolone treated group did not diminish. Autopsy on the prednisolone treated animals that died showed that infections and wound dehiscention were the major problems in these animals.

Thus, it is likely, that the doses of prednisolone should be kept as low as possible shortly after liver transplantation. One may then prevent impairment of connective tissue repair and also prevent a possible decrease of liver cell proliferation. Both side effects of prednisolone were noted, using the same immunosuppressive dosages, in a rat model of liver regeneration (Kort and v.d. Post 1973). Imuran 4 mg/kg added to ALG gave some deaths presumably caused by bone marrow depression.

The efficacy of the immunosuppressants used, could be evaluated through the results of repeated microbiochemical determinations involving liver synthesis, detoxication and excretion. It became evident that some immunosuppressive regimens, while giving adequate results in rat kidney transplantation, cannot be transferred without modifications to experimental rat liver transplantation. This probably holds true for man too.

The advantages of the use of a highly effective, non-toxic ALG (horse anti lymphocyte gammaglobulin) was confirmed in this rat liver transplantation model. It was clearly superior to all other immunosuppressants used. It was not hepatotoxic as described by others (Koumans et al. 1972) nor did it induce anaemia. All animals survived, hepatic function and histology were within normal limits and portal vein radiography showed no abnormalities. The fact that ALG, given alone during three weeks, was superior to the other immunosuppressive regimens tested, may however not be translated to the human situation without further research. In 1971, Myburgh and co-workers used a horse anti monkey lymphocyte globulin which had been tested in *vitro*. Using this ALG in baboons only 10 to 20 percent survivors were obtained 50 days after orthotopic liver transplantation. Apparently, a high lymphocytotoxic

titer of 1 : 8,000 and rosette inhibition titer of 1 : 8,000 and a seemingly adequate dosage scheme did not guarantee its effectiveness. A re-evaluation of ALG with known *in vivo* immunosuppressive activity in subhuman primates is of special importance, since this animal species is generally considered as the best for these sort of experiments. Indeed, it is well known that different batches of ALG may have quite different immunosuppressive activity and/or toxicity (Balner et al.1970, Koumans et al. 1972, Brendel 1973, 1975). The activity and toxicity of ALG for experimental use can be tested *in vivo* in well known animal transplantation models before it is used in a specific experiment. For instance skin allotransplants can be used in the rat or monkey for the purpose of testing anti-rat or anti-monkey lymphocyte sera (Balner et al. 1970). But, ALG cannot be tested in this way (*in vivo*) in humans. In heart transplantation therefore, an *in vitro* test system is used, based on the spontaneous rosette inhibition test of Bach et al. (1969). The results, this time, were promising. The dosages given in fact, could be adjusted to the circulating levels of ALG. It seemed further, that the levels of ALG, corrected for their rosette inhibition titers indeed positively correlated with the time interval between the operation and the onset of the first rejection of the transplanted heart (Schroeder et al. 1976). The provision of a highly effective, non-toxic anti-human lymphocyte globulin is however not the only problem encountered. The heterologous serum proteins namely invoke, apart from hypersensitivity reactions, antigen-antibody complexes which may get trapped, together with complement, in the renal glomerular filter and cause a pronounced glomerulopathy (Cohen et al. 1970). Recently these problems were also described in the human recipients of liver transplants (Cubilla et al. 1974). In conclusion, although ALG was highly effective in preventing liver allograft rejection and no adverse effects were noted in these rats, further experiments are required. Not only rats, but also the canine species and subhuman primates should be incorporated in these studies of liver transplantation and immunosuppression.

SUMMARY

The main indications for liver transplantation are acute or sub-acute liver necrosis, viral hepatitis and cirrhosis. From the literature one learns that also children with biliary atreasia are considered potential recipients of a liver transplant. However, according to Kasai (1974), patients with biliary atreasia have a prognosis of 80% cure when a modified hepato-portoenterostomy operation is performed before the patient is ten weeks old. This statement does however need confirmation.

Since only 15 patients with this type of diagnosis are to be expected in the Netherlands per year, centralisation of the efforts is a prerequisite. Only then, the necessary experience in treatment and operation of these children can be obtained. From the number of patients dying of the heretofore mentioned liver diseases, it is estimated that about 25 children under 15 years and 58 patients up to 50 years could be considered for liver transplantation per year, when, at least, the patients with biliary atreasia are incorporated in this group.

The results of liver transplantation compare unfavourably with those of heart transplantation (Bergan 1975). The reason why only 3.3% of the patients survive 3 years after the operation, is primarily caused by the high frequency of bacterial infections. In a series of 83 patients, 42% died of infectious complications. The intimate contact of the liver, via the biliary tract and the portal vein, with the intestines renders it particularly susceptible to bacterial and or viral invasion.

Immunosuppressive treatment after liver transplantation is largely based upon experience from clinical and experimental kidney transplantation. Investigations in dogs and monkeys proved that pronounced individual differences were obtained after liver transplantation when the animals received immunosuppressive treatment. Probably the unpredictable influence of individual differences in histocompatibility and immune responsiveness play a significant role in these results.

The purpose of the investigations in this thesis was to provide a liver transplantation model in which genetic influences would be controlled. Thus the inbred rat was chosen as the experimental animal. When the technique was developed, different immunosuppressive regimens were compared for their effectiveness in suppressing liver transplant rejection.

In chapter II a modified technique for auxiliary liver transplantation is described. Post-operative 4 day survival, improved from 27%

with the existing method, to 87% with the modified technique, which used the infra hepatic vena cava as the efferent vessel from the liver graft.

Chapter III describes a life sustaining (non auxiliary) liver transplantation technique. Operative mortality was about 20%. Several biochemical determinations were adjusted or developed on a micro-scale in order to assess the condition of the transplanted liver. These determinations were made first bi-weekly, later, once a week.

No immunosuppressive treatment was given to rats with isogeneic or allogeneic liver transplants. The rats with isogeneic grafts remained in good condition, and had concommittant good liver function. The following parameters were used: general (survival, weight, haematocrit), liver production (albumin, and clotting activity tested through Prothrombin, Thrombotest® and Normotest®), excretion (B.S.P. excretory capacity and bilirubin) and liver cell loss (SGPT). They all normalised during the seven week observation period. Portal vein radiography revealed that, after seven week observation period, blood flow through the grafted livers was normal.

Mean survival time of the allografted animals was 11.9 days. The results of the biochemical estimations correlated with the severity of the histopathological changes (indicative of graft rejection), noted after their death.

In chapter IV the effects of different immunosuppressive regimens given during three weeks after transplantation are compared. The liver function and histopathology of the allotransplanted rats are described. Through ALG (1 ml/week) rejection was effectively suppressed while the function of the livers was normal. When azathioprine (4 mg/kg/day) was added to the prior treatment, 4 out of 10 animals died of the consequences of bone marrow aplasia, a well known side effect of azathioprine. When azathioprine was given alone (4 mg/kg/day) the liver function progressively diminished and 50% of the animals died within 2 weeks. The surviving animals recuperated well from their rejection and liver function was restored during the following period. The postoperative mortality was high in the group treated with prednisolone (4 mg/kg/day) and azathioprine (2 mg/kg/day). General impairment of the animals' condition and retarded wound healing rather than impairment of liver function was the cause of death in these cases.

From these data it is concluded that treatment with ALG was clearly superior to the other immunosuppressive treatments, such as azathioprine, with or without ALG or prednisolone.

SAMENVATTING

De belangrijkste indicaties voor lever transplantatie zijn acute of subacute levernecrose, virus hepatitis en cirrose. Uit de literatuur blijkt dat kinderen met galgang-atresie ook als potentiele ontvangers van een levertransplantaat worden beschouwd. Kasai (1974) is echter van mening dat patienten met galgang-atresie een kans van 80% op genezing hebben mits, binnen 10 weken na de geboorte, een gemodificeerde hepatoporto-enterostomie operatie wordt uitgevoerd. Deze uitspraak dient nog te worden bevestigd.

Op grond van het aantal patienten die jaarlijks overlijden ten gevolge van de hierboven genoemde ziekten kan men een schatting maken van het aantal mogelijke candidaten voor levertransplantatie in Nederland. Deze groep zal bestaan uit ongeveer 25 kinderen tot 15 jaar en 58 patienten van 15 tot 50 jaar, indien patienten met galgang-atresie worden meegerekend.

De resultaten van levertransplantatie zijn slecht in vergelijking met die van harttransplantatie (Bergan 1975). Het feit dat slechts 3,3% van de patienten de operatie 3 jaar overleeft is voor een groot deel te wijten aan het hoge aantal bacteriele infecties. In een serie van 83 patienten stierf 42% ten gevolge van infectieuze complicaties. Het nauwe contact dat de lever via de galwegen en de vena porta met de darmen heeft verklaart de bijzondere gevoeligheid voor infecties.

De immunosuppressieve behandeling na levertransplantatie berust voornamelijk op ervaringen afkomstig van klinische en experimentele niertransplantatie. Onderzoek bij honden en apen heeft aangetoond dat er aanzienlijke individuele verschillen in overleving zijn wanneer de dieren na de levertransplantatie een immunosuppressieve behandeling krijgen. Waarschijnlijk zijn de onvoorspelbare invloeden van histocompatibiliteit en immuunreactiviteit daarbij van grote betekenis.

Het doel van het in dit proefschrift beschreven onderzoek was, een techniek voor levertransplantatie te verkrijgen waarin de genetische invloeden constant konden worden gehouden. Om deze reden werden ingeteelde rattestammen gebruikt. Toen deze techniek was ontwikkeld, kon worden overgegaan tot het vergelijken van verschillende immunosuppressieve regimes om hun waarde te bepalen voor het onderdrukken van de afstotingsreactie.

In hoofdstuk II wordt een gemodificeerde techniek voor auxiliaire transplantatie beschreven. Het overlevingspercentage, 4 dagen na de operatie, nam toe van 27% met de bestaande methode tot 87% met de gemodificeerde methode. Daarbij werd gebruik gemaakt van de vena cava infra-hepatica als afvoerend bloedvat van de lever.

Hoofdstuk III beschrijft een levertransplantatietechniek waarbij de ontvanger geheel afhankelijk is van de functie van de getransplanteerde lever (niet auxiliaire levertransplantatie). De mortaliteit rond de operatie was ongeveer 20%. Verschillende biochemische bepalingen werden aangepast of ontwikkeld om op microschaal de functie van de getransplanteerde lever te kunnen bepalen. Deze bepalingen werden eerst twee maal en later een maal per week uitgevoerd.

Aan twee groepen ratten met isogene of allogene levertransplantaten werd een immunosuppressieve behandeling onthouden. De ratten met isogene transplantaten bleven in goede conditie en hadden een daarmee overeenstemmende goede lever functie. De volgende parameters werden gebruikt: algemene (overleving, gewicht, heamatocriet), eiwit productie (albumine en stollingsactiviteit, gemeten via prothrombine, Thrombotest® en Normotest® bepalingen), uitscheiding (B.S.P. uitscheidingscapaciteit en bilirubine) en levercel verval (SGPT). Zij werden allen normaal gedurende de zeven weken durende observatieperiode. Met spleno-portografie werd aangetoond dat, zeven weken na de operatie, de bloed doorstroming door de levertransplantaten normaal was.

De gemiddelde overlevingsduur van de allogeen getransplanteerde dieren was 11,9 dagen. De uitslagen van de biochemische bepalingen weerspiegelden de ernst van de afstotingsreactie welke bij het postmortem onderzoek histologisch gevonden werd.

In hoofdstuk IV worden de resultaten vergeleken van verschillende immunosuppressieve behandelingen, die gedurende drie weken na de transplantatie werden toegepast. De leverfunctie en de histopathologie van de allogeen getransplanteerde ratten worden beschreven.

Door Anti Lymphocyten Globuline (ALG) (1 mg/week) werd de afstoting doeltreffend onderdrukt terwijl de levers normaal functioneerden. Vier van de 10 dieren stierven als gevolg van beenmerg-aplasie na behandeling met ALG en azathioprine (4 mg/kg/dag). Beenmergaplasie is een bekende bijwerking van azathioprine. Wanneer azathioprine alleen werd gegeven (4 mg/kg/dag) dan werd de leverfunctie geleidelijk aan minder en stierf 50% van de dieren binnen twee weken. Bij de dieren welke de afstotingsreactie overleefden, werd de leverfunctie geleidelijk aan normaal. De postoperatieve mortaliteit was hoog wanneer de dieren

met prednisolon (4 mg/kg/dag) en azathioprine (2 mg/kg/dag) werden behandeld. De doodsoorzaak was in deze gevallen eerder een vermindering van de algemene conditie van de dieren en vertraagde wondgenezing dan een vermindering van de leverfunctie.

Uit deze gegevens wordt geconcludeerd dat de behandeling met ALG duidelijk superieur was aan de andere immunosuppressieve behandelingen zoals toediening van azathioprine, al dan niet in combinatie met ALG of prednisolon.

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