XENO-ASSISTANCE OF THE FAILING LIVER

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XENO-ASSISTANCE OF THE FAILING LIVER

Xenogene ondersteuning van de falende lever

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To my parents

To Antoinette

To Eleonore, Carl, Constantijn and Paul

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Introduction and outline of the dissertation

INTRODUCTION

The liver is a highly complex organ in which many different metabolic processes take place. These include the metabolism of dietary carbohydrate, protein and fats, the storage of iron, the formation of hormones and blood coagulation factors and the removal of toxins from the bloodstream. Liver transplants are required when liver function has dropped to below 20% of normal[1]. This can be a result of chronic liver failure, where liver function declines progressively, usually over a period of years, or acute liver failure. The main chronic diseases in adults are cirrhosis, alcohol-induced and non-alcoholic, and hepatitis. In children, the main cause is biliary atresia and other inherited anatomic and metabolic disorders. Acute liver failure is normally caused by viral hepatitis or toxic drugs. Acute liver failure is less common than chronic liver failure, but results in brain damage through inflammation and fluid accumulation in the brain, and in 75% of cases the patient dies within a few days of onset[2,3]. In the few cases where the patient does survive, the liver appears to have an amazing ability to regenerate.

Thus far, patients with liver disease are handicapped by the lack of satisfactory means of artificial support comparable to renal dialysis. The transplanted liver must function efficiently from the time of transplantation, or the patient may be lost. Despite these and other difficulties, the therapeutic power and appeal of liver transplantation has had a considerate impact in the treatment of liver failure. Almost all patients with nonneoplastic chronic liver disease can at least be considered for liver transplantation, and even some of those with malignant tumors may benefit. Acute liver disease was rarely suggested as a reason to consider liver transplantation until the mid 1980s, but now transplantation of patients with fulminant hepatitis is common. Patient survival of more than 20 years after liver transplantation has been achieved.

The limiting factor for greater use of liver transplantation is the availability of donor organs. An estimated 80% of the patients with acute liver failure die while being on the waiting list[3], whereas another 15% of patients with chronic liver failure are not eligible for transplantation, because of other concomitant disease. The shortage of donor organs and the capacity of the liver to regrow after damage has led to a high level of interest in the development of liver assisting therapies. A method to assist the failing liver may keep the patient alive, either until

the patient's own liver regenerates or, in the case of irreparable liver damage, a human donor liver becomes available and the patient can receive a transplant.

Throughout the past 40 years many approaches to assist the failing liver have been developed and to date, most of them are still under investigation. The clinical outcome of the various approaches has been different as measured by temporary or permanent improvement of the patients' condition. When measured in overall survival rates, the equivalent of liver transplantation has never been achieved. This may be partly due to the fact that the mechanisms in liver failure remain unclear. Improvement of the biochemical parameters of the patient alone, e.g. a decrease in concentration of ammonia or bilirubin, is usually not related to the patients' clinical condition and vice versa[4]. Therefore, the methods involving extracorporeal perfusion through membranes which do not contain hepatocytes, called passive detoxification, do not eventually improve the patients state of disease[5-15]. However, the treatment of patients with liver assist devices containing hepatocytes, which are thought to replace the multiple and complex functions of the liver more completely, presents a whole new era of difficulties. First of all, human hepatocytes are sparse, because of the shortage of donororgans. Secondly, thus far, a culture system with stable viability and differentiated function of primary hepatocytes has not been established. Also, the use of human cancer cell lines (hepatoma cell lines), which does provide a stable culture system, introduces the danger of infecting the patient with cancer. Thirdly, an alternative source of hepatocytes, e.g. liver cells from other species, may be limited due to immunological barriers. Thus, in order to be able to improve the clinical condition of patients with liver failure even for a short period of time, these methods need further investigation[16-34].

It is known from studies as early as the 1960s, that the liver has a privileged position in xenogeneic circumstances. Extracorporeal liver perfusion studies have been performed taking livers from dogs and pigs without major immunological drawbacks[35] and liver replacement was successfully performed in dogs without long term immunosuppression, where a surprising number of dogs continued to thrive for years, some without signs of rejection[36]. An important advantage, when short term use of assisting liver therapies is considered, is the relative resistance of the liver to complement- and antibody-mediated rejection. In xenotransplantation, when organs are transplanted in a discordant situation, this complement mediated rejection, the hyperacute rejection, is a major barrier. For instance, kidneys and hearts may be hyperacutely rejected within hours in patients whose serum contains cytotoxic antibodies of the IgG class which are directed against HLA and other antigens in the donor. The pathogenesis of hyperacute rejection includes obstruction of the microvasculature of

nonhepatic grafts with clotting products and formed blood elements. However, livers are spared this fate in most cases[37-40], and what can be expected clinically is an unusually vigorous cellular rejection that can be treated with aggressive conventional immunosuppression. The practical implication is that a negative cytotoxic crossmatch, a necessary condition for transplantation of other organs, is not required for successful liver transplantation. Moreover, it has been established that the liver can provide a protective screen for otherwise vulnerable kidneys in highly sensitized recipients who need both a liver and a kidney. In such patients who receive a liver first, the titer of antidonor antibodies is drastically reduced during the first few hours after hepatic revascularisation, making it possible to safely insert a kidney from the liver donor[41]. However, overstatement of the case for the liver's privileged status could lead to erroneous conclusions about the practical requirements for immunosuppresive therapy following hepatic transplantation or the impact of xenogeneic temporary liver support. When routine biopsies are obtained in patients after orthotopic liver transplantation, histopathologic evidence of rejection can be found in more than two-thirds of patients and control of hepatic rejection may be difficult [42].

In short, mortality figures in liver failure remain high, because of the shortage of donor organs for liver transplantation and the lack of satisfactory means for temporary treatments of patients with liver failure to bridge to either liver regeneration or liver transplantation. Therefore, in this dissertation we concentrated on further investigation of the various approaches of temporary extracorporeal liver support considering the exceptional role of the liver in its xenogeneic environment.

OUTLINE OF THE DISSERTATION

Although liver assisting therapies have been around for several decades, thus far, no satisfactory temporary treatment for patients with acute liver failure has been found. *Chapter 2* evaluates the different approaches towards a solution for this problem and enlightens its immunological implications.

One of the possible solutions for temporary liver support therapy is the use of the biologic substrate, i.e. hepatocytes, in combination with artificial material to form a bioartificial liver. Critical to the successful development of this technique is the ability to foster long term viability and function of a large cell mass in a relatively small volume, since freshly isolated hepatocytes take as much as one week in culture to fully re-express differentiated functions, the dialysis volume should not exceed 500 ml and long treatment periods are anticipated. In *chapter 3*, a hepatocyte culture system is introduced, which uses the collagen sandwich culture system, that provides a long term stable culture of hepatocytes, scaled up through the use of microcarriers.

Another technique to support patients with liver failure using the biologic substrate is the extracorporeal pig liver perfusion, where the whole organ of the animal is connected in parallel to the patient with liver failure. In order to determine whether this technique may provide adequate support without the possible immunological disadvantages, we studied this method in an autogenous extracorporeal pig liver perfusion model. The results are described in *chapter 4*.

The absence of hyperacute rejection in the above mentioned discordant xenogeneic liver support techniques, may partly be due to the fact that patients with liver failure have by definition an impaired liver function, which results in a decreased amount of circulating complement, the major contributor to hyperacute rejection. In *chapter 5*, the possibility of prevention of the hyperacute rejection by inducing acute liver failure in a guinea pig heart to rat transplantation model is described.

The exceptional position of the liver regarding xenogeneic support of liver failure was shown by the absence of hyperacute rejection in a guinea-pig to rat liver transplantation model. In order to study the immunological pathways of a discordant xenogeneic rejection as may be found in a pig-to-human situation, a sensitized rat anti-hamster model was developed to examine the mechanism of hyperacute rejection of liver grafts, the results of which were compared to heart xenografting. The outcome of this study is described in *chapter 6*.

Although no hyperacute rejection in ex vivo whole liver perfusion has been shown, humoral damage to the liver tissue can be established. Transgenic organs are being used to diminish humoral damage to the ex vivo perfused liver and therewith possibly improving the long term

use of this liver support therapy. In *chapter 7* the function and the immunopathological features of the isolated normal and transgenic rat liver perfused with human serum are described.

The use of xenogeneic biologic material in the treatment modalities of patients with liver failure introduces several health hazards. In an already immunocompromized patient, short- and long-term immunological problems can be expected, and transmission of viral material to the patient is possible. In *chapter 8* the problems encountered in the interaction between the xenogeneic device and the patients with liver failure are discussed and directions for future research are given.

In *chapter 9* the content of the above mentioned chapters is summarized .

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2

Extracorporeal perfusion for the treament of acute liver failure

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ABSTRACT

Objective and background Because of the shortage of available donor organs, mortality rates due to liver failure remain high. Therefore, several temporary liver-assisting therapies have been developed. This paper offers a review of various approaches to temporary liver support as well as present immunological and metabolic developments towards a solution for this problem.

Methods and Results This paper is composed of a review of the literature, which has been obtained through searches in Medline and additional library searches to obtain further references. Only papers with a well-defined aim of the study and methodology as well as a clear description of the outcome of the experiments were included.

Conclusions Renewed interest has developed in old and new methods for an extracorporeal approach to the treatment of acute liver failure. Although temporary clinical improvement has been established, further research is needed to achieve a successful long-term clinical outcome. New developments in the field of genetic modification and tissue engineering await clinical application within the near future.

INTRODUCTION

Because of the life-threatening complications of acute liver failure (ALF), 75% of the patients die within a few days of onset. At present there is no satisfactory treatment for ALF except liver transplantation. Although liver transplantation is very successful, with 5-year survival rates ranging from 70 to 80 %, there is a shortage of available donor organs. It is estimated that in the United States only 20% of the patients with ALF receive a transplant; 80% die while on the waiting list [1]. The clinical need for an alternative is two-fold: first, to bridge the gap until liver transplantation in case of irreversible damage, and second, to gain time for regeneration of the damaged liver.

Various liver-assisting therapies have been introduced since the early 1960s, including plasmapheresis, hemodilution, and cross-circulation, but none of them led to a significant clinical improvement. At the same time, extracorporeal whole liver perfusion was developed, with promising results. This method was abandoned in the early 1970s, however, because of

the successful development of orthotopic liver transplantation and awareness of the possible immunological implications. At present, the consensus is that the multiple and complex functions of the liver can be replaced only by using the biological substrate (i.e. hepatocytes), whether in a whole liver or in combination with artificial material. This has led to increasing interest in the development of a bioartificial liver along with extracorporeal liver perfusion.

In addition to the worldwide shortage of available donor organs, new developments have made it worthwhile to pursue these solutions to the problem of ALF. The disadvantages and long-term effects of liver transplantation have become clear: a major and costly surgical procedure and lifelong immunosuppressive therapy. Furthermore, the possibility of genetically modifying donor animals creates an unique opportunity to overcome potential immunological barriers between species. In this review, we will assess the various approaches to a temporary solution for ALF; their shortcomings will be discussed and directions for future research suggested.

EARLY LIVER SUPPORT THERAPIES

Acute or fulminant hepatic failure, usually induced by chemical or viral hepatitis, is highly lethal, with a death rate of more than 75% as a result of life-threatening complications such as portal hypertension, variceal bleeding, ascites, and hepatic encephalopathy. While the patient is waiting for possible liver transplantation, current treatment consists of intensive clinical support: fluid and hemodynamic support, correction of electrolyte and acid-base abnormalities, respiratory assistance and treatment of cerebral edema. In the past, it was thought that encephalopathy and cerebral edema were caused mainly by dialyzable toxins such as ammonia, false neurotransmitters, phenols, aromatic amino acids and other substances with a molecular weight below 5,000 [2]; early liver support systems focused on blood detoxification. A kidney dialysis machine was used to remove blood ammonia but did not result in clinical improvement [3]. Because most of these toxins were thought to be protein-bound and traditional hemodialysis used cellulose membranes impervious to large molecules, polyacrylonitrile membranes were introduced that allowed passage of molecules up to a molecular weight of 15 kDa. Table I summarizes the results of the early liver support therapies introduced in the 1950s and 1960s. In two groups treated with hemodialysis, [4,5]

temporary improvement in mental status and duration of survival occured in more than half of the patients, suggesting at least partially effective removal of neurotoxins.

Hemofiltration has seen only limited application because of its major complication of thrombocytopenia, leading to bleeding in patients with an already compromised clotting system [6,7]. Exchange transfusion showed initial improvement in neurological status in several trials, but no effect on the death rate was found [8-12]. With the less laborious technique of plasma exchange or plasmapheresis, [13-15] significant improvement in neurological status and biochemical profiles and decreased bleeding episodes were demonstrated; control of the hemorrhagic diathesis was achieved through the addition of coagulation factors. With this method, a better overall survival rate was achieved (34% versus 14% in the non-treated group); however, this was a nonrandomized study and the groups were not controlled for cause or severity of the underlying disease [16].

Table 1. Patients treated with early liver-support therapies

		Neurologic improvement			
Support System	**No. of patients**	**Yes**	**No**	**Unkown**	**Survival**
Hemodialysis [4,5]	65	33	17	15	17 (26%)
Hemofiltration [6,7]	15	10	5	0	8 (53%)
Exchange transfusion [8-12]	48	24	16	8	9 (19%)
Plasma exchange [13-15]	32	15	17	0	7 (22%)
Resin hemoperfusion [17,18]	9	5	4	0	0 (0%)
Charcoal perfusion [20-23]	278	73	101	104	116 (42%)

In hemoperfusion or plasma perfusion, the blood or plasma of a patient with ALF is perfused through a column containing activated charcoal or anion exchange resins. The latter caused no significant improvement, mainly because of early saturation of the resins and non-selective binding of the cations removed [17,18]. After resolving the problems of platelet loss and hypotensive reactions by coating the columns and administering prostacyclin as a protective agent for the platelets [2,19], charcoal hemoperfusion yielded encouraging survival rates among patients with ALF [20-23]. However, none of these studies included a proper control group defined by both the cause of the hepatic failure and the stage of the disease. Thus, the effect of coated charcoal perfusion cannot be estimated conclusively. This technique is still in use because of the possible beneficial effect in combination therapies, where it is supposed to protect the adjacent liver support therapy from toxic substances.

The detoxifying function of the above-mentioned techniques may in itself not be enough to improve the survival of patients with ALF. It remains to be determined whether these techniques, in combination with other liver-support therapies will play a role in the future.

BIOARTIFICAL LIVER

The limited success of detoxifying liver support therapies and improved techniques for culturing isolated hepatocytes that express high levels of differentiated liver function in vitro have lead to the development of the hybrid or bioartificial liver (BAL). These systems consist of biologic components (freshly isolated pig hepatocytes or human hepatoblastoma cell lines) in a synthetic framework. Isolated hepatocytes have been used in two ways, extracorporeal hepatocyte bioreactors and implantable hepatocyte systems; the latter is beyond the scope of this paper. In the extracorporeal bioreactors the blood or plasma of the patient with ALF comes in contact with the cultured hepatocytes through an ex vivo perfusion system, as in the hemodialysis system for patients with renal insufficiency.
To create an artificial system containing viable hepatocytes, the procedure of culturing hepatocytes while maintaining their liver-specific function had to be improved. Culturing hepatocytes in suspension or in plastic tissue culture dishes causes the cells to loose their function within 24 hours and leads to cell death within days [24,25]. Two solutions to this problem are being investigated: the use of transformed cells or cell-lines, and optimization of the culture system for primary isolated hepatocytes. Another problem is how to expand the cell cultures to obtain sufficient quantities of hepatocytes in a relatively small volume to replace completely the function of a human liver [26]. Over the past 10 years, the development of clinically applicable BAL systems has focused on the volume of hepatocytes necessary to replace temporarily the liver function of the patient with ALF.

Hepatocytes in Suspension Cultures or Encapsulated in Hydrogels

Hepatocytes were first used in a liver support system in a clinical setting in 1987 by Matsamura et al. (Table 2) [27]. Hepatocytes in a suspension culture were separated from the patient's blood by a cellulose acetate dialysis membrane. In this single patient, some clinical

improvement was found. Because in vitro studies showed that hepatocytes function better when attached to a substrate, another attempt to establish a suspension system was made by Margulis et al. [28], who seeded liver cells on Biosilon microcarriers. They demonstrated improved survival rates in the treated group (63%) versus the group that received standard medical treatment (41%). However, randomization for the stage of liver disease was not performed in the different groups, and no follow-up cases have been reported. Further in vitro studies demonstrated poorly preserved hepatocyte function after several hours of application [28,29].

A rotating-disk type of artificial liver system, with hepatocytes entrapped within a calcium alginate hydrogel, showed ammonia metabolism and urea synthesis in in vitro studies and subsequently significant removal of ammonia in an ex vivo animal study [30]. Drawbacks of this system are the tendency of the gel phase to dissolve during hemoperfusion, with consequent loss of viability of the entrapped hepatocytes. This is why it was never applied in a clinical setting.

Table 2. Patients treated with artificial liver devices

		Neurologic Improvement				
Support System	**No. of Pts.**	**Yes**	**No**	**Unknown**	**Survival**	**OLT**
Suspension culture [27,28]	60	60	0		38 (63%)	0
ELAD [45,46]	23	18		5	14 (61%)	3
Bioartificial liver [52]	31	30		1	22 (71%)	21

OLT, orthotopic liver transplantation

Multiplated Hepatocyte Monolayers

The system, which best resembles an in vitro culture of hepatocytes, was designed by Uchino et al. in 1988 [31]. Hepatocytes were isolated from a canine liver and cultured on collagen-coated borosilicated glass plates in a monolayer. Stacking 200 plates in a transparant acryl resin module yielded about 80 g of hepatocytes, or one-third of a canine liver, with stable function of the hepatocytes for 14 days. When this device was used to treat anhepatic dogs, clearance of ammonia was demonstrated in the treated group (vs. the groups receiving no treatment or plasma perfusion) and led to a significant increase in overall survival (55 vs. 24 hours). The same system has been used in in vitro studies of pig hepatocytes, which were

perfused with plasma from patients with hepatic failure [32], and in the testing of in vitro hepatocyte culture techniques such as coculture and collagen-sandwich culture [33,34]. These studies yielded the same encouraging results in vitro, but thus far no report of a clinical application has been published.

Hollow Fiber Systems

Most devices used to create a BAL consists of a bundle of small-diameter tubes, made of compatible cellulose acetate, enclosed in a rigid polycarbonate module (Figure 1). This hollow fiber system has two compartments (intraluminal within the fibers and extraluminal outside the fibers) within the rigid housing; they communicate through the pores in the fiber walls. Due to the fact that most of the waste molecules and bilirubin are bound to albumin (molecular weight 65-70 kDa), the pore size chosen is usually a nominal molecular weight cutoff of 100 kDa; this excludes most complement factors and immunoglobins [35].

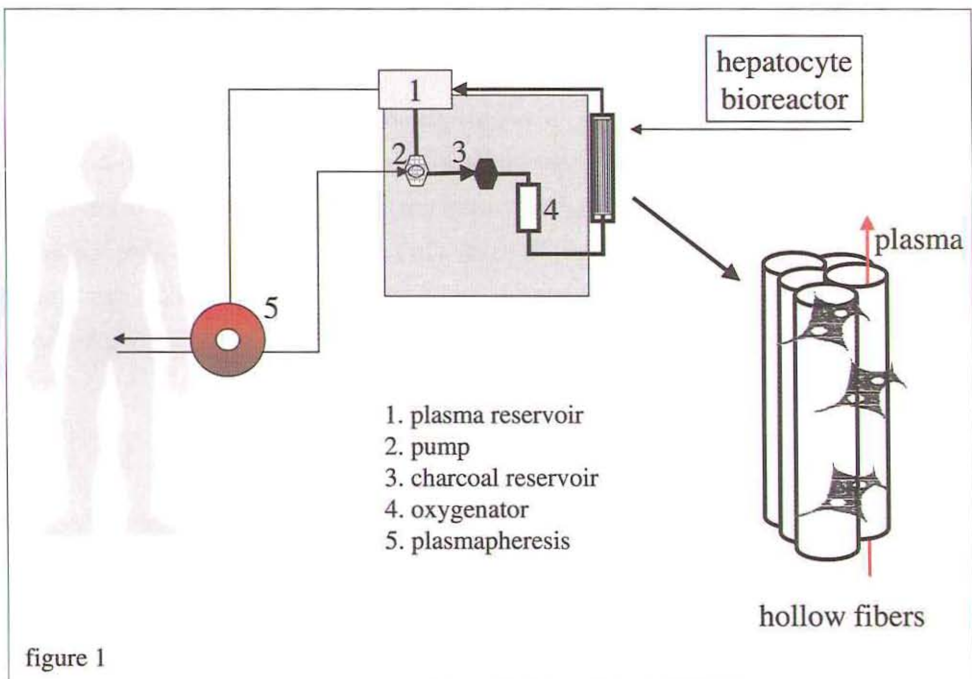

Figure 1. Liver support device. After plasmapheresis, plasma circulates through a hollow fiber system in which isolated pig hepatocytes are cultured in the extracapillary space.

Intraluminal gel-entrapment of porcine hepatocytes

Prolonged viability and functioning of the hepatocytes are difficult to maintain at a high seeding density in an artificial liver. The viability and liver-specific functioning of hepatocytes are maintained by meeting several conditions, such as culturing on specific extracellular matrices [36,37], using a hormone-enriched culture medium [38,39], and ensuring three-dimensional orientation of the cultured hepatocytes [37]. Gel-entrapment of hepatocytes allows most of the above. In these bioreactors, the primary isolated hepatocytes are inoculated into a collagen suspension within the intraluminal fiber space. The solution rapidly gels, resulting in hepatocytes entrapped in a three-dimensional matrix in the hollow fibers. The hepatocyte/collagen gel subsequently contracts to less than 50% of the original cross-section, resulting in three compartments: the contracted gel containing the hepatocytes, the rest of the intraluminal hollow fiber space, and the extraluminal space within the rigid housing. Hepatocyte culture medium is perfused through the intraluminal space to keep the hepatocytes viable and functional when another medium (in the future the patient's blood) is perfused through the extraluminal space. Proper functioning of this bioreactor has been demonstrated for 5 consecutive days in vitro, as measured by albumin synthesis, ureagenesis and oxygen consumption [40]. In an animal model of hepatic failure in small dogs (<30kg), improved hemodynamic stability, delayed or absent hepatic encephalopathy, and improved survival were shown [41]. Unfortunately, this concept yields only 0.5 to 1.0 g cells per bioreactor [42], which is not enough to treat humans. Although further characterization studies are being performed [43], the distance required for nutrients and exchangeable medium of the intraluminal gel-entrapped hepatocytes is not known, whereas the whole process of exchanging nutrients and toxic substances is dependent on passive diffusion through this gel.

Extracorporeal liver-assist device, ELAD

The C3A cell line is a highly differentiated, stable, and viral-free human cell line that performs a range of metabolic functions comparable to those of the normal liver. It has been shown to maintain viability and function for 4 to 8 months when seeded in a hollow fiber cartridge at high seeding density. In this concept, approximately 10 g of C3A cells is injected into the extraluminal space; the cells grow to confluence in a period of 3 to 4 weeks and do not outgrow their culture space. Maturity is determined by glucose use and albumin production. It is thought that approximately 200 g of C3A cells ($2x10^{11}$ hepatocytes) are

seeded in the bioreactor at confluence [44]. Animal studies of dogs with acetaminophen-induced hepatic failure showed a significant biochemical improvement and improved survival. However, although the fibers have a nominal molecular weight cut-off of 70 kDa, human proteins such as human transferrin (molecular weight 90 kDa) and α_1-antitrypsin were detected in dog serum, suggesting the transfer of human cell line material to the (canine) patient [44]. Initial experience was encouraging: 11 patients were treated with the extracorproeal device and no short-term safety problems were observed; 10 patients showed biochemical improvement and 6 patients survived (see Table 2) [45]. In a second study, a pilot controlled trial, Ellis et al [46] reported good survival for the treated group (78%), but surprisingly also for the group receiving conventional treatment (75%). The authors attribute the outcome of this trial to their choice of a group of patients with ALF at an earlier stage of the clinical course, when accurate predictors of prognosis are lacking. Further clinical studies will have to be performed to be able to justify the use of this system for patients with liver failure. More importantly, the long-term effects of leakage of human cell line material into the circulation of future patients, as previously shown [47], need to be assessed before the safety of this system can be established conclusively.

Bioartificial liver, BAL

Because hepatocytes are known to be anchorage-dependent for prolonged viability and functioning, and to increase the effective surface area for hepatocytes in the hollow fiber cartridge, Demetriou et al [48] used collagen-coated microcarriers for their BAL. After isolation of primary pig hepatocytes, the cells are incubated overnight with collagen-coated dextran microcarriers for attachment, and the microcarrier-attached hepatocytes are inoculated into the extraluminal compartment of the hollow fiber system. In vitro and in vivo studies showed hepatocyte viability and functioning on microcarriers by conjugation of bilirubin, uptake of ^{99m}Tc –DESIDA, and synthesis of liver-specific proteins (e.g. albumin) after implantation in albumin-deficient rats for 6 days. Moreover, similar functioning was measured after 2 weeks of storage of the microcarrier-attached hepatocytes at -80°C, suggesting that this part of the bioreactor can be prepared in advance. In vitro mass transfer studies were performed to determine the optimum operating parameters for a hollow fiber cartridge serving as a BAL. These studies demonstrated that cells attached to the microcarriers do not bind to the fibers, thereby allowing free convection of plasma/perfusion medium with an optimum cross-fiber pressure drop at high-flow recirculation of plasma [49]. Significant metabolic support was

shown when cryopreserved microcarrier-attached pig hepatocytes were used in the BAL to treat dogs with acute liver failure continuously for 6 hours, introducing plasmapheresis to avoid hemolysis and platelet depletion in the perfusion system. The viability of the cells after 6 hours of both allogeneic and xenogeneic perfusion was 90%. Unfortunately, survival was not one of the endpoints of this study [50]. A charcoal column was added to the extracorporeal perfusion system before perfusion through the BAL because it was assumed that this would protect the porcine hepatocytes from the toxic hepatic failure plasma [51]. This system, including plasmapheresis, charcoal column, and cryopreserved primary isolated microcarrier-attached pig hepatocytes (approximately 4-6 x 10^9) inoculated into the extraluminal space of a hollow fiber cartridge, was used for the first time to treat a patient in 1993, leading to a phase I clinical trial with 31 patients (see Table 2). Eighteen patients with fulminant hepatic failure, 3 patients with primary non-function after liver transplantation, and 10 patients with acute exacerbation of chronic liver disease were treated successfully, as indicated by significant neurologic improvement, decreased intracranial pressure, decreased plasma ammonia levels, and improvement of other biochemical parameters. Liver transplantation followed in 21 of the 31 patients; the other 8 patients were not eligible for transplantation [52].

Although this system gave promising results and a phase II/III clinical trial is being prepared to determine efficacy, further studies are needed to establish the immunologic consequences of perfusing human plasma through a device containing pig hepatocytes. In addition, the possible transfer of viral material from the bioreactor to the host must be monitored in detail.

Also, the long-term stable culture of human hepatocytes must be explored. Recent studies have shown normal proliferation of human blastocyst-derived, pluripotential embryonic stem cell lines, which differentiate into derivatives of all three embryonic germ layers [53]. These cell lines could possibly be used to develop noncarcinogenic human hepatocytes. When incorporated into a BAL, this could result in a functional device without the immunologic drawbacks and the potential danger of a human hepatoblastoma cell line.

EXTRACORPOREAL WHOLE LIVER PERFUSION

The need for hepatic tissue for effective treatment of patients with ALF led to the concept of using the whole liver in an extracorporeal perfusion system. Isolated liver perfusion studies date back to the 19th century, when they were performed to study liver metabolism,

demonstrating complex metabolic liver function outside the natural environment of the liver [54]. Not until 1958 was the first experimental study carried out with allogeneic livers to clear ammonia given to dogs. The study showed a significant decrease in ammonia levels in animals treated by ex vivo liver perfusion compared with control animals [55]. The first clinical study was performed in 1965 by Eiseman et al [56], who tested several perfusion circuits in pigs as well as humans with ALF. During the past three decades, approximately 141 ex vivo pig liver perfusions have been performed to treat 87 patients with liver failure in usually poor clinical condition (Table 3). The technique and clinical outcome of extracorporeal whole pig liver perfusion, as reported in well-documented studies comprising more than one patient, are described below.

Table 3. Clinical studies using extracorporeal pig liver perfusion

Investigators	Publication year	No. of Patients	No. of Perfusions
Eiseman*	1965/1966	8	11
Norman	1967	5	13
Watts*	1967	3	6
Bertrand	1968	10	15
Beveridge	1968	1	1
Mieny	1968	1	1
Ham	1968	1	2
Abouna*	1968/1969	4	7
Watts	1969	5	8
Schleifer	1969	2	2
Häring	1969	3	4
Chevrel	1969/1970	4	4
Abouna	1970	1	10
Ranek*	1971	5	7
Pharboo*	1971	5	6
Lempinen	1971	1	3
Nielubowicz	1972/1973	5	10
Margulis	1975	10	10
Tung*	1981	9	13
Chari*	1994	4	8
	Total:	87	141

*Studies analyzed for Table 4.

Experimental setup

The pig liver has been used in most cases because it is readily available. Immediately before perfusion, hepatectomy is performed on pigs weighing between 20 and 45 kg. In all studies, the cystic duct is ligated, the common bile duct cannulated to collect the bile produced during

perfusion, and the hepatic artery and the portal vein are cannulated for inflow of human blood. The inferior vena cava is either left open, in which case outflow goes to a reservoir where the patient's blood is mixed with ascitic fluid and pumped back into the circulation, or cannulated for immediate return of blood to the patient. The isolated liver is placed in a sterile perfusion chamber and connected to the patient's circulatory system within no more than 30 minutes. Bile flow proved to be the single most reliable index of hepatic function and was assessed in all cases of extracorporeal liver perfusion.

Many perfusion configurations have been tested [56]. In general, either blood is drawn from the femoral artery and returned to the patient through the saphenous vein, or a direct arterio-venous shunt in the upper arm is used [56-58,60,61]. In other studies, a veno-venous shunt was used, placing a double lumen catheter in the femoral vein [59] or using the femoral and jugular veins [62]. As is known from isolated liver perfusion studies, oxygen supply to the liver is vital. Therefore, the addition of a heater-oxygenator unit to the perfusion system ensures a blood supply with a temperature of 37 to 39°C and 100% oxygen saturation. Thus, there is no difference in performance of the ex vivo liver between perfusing the hepatic artery and the portal vein or the portal vein alone [56]. A pump is used in the perfusion circuit, either to return the blood from the reservoir containing 'post'-ex vivo liver blood to the patient [56,58,60,61] or to create stable and constant pressure and flow into the liver when a veno-venous bypass was used [59,62]. Length of extracorporeal pig liver perfusion varied between 1.5 and 9 hours; one to four perfusions were performed per patient. Regional or systemic heparin was administered.

Clinical outcome

For the literature review, references 56 to 62 were chosen because their documentation is the best and the techniques used for extracorporeal pig liver perfusion are comparable. The variation in the cuase of the hepatic failure among individual patients from each study makes it hazardous to compare the success rate of the ex vivo liver perfusion technique. In addition, one is tempted to assume that groups of patients (e.g., those with ALF compared with those with chronic liver disease) may exhibit a better outcome; therefore, we determined neurologic improvement, the percentage decrease in levels of NH_3 or bilirubin before and after perfusion, as well as the mean survival (in days) to compare the results of patients with the same cause from the different groups. Five patients who received eight perfusions have been excluded because they had single-case diseases such as hepatic failure from septic shock,

Table 4. Patients treated with extracorporeal liver perfusion

Cause	No. of Patients	No. of Perfusions	Neurologic Improvement /Perfusion	NH_3 Decrease (%)	Bilirubin Decrease (%)	Survival (mean)
Cirrhosis	11	15	14/15	55	36	4.8 days
Viral Hepatitis	10	16	10/16	43	41	4.8 days + 1 complete recovery
Liver failur not otherwise specified	12	19	16/19	25	47	4.3 days + 2 complete recoveries

Data from references 56-62.

hemochromatosis, acetaminophen-intoxication, and primary graft non-function. Of the remaining 33 patients, 11 had cirrhosis from alcohol abuse, 10 had viral hepatitis, and 12 had hepatic failure not otherwise specified (Table 4). All patients had coma hepaticum grade IV to V (Table 5), and all other treatments had failed to that point.

The results show neurologic improvement to at least coma hepaticum grade III to II in most patients, which does not seem to be related to the cause of the hepatic failure. Moreover, no relation between clearance of NH_3 or bilirubin and neurologic improvement or survival and cause was demonstrated. This underlines the fact that the biochemical parameters used to describe the function of the isolated liver and to follow the clinical condition of patients with hepatic failure do not reveal the mechanism of hepatic failure and hepatic encephalopathy. Although controlled trials have not yet been carried out, the complete recovery of three patients and the prolonged survival of some patients strongly suggest improvement over conventional treatment.

Table 5. Clinical stages of acute fulminant hepatic failure (Hepatic Coma)

Grade	Description	Clinical symptoms
I	Neurasthenic syndrome	Abnormal fatigability, depression, confusion, altered mood of behavior, appropriate response, slight tremor
II	Somnolence	Drowsiness, inappropriate behavior, flapping tremor
III	Sopor	Reduced responsiveness, ability to obey simple commands, incoherent speech, Babinski
IV	Coma	No response to verbal stimuli, response to painful stimuli, decerebrate, grossly abnormal EEG
V	Deep coma	No response to pain, little or no EEG activity

Most complications that accompany extracorporeal whole liver perfusion are due to preexisting cardiopulmonary disease, although hemodynamic changes during perfusion were highly limited. Uncontrolled bleeding secondary to thrombocytopenia as aresult of the oxygenator in the circuit or the development of disseminated intravascular coagulation is another common complication. In three patients, a relapse to stage V hepatic encephalopathy eventually led to death.

Most ex vivo liver perfusion studies were performed in the late 1960s and early 1970s; the method was then abandoned because of the successful development of orthotopic liver transplantation (Table 3). However, with the present shortage of available donor organs, there is renewed interest in this method [62-64]. Although the results are encouraging, a controlled study has not yet been performed to predict conclusively the outcome of extracorporeal whole liver perfusion. Further, the immunologic implications of human blood perfused through hepatic endothelial tissue are unknown. This aspect will be considered in the next section.

IMMUNOLOGICAL IMPLICATIONS

The use of extracorporeal pig liver perfusion or an artificial liver device with pig hepatocytes implies contact of human blood or plasma with porcine material. When such an xenogeneic contact is established, whether it be only once or several times per patient for limited periods of time, immunologic problems should be anticipated. In the first clinical study on extracorporeal liver perfusion by Eiseman et al. in 1965 [56], C^{14}-tagged glycine and lysine were added to the perfusion system and synthesized into protein by the pig liver that probably contained the porcine amino acid code, suggesting exchange of biologic material between graft and host. Renewed interest in the application of xenogeneic cells or tissues has drawn attention to the possible immunologic consequences. Although a cell-mediated reaction is seen at an early stage, as indicated by activation of neutrophil granulocytes and natural killer cells after suppression of complement [65,66], these mechanisms are probably less significant because therapy is intended to last only several days. For this reason, we will focus on the humoral response.

The humoral response

Xenotransplantation normally leads to hyperacute rejection of the xenograft mediated by preformed naturally occurring antibody (pNAb) and complement. These antibodies, also called xenogeneic pNAb, are directed against porcine antigens and are present in most primates without preceding sensitization. In man, xenoreactive pNAbs, which consist of IgG, IgA and mainly IgM, form 2 to 4% of the total immunoglobulins. Approximately 80% of these pNAbs are directed against the GAL-alpha [1,3] membrane epitope, the most important target antigen of the human humoral defense against porcine organs [66]. pNAbs are mainly produced by splenic B-lymphocytes [67], and several theories about their development have emerged. For instance, they may be induced by contact between mucosal immune cells and intestinal flora [68] or food-derived antigens with epitopes similar to GAL [69]. Whether IgG, with a higher affinity for pig antigens, or IgM, which is supposed to act as an 'early' antibody, plays the predominant role in complement activation and cytotoxicity has yet to be established [70]. In a recent study by Schraa et al. [71], rats were sensitized against hamsters 1 and 5 weeks before hepatic xenografting; high titers of IgM and IgG, respectively, were found. Hyperacute rejection was demonstrated in the second group but not the first group, indicating the importance of IgG but not IgM-mediated cytotoxicity in hepatic xenografting. As for the direct effect of complement, both the classical and the alternative pathways seem to be involved in the activation and cytotoxicity of a xenogeneic reaction [69].

Immunological aspects in liver perfusion

In patients with ALF, the levels of total CH50 and C3, C4, C5, and the regulatory proteins factor I and beta-1H are significantly lower compared with controls [72], whereas the naturally occurring antibody levels do not differ. It is not clear whether this complement depletion is the result of impaired synthesis because of the diseased liver or ongoing activation of the complement system, or a combination of the two. Hyperacute rejection after xenogeneic kidney perfusion can be prevented only by complete inactivation of complement [73], whereas the perfusion of a liver with human blood is characterized by normal liver function for several hours [74]. At the same time, the drops in the levels of pNAbs during kidney and liver perfusion are similar. However, immunohistochemical analysis of the kidneys after perfusion with human blood demonstrates the binding of both IgM and IgG xenoantibodies and complement (C3) to kidney vascular endothelium, whereas staining was minimal for liver vascular endothelium[75].

Tector et al. [76] confirmed these data in an isolated liver perfusion study of blood from patients with liver failure. They showed significantly reduced complement levels and activity compared with normal subjects, resulting in an impaired ability to lyse both aortic and sinusoidal endothelial cells. Immunofluorescent analysis showed little or no staining for sinusoidal deposition of IgM, IgG or C3, suggesting that the sinusoidal endothelium of the liver can remove antibody and complement without cell lysis, possibly by endocytosis, compared with aortic or other endothelium.

Tabel 6. STRATEGIES TO OVERCOME HYPERACUTE REJECTION OF PIG-TO-HUMAN XENOGRAFTS

Antibody
- Removal by plasmapheresis, immunoapheresis
- Removal by absorbtion with pig organs
- Removal by absorbtion on gal columns
- Block with high avidity chicken anti-gal
- Block with peptides

Antigen
- Gene "knock out" (not in pig)
- Anti-sense constructs
- Enzymatic destruction: External Galactosidase transgene
- Suppress GAL: H transferase transgene
- Secretor gene

Complement
- Cobra venom factor
- Antibodies
- Solule complement receptor receptor type 1
- Transgenes (CD46[MCP], CD55[DAF], CD59)

Adapted from McKenzie et al [79]

The same results were obtained in a clinical study in which no adverse effects on ex vivo organ function were measured during treatment of patients with ALF by extracorporeal pig liver perfusion. Again, low complement levels, normal amounts of pNAbs, and, after perfusion, only trace deposits of pNAb and C3 on the sinusoidal endothelium with a decrease in the level of pNAb were demonstrated [77]. Long-term follow-up of these patients showed an increase in xenoreactive IgM and IgG within 10 days of perfusion and a subsequent return to normal [78]. These concepts are being used to study ways to delay hyperacute rejection (Table 6) [79]. Several strategies are being tested in different organs, with encouraging results. The efficacy of humoral mediators present in serum has been reduced by means of porcine livers

transgenic for human decay-accelerating factor (DAF/CD55) [80]. Reduced humoral injury was found with soluble complement receptor type 1 [81], and immunopheresis was successfully used to remove xenogeneic pNAb before perfusion of the liver, which resulted in improved functioning of the perfused liver [82].

The role of the immunologic defense in the treatment of patients with liver failure is far from clear, but no immunologic adverse reactions have been detected during treatment in the clinical studies reported thus far. In one patient with chronic aggressive hepatitis who underwent 16 extracorporeal liver perfusions in 2.5 months, a decrease in antibodies could be shown after every perfusion. This was followed by a progressive rise in the titer of these antibodies, which peaked during the fifth perfusion, when an anaphylacic reaction was recorded; the next five perfusions with a pig liver did not cause detrimental effects [83]. Whether prolonged or repetitive treatment leads to sensitization of the patient to porcine antigens remains to be determined.

Immunological aspects of artificial liver devices

Compared to extracorporeal whole liver perfusion, where human blood is in direct contact with porcine liver endothelium, artificial liver systems contain only pig hepatocytes and are usually perfused with human plasma, because a plasma separation unit is used in the perfusion circuit. As described before, the hollow fiber bioreactor consists of several compartments that communicate through the pores in the fiber walls, allowing mass transfer between the patient's plasma and the heterologous hepatocytes. The size of the pores is determined by the balance between sufficient waste removal and potential immune activation. Removal of ammonia, aromatic amino acids, and red blood cell breakdown products such as bilirubin (MW 0.6 kDa) is desirable. Coagulation proteins such as prothrombin (MW 70 kDa) and thrombin (MW 40 kDa) could potentially benefit the patient, which is why they should be allowed to pass through the fiber membrane. However, immunoglobulins (MW 150-900 kDa) and cytokines (MW 100-400 kDa) preferably do not make contact with the hepatocytes. The ideal molecular weight cutoff therefore may lie between 50 and 100 kDa [35], which should theoretically prevent a cellular as well as a humoral immune response.

However, te Velde et al. [84] showed that rats infused with the supernatant of in vitro cultured pig hepatocytes raised antibodies against the very small amounts of pig hepatocyte-derived proteins in the culture medium. Another study showed an increase in tumor necrosis factor-alpha after a 4-day perfusion of pigs connected to a hybrid liver support system containing

homologous hepatocytes [85], although no typical side effects were observed. In a clinical study on patients with ALF treated with the BAL, anti-pig xenoantibodies (IgM and IgG) remained at the same level 10 days after treatment, when one treatment was carried out. If more than one treatment was given, the levels increased two to threefold and decreased only if immunosuppression was administered because of subsequent liver transplantation. [86]. Thus, either through contact of human plasma with the pig hepatocytes during perfusion or because of pig hepatocyte material entering the human circulation, an immunological reaction can be induced. Whether this is of clinical relevance remains to be seen, since so far no side effects have been noticed.

Cellular and humoral graft-versus-host reactions also must be considered, although thus far there is no proof of this type of reaction in the literature. In a baboon-to-human liver transplantation, no baboon antibodies were detected in the human serum [87]. Another problem is the potential risk of zoonosis. Patience et al [88] showed that porcine kidney cell lines release particles of pig endogenous retrovirus (PERV), which can infect human cell lines in vitro. However, transmission of PERV from transplanted porcine endothelial cells to baboons [89] or humans [90] in vivo has not been detected. Obviously, these issues need further attention if artificial liver systems are to be used in clinical trials.

CONCLUDING REMARKS

Two major concerns are noted in the literature on extracorporeal liver perfusion for the treatment of liver failure. First, there is still no clear understanding of the biologic mechanisms of liver failure; therefore, the various approaches to temporary treatment of this disease lack a well-defined basis. Second, no uniform definition of liver failure is given. Within studies and between studies, there is a wide variety in the severity of liver disease, and success of treatment is defined in many ways. Although most studies report temporary improvement of the patient's clinical neurologic condition, evidence-based research indicating successful treatment has not yet been reported. Therefore, in our opinion, randomized studies that focus on a well-defined method and include sufficient patients to allow adequate statistical analysis are needed to determine the efficacy of this approach. Only then can conclusive evidence be gathered to benefit patients with liver failure who undergo either extracorporeal whole liver perfusion or treatment with a BAL.

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3

Expression of long-term liver-specific function by adult rat hepatocytes cultured on microcarriers

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ABSTRACT

The successful development of a bioartificial liver relies, in part, on the ability to maintain viability and differentiated function of a large number of hepatocytes *in vitro* for extended periods of time. Microcarriers have been widely used to scale up anchorage-dependent mammalian cell cultures. The goal of the present study was to seek optimal culture conditions for hepatocytes attached to microcarriers. Hepatocytes were seeded onto gelatin-coated polystyrene microcarriers, and the composite was cultured in suspension or embedded in an agarose or type I collagen gel for up to 14 days. We were able to seed up to 130 hepatocytes per microcarrier. Viability and function of hepatocytes on microcarriers cultured in suspension or in agarose decreased as a function of time. On the other hand, microcarriers embedded in a collagen gel exhibited specific albumin and urea secretion rates of approximately 3 $\mu g/h/10^6$ cells and 10 $\mu g/h/10^6$ cells during the second week of culture, respectively. These rates were similar to those obtained in control cultures of collagen-sandwiched hepatocytes in the absence of microcarriers. Thus, long-term liver-specific function can be induced by surrounding hepatocytes seeded on microcarriers with a collagen gel. This may be a viable approach to scale up the sandwich hepatocyte culture system.

INTRODUCTION

A promising approach for the treatment of hepatic failure is the use of an extracorporeal bioartificial liver device containing xenogeneic hepatocytes. Critical to the successful development of this technology is the ability to foster long-term viability and function of a large cell mass (~10% of liver) since freshly isolated hepatocytes take as much as 1 week in culture to fully re-express differentiated functions, and long treatement periods (days to weeks) are anticipated. Current systems being evaluated include rat or pig hepatocytes entrapped in hollow fiber devices, cultured on flat plates, cultured on microcarries, or combinations thereof [1-7]. The level of expression of liver-specific function in these large-scale systems is often lower than that observed under optimal conditions in small-scale cultures. Potential explanations include (a) nutrient transport limitations, (b) a microenvironment that

does not induce hepatocyte differentiation, and (c) insufficient time in culture to allow full recovery of the expression of liver-specific function after hepatocyte isolation.

Long-term viability and function of hepatocytes can be induced by (a) specific medium formulations [8,9], (b) complex extracellular matrices [10], (c) feeder cell layers [11,12], (d) culture as aggregates or spheroids [6,13] and (e) culture in collagen gels [14-17]. In the latter approach, cells can be either directly suspended in a type I collagen gel or "sandwiched" by placing a collagen gel overlay on top of a monolayer of hepatocytes preseeded onto another collagen layer [14,17]. Culture in collagen gels is an attractive option that is suitable for scaleup and eventual use as the basis for a bioartificial liver for several reasons. First, a physiologically compatible medium can be used. Second, no complex, poorly defined, or expensive extracellular matrix materials are required. Third, the cumbersome task of propagating a feeder cell line is obviated. Finally, hepatocytes sandwiched in type I collagen adopt a monolayer morphology, which facilitates the optimization and control of nutrient and metabolic transport between the cells and the medium. However, the sandwich culture system in its current form is not easily amenable to scaleup because it requires a flat surface to lay down the collagen layers. Scaleup of cultures with a flat-plate geometry is cumbersome and has previously yielded relatively low cell concentrations (~0.5 x 10^6 cells/ml) [2].

Microcarriers have been widely used for scaling up mammalian cell culture and provide a large biocompatible surface area per unit volume. In addition, sampling of the cells from the culture is possible, which facilitates characterization of a large-scale culture system under development. Microcarriers seeded with hepatocytes have been used in suspension, in a perfused packed-bed system as well as packed in the extraluminal (or shell) compartment of hollow fiber modules [3,4,18]. In these cases, liver-specific function was evaluated on a short-term basis (< 70 h). In one study, Shnyra et al [19] examined hepatocyte function on microcarriers cultured in suspension over 1 week. They observed a stable phase of albumin secretion for up to 5 days followed by a decrease, with a concomitant increase in fibronectin secretion. Similar findings were made with hepatocytes cultured in plastic dishes or on a single collagen gel and are indicative of dedifferentiation [10,14,20]. In these systems, the necessary microenvironment to induce and maintain liver-specific function was not provided.

In the current investigation, we sought to establish long-term function of hepatocytes on microcarriers. Thus, we examined the effect of embedding microcarrier-attached hepatocytes in a collagen gel, thereby creating a sandwich configuration between the microcarrier surface and the surrounding collagen, on the morphology and expresion of two major liver-specific functions (albumin and urea synthesis) by hepatocytes.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle medium (DMEM) with 4.5 g/l glucose was purchased from Gibco (Gaithersburg, MD, USA); fetal bovine serum (FBS), penicillin, and streptomycin from Hazleton (Lenexa, KS, USA); hydrocortisone from Upjohn (Kalamazoo, MI, USA); epidermal growth factor (EGF) from Collaborative Research (Bedford, CA, USA); and insulin from Squibb (Princeton, NJ, USA). Seaplaque low gelling temperature agarose (for a 1% w/v agarose gel, the gelling temperature is 26-30°C and the melting temperature is 65°C) was obtained from FMC Bioproducts (Rockland, ME, USA). Type I collagen (1.2 mg/ml in 1 mM HCl) was prepared from rat tail tendons as described previously [16]. Calein acetoxymethyl ester (Calcein AM) and Hoechst 33258 were obtained from Molecular Probes (Eugene, OR, USA). Cytodex 2 and Cytodex 3 microcarriers were obtained from Pharmacia (Piscataway, NJ, USA) and gelatin coated plastic microcarriers (cat. no. M-9650) from Sigma (St. Louis, MO, USA). All other chemicals, including calf thymus DNA (cat. no. D-5668), were obtained from Sigma (St. Louis, MO, USA).
The culture medium consists of DMEM supplemented with 10% FBS, 200 units/ml penicillin, 200 μg/ml streptomycin, 50 μg/ml gentamycin, 20 ng/ml EGF, 7.5 μg/ml hydrocortisone, 20 ng/ml glucagon, and 0.5 unit/ml insulin. SDS buffer consists of 0.1% w/v sodium dodecyl sulfate and 1 mM ehtylenediamine tetraacetic acid (EDTA) in 100 mM Tris-HCl (pH 7.4). TEN buffer consists of 10 mM Tris-HCl (pH 7.4) with 1 mM EDTA and 2 M NaCl.

Isolation of hepatocytes

Hepatocytes were isolated from 2- to 3-month-old female Lewis rats (Charles River Laboratories, Boston, MA, USA) by a modified procedure of Seglen [21]. Details of the isolation procedure are described elsewhere [16]. The average yield was 200-250 x 10^6 hepatocytes with a viability of 80-90% as determined by trypan blue staining.

Microcarrier Preparation and Characterization

Plastic microcarriers were suspended in 50 ml of culture medium and allowed to sit overnight at 4°C. The concentration of microcarriers was determined by placing aliquots of appropriate dilutions of the stock suspension in a 96-well plate and counting the number of microcarriers per well using a stereoscope (Nikon SMZ 2B). This stock solution was kept at 4°C until use for a maximum of 2 months.
To determine the microcarrier size and distribution, microcarriers were visualized through a Plan Neofluar 10x/0.30 NA objective on a Zeiss Axiovert 10 inverted microscope (Zeiss, Thornwood, NY, USA) equipped with a Nuvicon camera (Hamamatsu, Japan). Phase contrast images were acquired and digitized using Image/1 software (Universal Imaging Inc., West Chester, PA, USA) installed on a 486/66 MHz IBM compatible personal computer, recorded on an optical laser disc, and trasnferred onto Kodak Tmax 100 35-mm film using a LFR^+ film recorder (Lasergraphics, Irvine, CA, USA). The diameter of at least 100 microcarriers was measured on printed images. The length scale was determined form images of a hematocytometer grid processed in an identical fashion. The average microcarrier diameter was found to be 163 ± 19 μm. Thus, the average surface area of a microcarrier, calculated assuming spherical geometry, is 8.3×10^4 μm^2.

Hepatocyte Attachment Procedure

T25 square flasks (Corning Glassware, Corning, NY, USA) were made nonadherent by incubating them overnight with 5 ml of 0.1% w/v bovine serum albumin (BSA) in phosphate buffered saline (PBS) at 37°C. The flasks were rinsed with PBS three times, and 5×10^4 microcarriers and $2.5\text{-}10 \times 10^6$ viable hepatocytes (i.e., 50-200 cells per microcarrier) suspended in 5 ml of culture medium were added. The flasks were gassed for 2 min with 10% CO_2 + 90% oxygen (Northeast Airgas, Nashua, NH, USA), closed thight, placed on a linear shaker (New Brunswick Scientific, Edison, NJ, USA) at 37°C, and subjected to a 5-sec shaking cycle (amplitude: 1 in., speed: 150 rpm) every 5 min for 24 h. Based on our previous experience with seeding hepatocytes onto microcarriers, a high oxygen concentration in the gas phase and intermittent shaking are optimal for cell attachment [23]. To seperate unattached cells from hepatocytes attached to microcarriers, the total content of each T25 flask was

filtered through a nylon filter (mesh size 100 μm), and the retained microcarriers with attached hepatocytes were used in the subsequent procedures.

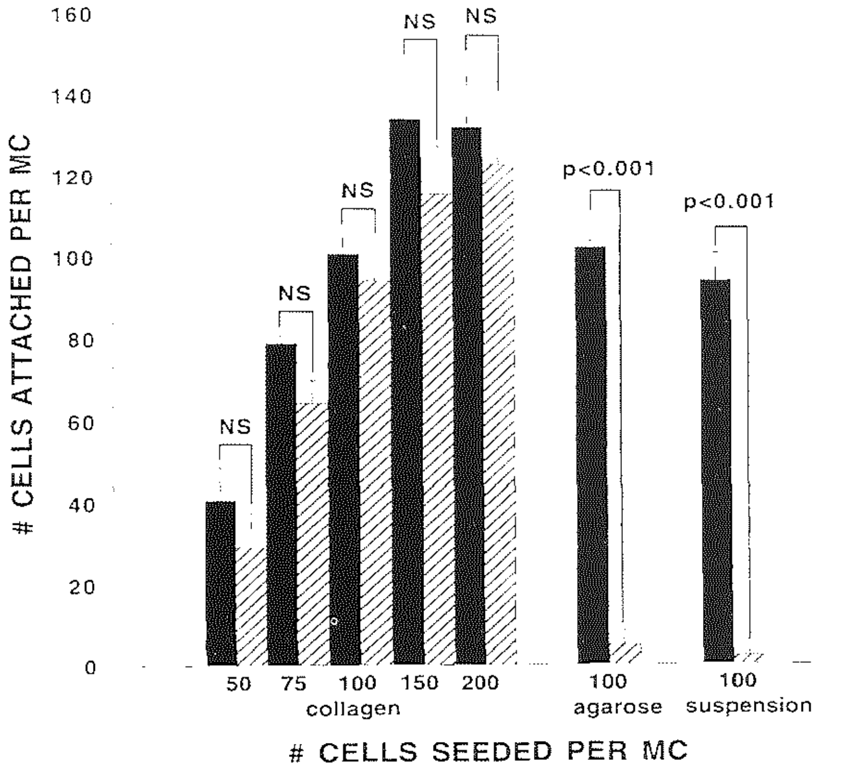

Figure 1. Number of cells per microcarrier before and after long-term culture in collagen, agarose, or in suspension. Cells were seeded onto plastic microcarriers using a cell/microcarrier ratios of 50:1 to 200:1. The amount of microcarrier-bound DNA normalized to the number of microcarriers was determined after seeding (solid bars) and after culture between two layers of collagen gel, agarose gel, or in suspension in agarose-coated dishes for 14 days (shaded bars). The amount of DNA is expressed as a cell number based on DNA standards containing known concentrations of freshly isolated hepatocytes. Each data point shown is the average of at least three replicate dishes. NS = not statistically significantly different.

To estimate the number of attached cells per microcarrier, the filtered microcarriers from each flask were resuspended in 4 ml of SDS buffer, and the samples were incubated overnight at 37°C. Total DNA was measured using a method described elsewhere [22]. Briefly, 40 μl of the sample were added to 2 ml of a Hoechst 33258 dye solution (0.1 μg/ml of Hoechst 33258 in TEN buffer), and the fluorescence (settings: excitation, 365 nm; emission, 458 nm) was

measured in a SPF 500C spectrofluorometer (SLM Instruments, Urbana, IL, USA). DNA standards consisted of solutions containing known concentrations of calf thymus DNA (0-50 μg/ml in SDS buffer). The resulting amount of DNA was converted to a cell number using an appropriate standard curve prepared using cell suspensions containing 0-2 x 10^6 cells/ml of freshly isolated hepatocytes in SDS buffer. On the average, the specific DNA content of freshly isolated hepatoyctes was 25.4 ± 39 μg DNA/10^6 cells. The cell number was then divided by the total number of microcarriers in each sample.

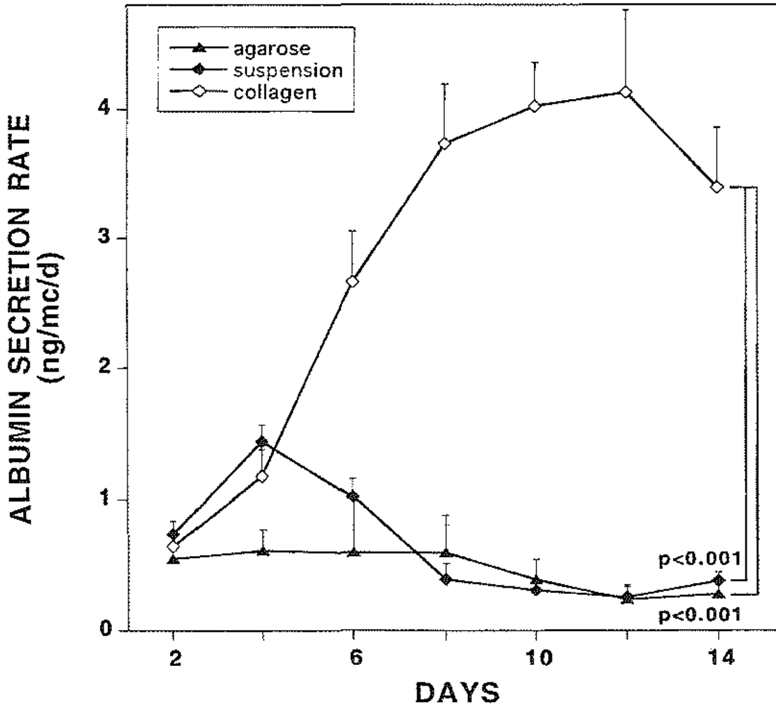

Figure 2. Effect of extracellular matrix composition on albumin secretion of hepatocytes cultured on microcarriers. Hepatocytes were seeded onto plastic microcarriers using a cell/microcarrier ratio of 100:1. The microcarriers were cultured in suspension in 100-mm dishes (15,000 ± 900 microcarriers/dish), or embedded between two gels of agarose or collagen in 60-mm dishes (3,700 ± 300 microcarriers/dish). Specific albumin secretion rates were calculated by multiplying the albumin concentration values by the volume of daily medium change (5 mL for suspension cultures, 2 mL for the others) and normalizing to the the number of microcarriers in the dish and the sampling time (1 day). Each data point shown is the average of at least three replicate dishes.

Long-Term Culture of Hepatocytes on Microcarriers

The microcarrier-attached hepatocytes were cultured in three different ways: (a) in suspension in medium on top of an agarose gel, (b) embedded in agarose, and (c) embedded in type I

collagen. For each suspendion culture, the content of one T25 used for seeding was filtered, the retained microcarrier cells resuspended in 10 ml of culture medium, and then spread into a 100-mm bacterial Petri dish precoated with an agarose gel. The coating was prepared by pouring 3 ml of an agarose solution (prepared by dissolving 1% w/v agarose powder in PBS at 90-100°C) into each dish followed by incubation at 4°C for 30 min. Every day, 5 ml of medium were replaced with fresh medium.

For the cultures embedded in agarose or collagen, 60-mm dishes were precoated with 1 ml of a 1% agarose gel (prepared as above) or 0.1% collagen gel, respectively. The collagen gel was prepared by mixing 9 parts of a collagen stock solution (1.2 mg/ml in 1 mM HCl) with 1 part of serum-free 10x DMEM on ice. One milliliter of this solution was poured into each dish, and gelation was induced by incubating at 37°C for 30 min. The contents of a T25 flask used for seeding microcarriers was filtered, and the retained microcarrier cells resuspended in 8 ml of culture medium. Two milliliters of this suspension were added per agarose- or collagen-coated 60-mm dish. This yields ~3,700 microcarriers/dish, the standard seeding density used; larger or smaller densities were obtained by adding a proportionally greater or smaller amount of microcarrier cell suspension. These cultures were incubated overnight at 37°C, and, after aspirating the culture medium, they were overlaid with 1 ml of either liquid 1% agarose in PBS at 37°C or a collagen solution. Gelation of the agarose and collagen overlays was induced by placing the cultures at 4°C or 37°C for 30 min, respectively, prior to adding 2 ml of fresh culture medium per dish.

Control cultures conisted of 2 x 10^6 viable hepatocytes seeded between two 1-ml collagen gels as descreibed previously [16]. All cultures were kept at 37°C in a humidified atmosphere with 10% CO_2, balance air. Culture medium was replaced daily. Medium samples were saved every other day for albumin and urea level determination.

To estimate the number of viable cells remainng at the end of the culture period (14 days), the amount of DNA per dish was determined. Suspended cultures were removed from the dishes, washed in PBS, and resuspended in 4 ml of SDS buffer. To liberate cells from collagen-embedded cultures, the medium was removed, and the dishes were rinsed with PBS and 1 ml of 0.1% collagenase in Krebs-Ringer buffer supplemented with 0.2 mg/ml $CaCl_2$. After 15 min at 37°C, the freed cells were removed, washed in PBS, and resuspended in 4 ml of SDS buffer. To liberate cells from agarose-embedded cultures, agarose was liquefied by heating the cultures to 65°C. The cells were removed, washed in PBS, and resuspended in 4 ml of

SDS buffer. The amount of DNA in each sample was determined and the corresponding number of viable cells calculated as described previously.

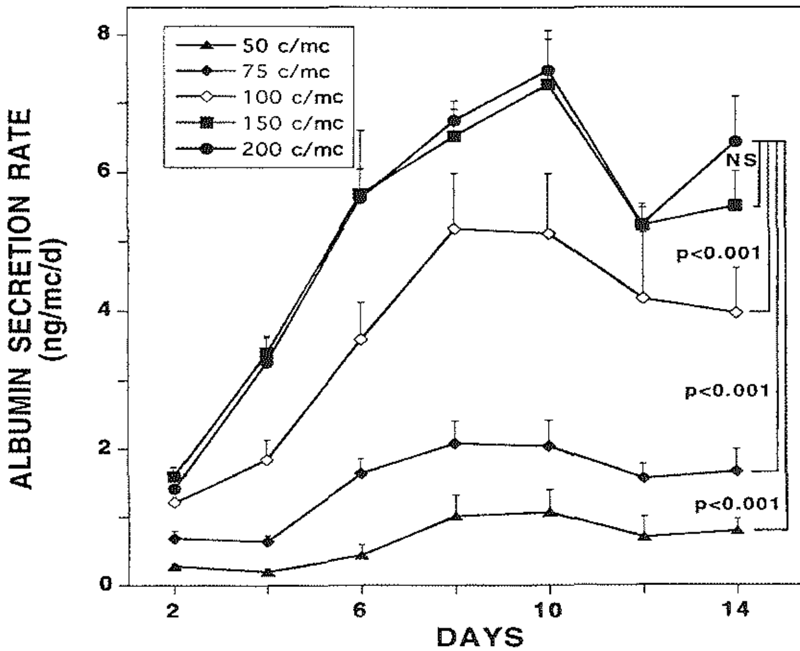

Figure 3. Effect of number of cells seeded per microcarrier on albumin secretion by hepatocytes. Hepatocytes were seeded onto plastic microcarriers using cell/microcarrier ratios of 50:1 to 200:1. The microcarriers were embedded between two gels of collagen in 60 mm dishes (3,700 ± 270 microcarriers/dish), and the culture medium was changed daily (2 ml/d). Each data point shown is the average of at least three replicate dishes.

Albumin and Urea assays

Albumin concentration in medium samples was measured using an enzyme-linked immunosorbent assay in 96-well plates using purified rat albumin standards (0-100 μg/ml) and a peroxidase-conjugated anti-rat albumin antibody (sheep IgG) that does not cross-react with BSA (cat. #55776, Cappel, Cochranville, PA, USA). The absorbance was measured with a Dynatech MR600 microplate reader (Chantilly, VA, USA). Urea concentrations were assayed using an assay kit (Sigma Chemical, St. Louis, MO, USA; cat no. 535-A). Albumin and urea concentration measurements were converted into specific secretion rates (either per microcarrier or per cell).

Viability Staining

At the beginning (day 2) and at the end (day 14) of the culture period, some cultures were stained using the viability dye calcein AM. Calcein AM specifically labels viable cells with a green fluorescence while nonviable cells remain nonfluorescent. Calcein AM was added to the culture medium to a final concentration of 1 μM and the dishes incubated at 37°C for 3 min. The stained cultures were visualized through an Achrostigmat 20x/0.30 NA objective on a Zeiss Axiovert 10 inverted microscope. A mercury lamp and standard fluorescein optics were used for observation of calcein fluorescence. Images were acquired, digitized, and transferred onto film as described previously.

Statistical Analysis

Analysis of variance was used. Statistical significance between groups was determined by the Tukey Honest Significant Difference Test for unequal sample sizes. The level of statistical significance was set to $p < 0.05$. Data shown are the average ± SEM unless otherwise specified.

RESULTS

Attachment and Viability of Hepatocytes on Microcarriers

To determine the maximum number of cells that could be attached onto microcarriers, hepatocytes were seeded with cell/microcarrier ratios of 50:1 to 200:1. DNA analysis of microcarriers after seeding shows a saturation coverage at approximately 130 cells/microcarrier (Fig.1). Furthermore, viability was preserved after 14 days of culture in collagen, based on the amount of DNA remaining at that time. In contrast, cultures of microcarriers seeded with a cell/microcarrier ratio of 100:1 and maintained in suspension retained less than 10% of the initial amount of DNA. Because suspended microcarrier cultures formed large aggregates wherein nutrient transport limitations may occur, microcarriers were also immobilized in agarose. Again, the amount of remaining DNA after 14 days was less than 10%, suggesting a specific requirement for collagen to maintain hepatocyte viability.

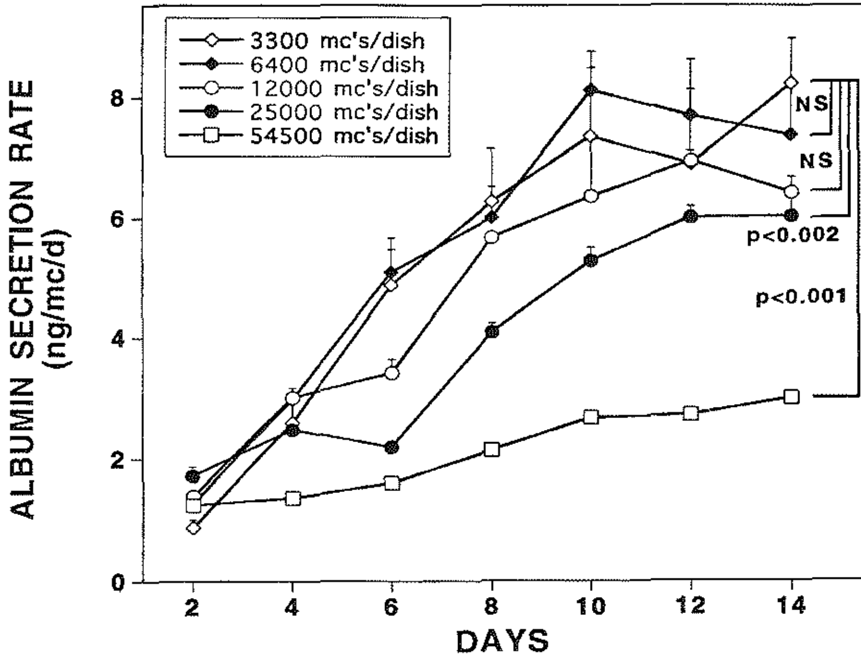

Figure 4. Effect of number of microcarriers per dish on albumin secretion by hepatocytes. Hepatocytes were seeded onto microcarriers using a cell to microcarrier ratio of 100:1. The microcarriers were embedded between two gels of collagen in 60 mm dishes, and the culture medium was changed daily (2 ml/d). Each data point shown is the average of at least three replicate dishes.

Expression of Liver-Specific Functions

To investigate the effect of the culture conditions with respect to the expression of liver-specific function, albumin secretion rates were determined (Fig. 2). In collagen-sandwiched microcarriers, the albumin secretion rates increased approximately 7-fold between days 2 and 8 and were relatively stable at 3-4 ng/mc/d from days 8 to 14. Microcarriers cultured in suspension or embedded in agarose exhibited albumin secretion rates below 0.5 ng/mc/d after day 6. Thus, long-term albumin secretion levels (i.e., at day 6 of culture and afterwards) paralleled the DNA data obtained earlier.

To optimize the culture conditions in collagen and to ensure that no significant nutrient limitations were present, the seeding density was varied in two ways. First, microcarriers were seeded with cell/microcarrier ratios of 50:1 to 200:1. The albumin secretion rate per microcarrier increased as a function of seeding density (Fig. 3). No further increase in function was observed above a seeding ratio of 150 hepatocytes/microcarrier, consistent with

the maximum number of cells that could be attached to each microcarrier. Second, microcarriers were plated between 3,300 and 54,500 microcarriers per 6-mm dish, keeping the cell/microcarrier ratio constant at 100:1. Increasing the total numer of microcarriers up to 12,000 microcarriers per dish did not affect the albumin secretion rate per microcarrier, but a significant reduction was observed at 25,000 and 54,500 mc/dish (Fig. 4). These densities correspond to 2.5 and 5.5 x 10^6 cells/dish. We have observed a similar reduction in albumin secretion in standard sandwiched hepatocyte cultures above a seeding density of 2 x 10^6 cells per 60-mm dish (unpublished observations), suggesting that this effect is not due to the presence of microcarriers but may arise due to possible nutrient limitations.

The function of hepatocytes on microcarriers was compared with the previously established sandwich culture system. Hepatocytes were seeded onto microcarriers and cultured in the collagen sandwich in parallel with hepatocytes directly placed in the collagen sandwich in the absence of microcarriers. Specific albumin and urea secretion rates of hepatocytes on microcarriers, expressed on a per-cell basis, were approximately 3.0 μg albumin/h/10^6 cells and 9.5 μg urea/h/10^6 cells in the second week of culture, and were similar to that of hepatocytes in the absence of microcarriers throughout the course of the experiment (Fig. 5).

Morphology and Spatial Localization of Hepatocytes on Microcarriers

To confirm our viability results and to assess the morphology of hepatocytes after embedding, microcarrier-hepatocyte cultures embedded in agarose or collagen were visualized by phase contrast microscopy and stained with the viability marker calcein AM. Two hours after placing the second gel layer (day 2 after isolation), agarose-embedded microcarriers bear viable cells over much of their surface (Fig. 6, panels A & C). On day 14, cells on agarose-embedded microcarriers still appear to be evenly distributed. There is little calcein fluorescence, however, suggesting a considerable loss of cell viability (panels E & G). On day 2, microcarriers embedded in collagen show a tendency to spread away from the microcarrier surface (panels B & D). On day 14 of the culture, hepatocytes have formed a belt that appears to be located approximately halfway through the microcarrier as determined by focal plane. The bright calcein fluorescence suggests that many of these cells are alive (panels F & H). Similar findings were obtained even if the overlay was placed as early as 2 h after plating the microcarriers (not shown).

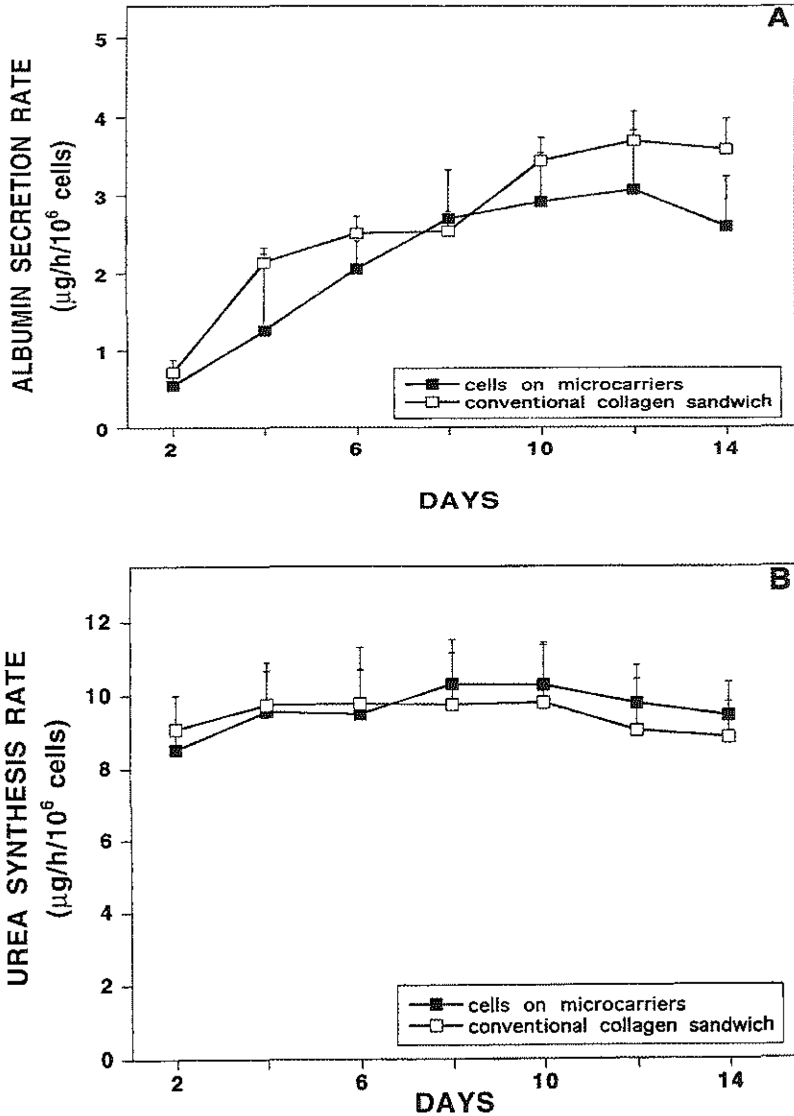

Figure 5. Comparison of liver-specific functions between hepatocytes cultured on microcarriers embedded in collagen and hepatocytes embedded in collagen in the absence of microcarriers. Hepatocytes were seeded onto microcarriers using a cell to microcarrier ratio of 100:1. The microcarriers were embedded between two gels of collagen in 60-mm dishes (3,460 ± 860 microcarriers/dish). In addition, freshly isolated hepatocytes were seeded at 2 x 10^6 cells per 60-mm dish and cultured between two gels of collagen. The culture medium was changed daily (2 ml/d). Secretion rates for albumin (panel A) and urea (panel B) were normalized to the number of cells (based on the DNA content) per dish at the end of the culture (14 days post seeding). Each data point shown is the average of at least nine replicate dishes.

DISCUSSION

Microcarriers have been used previously for large-scale culture of hepatocytes; however, long-term and stable expression of liver-specific function had not been demonstrated before. Furthermore, it was not possible to determine whether cell viability and function were compromised due to nutrient limitations or to an inherent inability of hepatocytes to function on microcarrier surfaces. In this study, we assessed the potential of microcarriers to serve as attachment surfaces for future large-scale hepatocyte culture systems by optimizing the seeding and culture of hepatocytes on microcarriers. Liver-specific function was induced by embedding the hepatocytes attached to microcarriers in a collagen gel, thereby sandwiching the hepatocytes between the microcarrier surface and the surrounding collagen. In the absence of significant transport limitations, we show that these hepatocytes exhibit a high level of albumin and urea secretion for at least 14 days in culture.

We were able to seed approximately 130 hepatocytes per microcarrier, which corresponds to a seeding density of 1.6×10^5 cells/cm^2 of bead surface. In our experience, the maximal cell coverage in collagen-coated dishes corresponds to 2×10^5 cells/cm^2, suggesting that approximately 80% coverage of the microcarriers surface was obtained in the current studies. Microcarriers examined in the seeding flasks appeared to be entirely covered with cells, while those that were transferred to the culture dishes often exhibit bare spots. Foy et al [23] previously suggested that cells may be subjected to significant detachment forces during postseeding manipulations (e.g. pipetting, filtration) that may limit the ultimate seeding density on microcarriers. Thus, coverage approaching 100% may be possible if more gentle postseeding manipulations are used. Using microcarriers made of cross-linked dextran coated with denatured collagen (Cytodex 3, diameter = 133-215 μm), Foy et al [23] could seed no more than 100 cells per microcarrier, which corresponds to a seeding density of 1.2×10^5 cells/cm^2 of bead surface. In a preliminary evaluation of different microcarriers, we found that hepatocytes seeded on gelatin-coated polystyrene microcarriers and cultured for 14 days in suspension remained evenly distributed around the polystyrene microcarriers. Hepatocytes seeded onto Cytodex 3 inititally attached and spread somewhat but eventually detached to form aggregates (unplubished observations). This finding is consistent with prior studies suggesting that denatured collagen, by virtue of its lack of rigidity, is not conductive to hepatoyte spreading and instead, promotes aggregate formation [24]. On the other hand, the plastic surface does not yield to cell-generated forces an thus allows cells to spread. Because

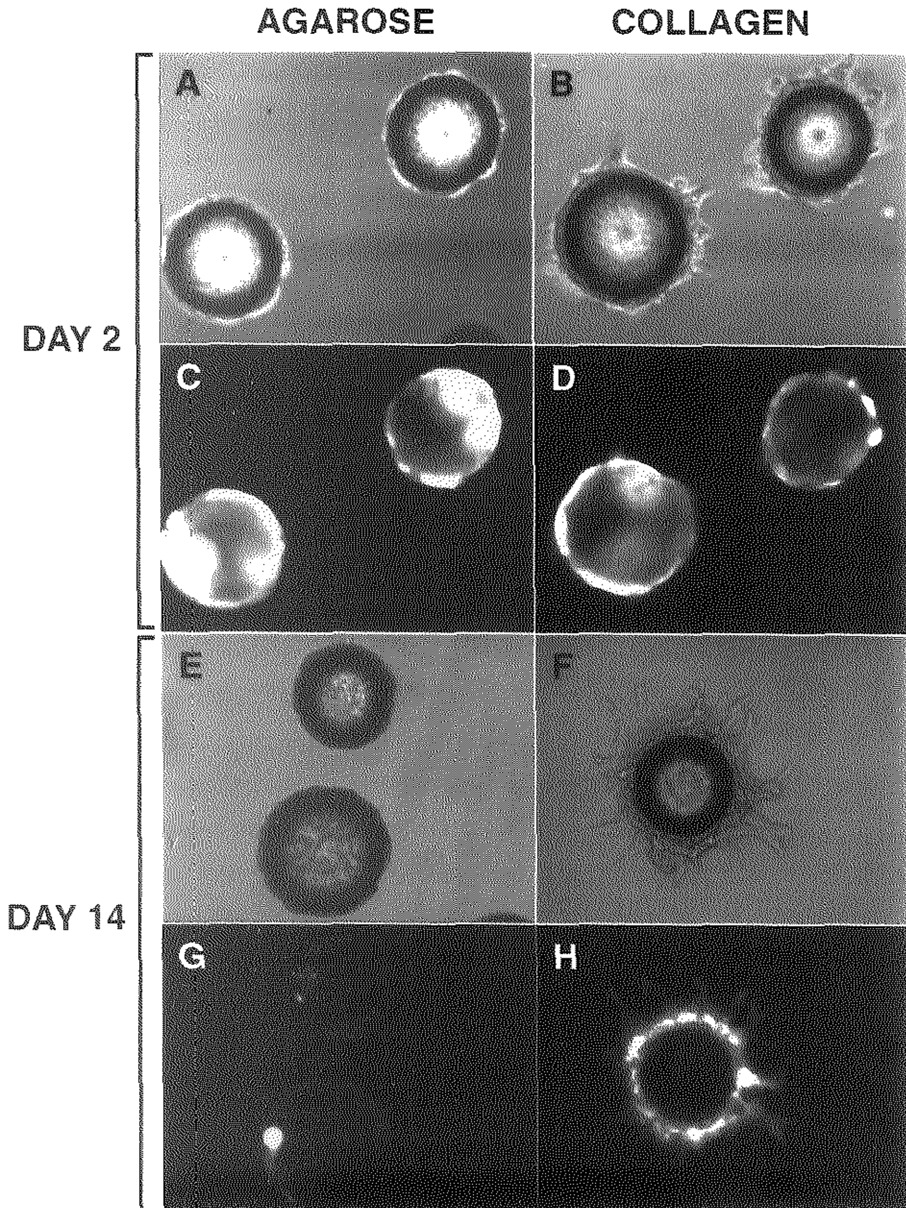

Figure 6. Morphology and distribution of hepatocytes cultured on microcarriers embedded in agarose and collagen gels. Hepatocytes were seeded onto plastic microcarriers using a cell/microcarrier ratio of 100:1 and embedded between two agarose or collagen gels. The cells were stained with calcein AM 2 h after placing the second gel (day 2 after hepatocyte isolation) as well as after 14 days in culture and visualized. Panels A, B, E, and F are phase contrast images. Panels C, D, G, and H show the corresponding calcein fluorescence distribution.

cell spreading is generally required for strong attachment to the substrate, it is possible that cell loss is less likely to occur during handling of plastic rather than Cytodex 3 microcarriers.

Albumin and urea secretion were evaluated as two major representative liver-specific functions. Albumin secretion was used as a marker for protein synthesis because it requires liver-specific gene expression and intact translational and secretory pathways in the cell [25]. Both cultures on microcarriers embedded in collagen and control sandwich cultures exhibited a time-dependant increase in the rate of albumin secretion during the first week to remain stable thereafter. A similar behavior has been observed in various long-term culture systems, and it has been suggested that this dynamic behavior reflects the repair and recovery process that hepatocytes must undergo after isolation [14,16]. The albumin secretion rate in the second week of culture (approximately 3 μg albumin/h/10^6 cells) falls within the range reported for other optimal long-term hepatocyte culture systems (1-10 μg albumin/h/10^6 cells) [9,10,12,15,16] and compares well with the *in vivo* rate of ~7 μg albumin/h/10^6 cells [16,26]. Suspended cultures or agarose-embedded cultures did not exhibit a sustained upregulation of albumin secretion. This trend has also been observed in spinner flask cultures of hepatocytes on Biosilon microcarriers [19]. The low level of albumin secretion obtained in these cases is consistent with the previous observations that hepatocytes cultured on a single surface (either tissue culture plastic or a single collagen gel) and hepatocytes overlaid with an agarose gel exhibit low albumin secretion rates in long-term cultures [10,16,20]. Overall, these data suggest that a surrounding collagen matrix was required for hepatocyte differentiation on microcarriers and that hepatocytes are functionally similar on these surfaces as in standard cultures in the collagen gel sandwich. This conclusion does not hold solely with respect to albumin secretion since urea secretion in cultures of microcarrier-attached hepatocytes in collagen, used as a marker of detoxification functions, was similar to that in the control sandwich cultures.

One interesting observation is that, after embedding, hepatocytes appeared to extend progressively into the surrounding collagen on a plane approximately halfway through the microcarrier when placed on top of a collagen gel. Hepatocyte aggregates have been shown to dissociate into individual cells when placed on a collagen gel [13]. This behavior may indicate a higher affinity of the cells for the collagen matrix as opposed to the microcarrier surface or other cells. We were unable to prevent this phenomenon even if microcarriers were directly suspended in a collagen solution or when the second gel added after a shorter period of time

(unpublished observations). Hepatocyte migration always occurred in a plane parallel to the surface of the dish, suggesting that certain macrosocopic features of the gel (e.g. geometry, attachment to the bottom plastic surface) may import some directionality to this process.

Current approaches for the development of bioartificial liver devices using primary hepatocytes involve microcarrier-attached hepatocytes [3,4], hepatocytes embedded in a collagen gel within hollow fibers [1,7], hepatocyte aggregates [6], and hepatocytes sandwiched between collagen layers on parallel plates [2]. In these systems, hepatocytes are introduced in the unit shortly after isolation, and the performance of the device is measured over a period of a week or less. This period of time corresponds to that normally required for the functional recovery of hepatocytes after isolation [25]. Nutrient limitations and the lack of an appropriate microenvironment conductive for hepatocyte differentiation may also explain the poor functional results obtained in some of these systems. One potential approach to facilitate the development of large-scale hepatocyte culture systems is to allow hepatocyte differentation and recovery from the isolation procedure prior to introduction into a large-scale device. The availability of fully functional and differentiated hepatocytes would facilitate the optimization of bioreactor performance with respect to providing an adequate nutrient supply. Microcarriers may provide a convenient scaleable unit for the devolopment of large-scale hepatocyte culture systems. Although in the current studies the microcarriers were embedded in a collagen gel, the next step would likely involve placing a thin collagen layer around each microcarrier. These microcarriers could then be cultured in suspension in a well controlled homogeneous environment to allow sufficient time for the re-expression of differentiated function. Furthermore, microcarrier-attached hepatocytes can be stored frozen until needed. The survival of cultured hepatocytes to a freeze-thaw cycle is significantly enhanced compared with freshly isolated hepatocytes [27], and hepatocytes have been successfully cryopreserved on microcarriers [4]. Cell survival after freeze-thaw requires precise control of cooling and warming rates, which may not be possible over the length scales of large devices (mm to cm).

Potential formats for a bioartificial liver based on microcarriers are packed or fluidized systems, or incorporation into hollow-fiber devices. The minimum cell mass required for a bioartificial liver has been estimated to be approximately 10% that of the intact human liver [28]. Assuming that each microcarrier with 130 cells occupies a 250 μm x 250 μm x 250 μm volume in a bioreactor, the resulting cell density is $\sim 10^7$ cells/cm^3, and thus the total volume required to accommodate the target number of 10^{10} cells would be about 1 liter. Assuming

that the void volume of the reactor would be of the same order of magnitude, it would be significantly larger than commonly used extracorporeal devices such as kidney dialysis equipment (90-250 ml) [29]. However, the use of smaller microcarriers could eventually bring this volume down.

In conclusion, we have found that hepatocytes cultured on gelatin-coated plastic microcarriers and embedded in type I collagen secrete albumin and urea at rates comparable to that seen in small-scale long-term culture systems. In addition, the specific albumin secretion rate obtained was similar to that found *in vivo*. These findings suggest that microcarriers might be useful in the development of a large-scale hepatocyte culture system expressing high and stable levels of liver-specific function.

ACKNOWLEDGEMENTS

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Non-immunological factors limiting extracorporeal liver perfusion

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Submitted.

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ABSTRACT

Background At present, transgenic pig livers are being used to study the feasibility of extracorporeal liver support in the management of acute liver failure. However, it remains to be determined whether non-immunological aspects may limit the quality of extracorporeal liver perfusion. For this reason the present study was designed using autogenous extracorporeal liver perfusion.

Materials and Methods After making a porto-caval shunt in pigs (n=5), hepatectomy was performed, the liver preserved and the circulation restored with a cavo-caval shunt. Three hours after hepatectomy the liver was placed into the extracorporeal perfusion system connecting the venous outflow of the left iliac vein with the left jugular vein. Haemodynamics of the pig were studied, and ex-vivo liver function was determined by O_2-consumption, bile-production (bilirubin in bile), clearance of bromo-sulfophtalleine (BSP) and NH_3, and pathology.

Results Macroscopically, the best achievable perfusion was realised with a pre- and posthepatic perfusion presure of 12-15 cm H_2O and a flow-rate of 350 ml/min. However, microscopic examination of the liver demonstrated venous stasis mainly in the peripheral parts of the liver. Although a good function of the liver was established, an increasing lactate-acidosis was measured, correlating with haemodynamic instability of the pig after approximately 3 hrs of ex vivo perfusion.

Conclusions Although the liver in an extracorporeal circuit may give adequate hepatic support, this autogenous model demonstrates that irrespective of immunological responses, physiological parameters limit the period of effective use of extracorporeal liver perfusion. This finding has important consequences in the perspective of using xenogeneic livers for extracorporeal perfusion.

INTRODUCTION

Extracorporeal liver perfusion has been used in the past to treat patients with acute liver failure [1,2]. Although encouraging results have been obtained, this technique was abandoned in the early seventies, because of the successful development of the orthotopic liver

transplantation. However, mortality rates in liver disease remain high, because of the shortage of –in time- available donororgans. Patients with fulminant hepatic failure usually develop multiple organ disease, haemodynamic instability and hepatic encephalopathy. Because the liver has an enormous regenerative capacity after massive necrosis or partial hepatectomy, several alternative therapies are currently being investigated to gain time to either liverregeneration or a livertransplantation. Most of these techniques focus on the perfusion of the patients' blood or plasma outside the body through a device containing xenogeneic hepatocytes, since it is thought that the multiple and complex function of the liver can only be adequately replaced by metabolically active liver tissue itself. Although this technique shows promising results [3], it is a complex, time-consuming and expensive procedure.
Extracorporeal liver perfusion (ECL) has been shown to stabilize the condition of patients with acute liver failure [4]. At present transgenic pig livers are being used to study the immunological implications of extracorporeal liver support in the management of acute liver failure [5]. However, it remains to be determined whether non-immunological aspects may limit the quality of extracorporeal liver perfusion. For this reason the present study was designed using an autogenous liver perfusion model.

MATERIALS & METHODS

Surgery. In pigs weighing 35-45 kg (n=5) anaesthesia was induced with an intramuscular injection of ketamine chloride (35mg/kg). The animal was intubated and ventilated, keeping the pig anaesthetized throughout the entire experiment. The end-expiratory CO_2 was kept between 4 and 5%. Haemodynamic instability was only corrected using prewarmed NaCl 0.9% or Haemacel; no whole blood or inotropica were used. Continuing haemodynamic monitoring was performed using a Schwann-Ganz catheter in addition to systemic measurement of central venous pressure and arterial blood pressure. Median laparotomy was performed and the liver was freed by dissecting the triangular ligaments, the falciform ligament and all peritoneal attachments of the liver. The cystic duct and artery were ligated. A side-to-side portacaval shunt was made, followed by ligation and transection of the portal vein close to the hilum to create a functional end-to-side shunt. Then, the internal iliac vein and the internal jugular vein were cannulated using Gott-shunts (Sherwood, Davis & Geck, Veghel, the Netherlands), which were connected with a three-way connector (Baxter, Uden, the Netherlands) by heparin coated Tygon tubing (Baxter, Uden, the Netherlands) in order to

have a venovenous bypass (VVB); the third exit of the three-way connector was used for the perfusion system to the ex vivo liver. The VVB was filled, partially with the pigs' blood, partially with NaCl 0.9% and freed of air. Hepatectomy was performed. The isolated liver was immediately perfused with ice-cold (0-4°C) UW-solution and put aside on ice for approximately 3,5 hours. The circulation in the pig was restored by a cavo-caval shunt using a GORE-TEX prothesis (Gore-tex, Flagstaff, Arizona, USA) of 12 mm diameter. A catheter was left in the portal vein in order to measure the portal blood pressure, after which the abdomen of the pig was closed.

Table I	**0 hr**	**2 hr**	**4 hr**	**6 hr**	**8 hr**	**10 hr**
HR (beats/min)	90	95	130	110	130	160
BP (mmHg)	110/80	120/90	110/60	110/80	100/60	110/70
CO (L/min)	4.1	6.2	4.3	3.5	4.6	5.4
Wedge (mmHg)	9	10	10	12	9	10
CVP (mmHg)	9	11	11	10	9	9
Portal P (mmHg)				27	25	24
Hb (mmol/L)	5.3	5.0	5.0	4.5	3.8	3.5
Ht	0.30	0.29	0.31	0.25	0.21	0.19
Thr ($*10^9$/L)	243	234	230	169	140	130

Table I. The haemodynamic changes and haematological results every two hours throughout the experiment: 0-3 hours: surgery; 3-6 hours: anhepatic phase; 6-10 hours ECL (means of 5 experiments).

Preparement of the perfusion circuit. The perfusion circuit consisted of heparin coated Tygon tubing coming from the left internal iliac vein, through a roller pump entering the extracorporeal liver via the portal vein. A separate tube went directly from the carotid artery to the hepatic artery. The infrahepatic vena cava inferior was closed and the suprahepatic vena cava was cannulated using a Sump-drain (Sherwood, Davis & Geck, Veghel, the Netherlands) entering the anhepatic pig through the left internal jugular vein. Three-and-a-half hours after hepatectomy the liver was put in a sterile bag and put in a hot water bath set at 39°C, keeping the liver at a constant temperature of 37.5°C. The hot water bath was put approximately 20 cm above the anhepatic pig. The anhepatic pig was 'treated' with the autologous ex vivo perfusion keeping a constant perfusion pressure of 12-15 cm H_2O and a constant flow-rate of 375 ml/min. A time period of 4 hours was chosen because stable function was obtained within this time frame.

Haemodynamics, ex vivo liver function and histology. Continuous monitoring of the haemodynamic parameters was performed and samples for biochemistry were taken every

half hour throughout the experiment and processed the conventional way. The function of the ex vivo liver was established in the following ways: systemic measurement of the clearance of bromosulphophtalleine (BSP)(Janssen, Geel, Belgium), which was administered (5mg/kg) four times, with the liver in situ, in the anhepatic phase and twice in the ECL-phase; systemic measurement of the clearance of NH_3 throughout the experiment; oxygen consumption of the liver during perfusion was calculated substracting the oxygen-saturation in samples taken from the perfusion system directly pre- and postliver; bileproduction was monitored during perfusion and bilirubin in the bile was determined. Core needle biopsies were taken for histological examination.

RESULTS

Haemodynamics and haematology. Table I shows the haemodynamic changes and haematological data every two hours as measured throughout the experiment. Pigs were haemodynamically stable throughout the surgical procedure and the anhepatic phase without the need of inotropica. While blood pressure (BP), cardiac output (CO) and pulmonary wedge remained unchanged during the extracorporeal liver perfusion, there was significant increase in heart rate (HR) from the fourth hour on. The portal pressure was 24 mm H_2O, indicating

Table II	**0 hr**	**2 hr**	**4 hr**	**6 hr**	**8 hr**	**10 hr**
Ureum (mmol/L)	4.2	4.0	3.6	3.1	2.2	2.7
Creat (µmol/L)	133	129	132	130	125	124
Bili (µmol/L)	2.4	3.3	5.7	4.6	5.0	4
AF (u/L)	92	70	70	87	81	87
ALAT (u/L)	39	30	27	23	18	20
ASAT (u/L)	26	48	86	74	103	230
γGt (u/L)	36	29	21	21	16	16
LDH (u/L)	720	590	578	525	440	660
Glucose (mmol/L)	9	10.7	6.7	7.8	8.4	7.1
pH	7.4	7.38	7.4	7.32	7.15	7.19
Lactate (mmol/L)	2.3	2.7	2.9	2.0	6.3	9.6

Table II. The biochemistry of the animal and the liver every two hours throughout the experiment: 0-3 hours: surgery; 3-6 hours: anhepatic phase; 6-10 hours ECL (means of 5 experiments).

the existence of portal hypertension. No blood was lost in surgery, or during the anhepatic phase. There was a 30% decrease in the level of hemoglobin (Hb) and a 36% decrease in haematocrit (Ht) during the perfusion period, whereas the amount of thrombocytes (Thr) decreased until half the initial value during the 4th hour of perfusion.

Biochemistry. The levels of ureum and creatinine did not change during the experiment accounting for a good renal function (Table II). Liver enzymes including AF, ALAT, γGt, and LDH remained unchanged throughout the experiment. . ASAT, however, increased gradually up to 10 times the initial level during the last two hours of perfusion. Systemic glucose and bilirubin concentration did not change significantly during the course of the experiment. Finally, during the ex vivo perfusion a decrease of 0.2 in the pH was observed, due to a metabolic acidosis; a 4-fold increase in lactate levels was observed.

Function of the ex vivo liver. As can be seen in figure 1, oxygen consumption, measured by substracting the pre- and postperfusion saturation of the portal blood, was stable throughout the experiment. During a period of more than four hours oxygen consumption was 45% ± 4.8. In figure 2 bile production is shown during the 4 hours of perfusion, showing 4 ml of bile in the first hour and in the next three hours a mean production of 12 ml; no significant difference in production is found between the 2nd, 3rd and 4th hour. The amount of bilirubin in bile was 2120 ± 466 μg/ml in the first three hours of perfusion and 3895 ± 748 μg/ml in the fourth hour accounting for functional liver metabolism. Clearance of BSP was measured 4 times (figure 3). The first time when the liver is in situ, with a normal $t_{1/2}$ of 3.7 min; the second time during the anhepatic phase, in which BSP is cleared by excretion in urine (figures not shown) and $t_{1/2}$ is 27.8 min; the third measurement was performed during the first hour of perfusion and the fourth during the last, demonstrating a $t_{1/2}$ for BSP of 19.4 min and 18.9 min respectively.

The NH_3-clearance is shown in figure 4. NH_3 was found to increase moderately in the first three hours of the experiment. From the beginning of the anhepatic phase on there is a significant increase of the concentration of NH_3, which is about 9 times as high as preoperative at the highest point. The NH_3 concentration returns to about 5 times the original level in 4 hours of ex vivo liver perfusion.

Histopathology of the liver. Macroscopically, optimal perfusion of the ex vivo liver was obtained with a constant flowrate of 375 ml/min and a perfusion pressure of 12-15 cm H_2O. The central parts of the liver remained unchanged throughout the experiment: normal light brown liver tissue. However, towards the end of the experiment, the peripheral parts of the

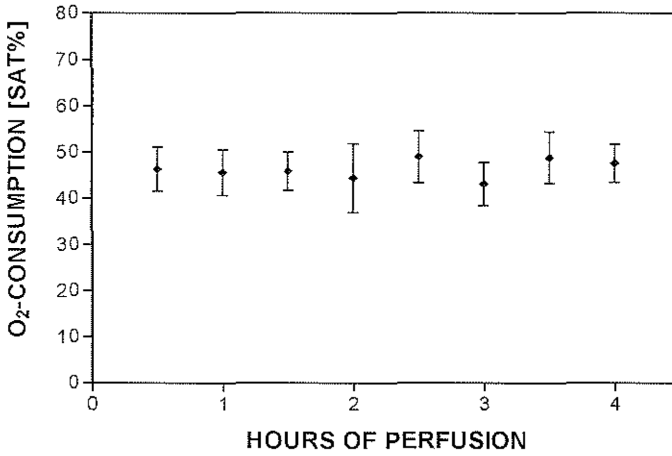

Figure 1. Oxygen consumption during the perfusion (means of 5 experiments with sem)

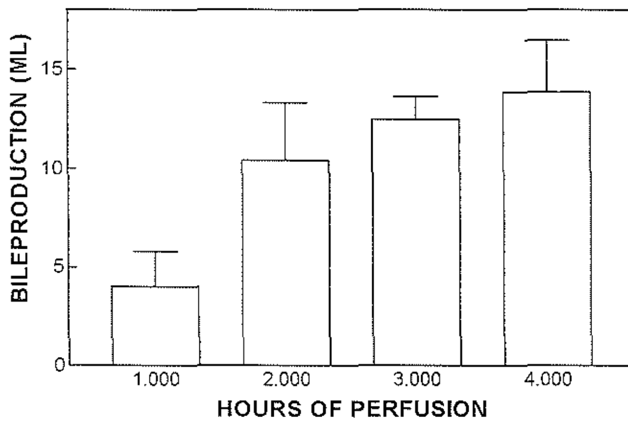

Figure 2. Bileproduction during the perfusion (means of 5 experiments with sem)

liver showed a color change from light brown into purple and a loss of softness. On histopathological examination, normal liver tissue was shown in the central part of the liver, although a slight neutrophil invasion and some steatosis could be noticed. (figure 5a). The peripheral part, on the other hand, clearly showed stasis of blood: enlarged sinusoids filled with erythrocytes (figure 5b), therewith confirming our findings during perfusion.

DISCUSSION

In the past three decades, a number of studies using extracorporeal liver perfusion for the treatment of patients with acute liver failure have been performed; the results were promising as to temporary enhancement of the patients' neurological condition, although no improvement in survival was obtained [1,2]. Most of these studies were done before large scale introduction of orthotopic liver transplantation. At present, liver transplantation is very successful with survival rates of 70-80%. However, because of the shortage of available donororgans in the United States, only 20% of the patients with liver failure receive a liver transplantation, whereas 80% dies while being on the waiting list [5]. Therefore, there is a clinical need for a temporary liver function replacement, which renewed the interest in the extracorporeal liver perfusion [4,7].

After initial experiments in animals, most experience has been obtained from clinical studies, in which very sick patients with hepatic failure are usually managed in the ICU. In order to be able to asses the haemodynamic and metabolic changes and the actual liver function during the use of an ex vivo liver perfusion system, we studied these parameters in an anhepatic pig model, in which no major correction of haemodynamic or metabolic parameters occured.

As has been shown, pigs remain haemodynamically stable during more than 5 hours after hepatectomy and survival of anhepatic pigs is at least 24 hours [8]. In the experiments performed in our laboratory, no haemodynamic instability was observed during surgery or in the anhepatic phase. However, more than three hours of extracorporeal liver perfusion resulted in gradually progressive haemodynamic and metabolic changes. Part of this haemodynamic instability may be due to dilution of blood as can be concluded from the decrease in hemoglobin and haematocrit, and part of it may be due to the changed physiology of the abdomen in our model inducing portal hypertension. Most experiments did end with a positive fluid balance, with a considerable amount of ascitic fluid in the abdomen as well as in

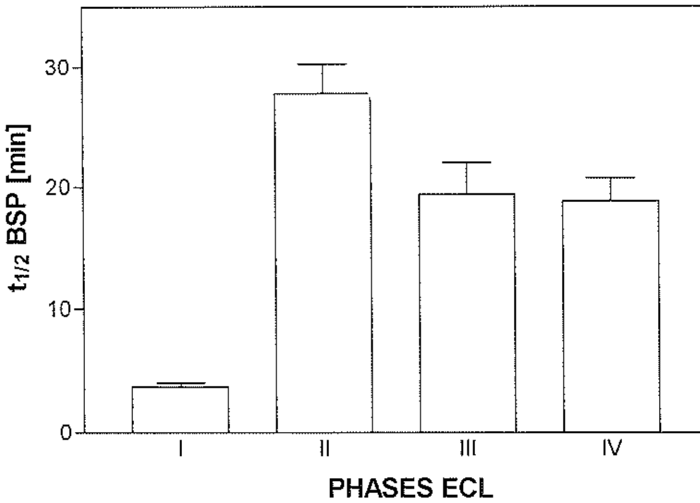

Figure 3. Clearance of BSP throughout the experiment (phase 1: liver in situ; Phase II: anhepatic pig; phase III: 1st hour of ECL; phase IV: last hour of ECL; means of 5 experiments with sem)

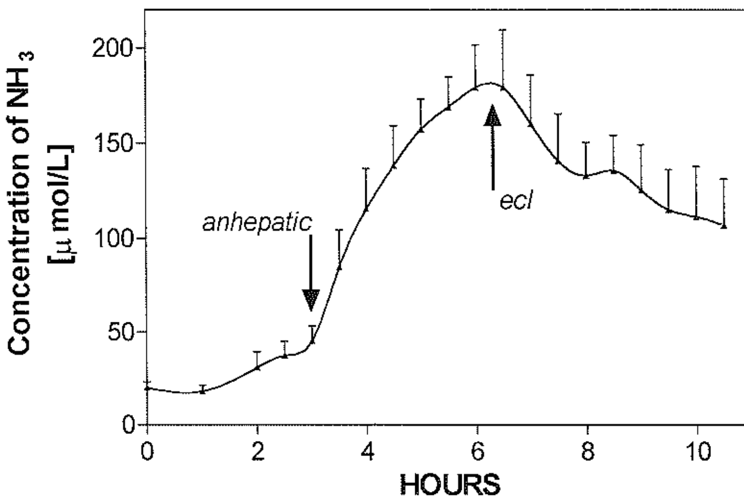

Figure 4. [NH3] throughout the experiment (means of 5 experiments with sem)

the liver perfusion bag. As has been demonstrated before, the ascitic fluid from the ex vivo liver should either be collected into a reservoir and returned to the patient after mixing with the patients blood [1], or discarded and replaced by reconstituted human plasma [9].

Although haemodynamic changes were noticed, good function of the liver was measured during the four hours of ex vivo perfusion as determined by oxygen consumption and clearance of BSP and NH_3. Bile production was 4 ml in the first hour and increased to a fairly constant production of 12 ml in the next three hours. This indicates, that the liver is immediately capable of metabolic processes requiring oxygen such as detoxification, but the conjugation of bilirubin and the production of bile takes more time, which is comparable with literature and our experience with bileproduction in orthotopic livers transplanted into humans. Although the level of NH_3 decreased most in the first hour, based on our experience at least several hours of perfusion are necessary for optimal treatment with ex vivo liver perfusion, despite the haemodynamic instability that occured in the last hour of perfusion.

On macroscopic and microscopic examination of the ex vivo liver, hypoperfusion of the peripheral parts of the liver were observed. Acidosis may have been the consequence of organic acid accumulation, e.g. as a result of hypoperfusion of the gut due to portal hypertension, or due to ex vivo hepatic hypoperfusion of the peripheral liver. At the end of the experiments, a 10-fold increase in ASAT was measured, the liver enzyme known to be the most sensitive indicator for liver cell damage. In our study we were not able to determine the cause of the metabolic acidosis, although improved ex vivo liver perfusion could diminish the metabolic problems encountered in ex vivo liver perfusion. Improved perfusion of the liver may be obtained when the liver is put on a diaphragm in a perfusion chamber ventilated with IPPV, thereby mimicking the physiological conditions of the liver [10].

Bertrand et al claimed from their study performing 15 short term perfusions on 10 patients the same outcome for perfusions less than one and a half hour as compared to more than two and a half hours [11]. Whether patients with acute liver failure would benefit most from long term perfusion with one liver or multiple short term liver perfusions remains to be determined. It should also be noticed that biochemical improvement as measured in our experiments, does not necessarily correlate with neurological improvement [1,9]. In a clinical situation hepatic encephalopathy with increased cranial pressure is one of the major causes of death. Moreover, bleeding disorders causing major complications in patients with acute liver failure might not be prevented or may even be enhanced in extracorporeal liver perfusion, because of the damage done to platelets in the extracorporeal perfusion system.

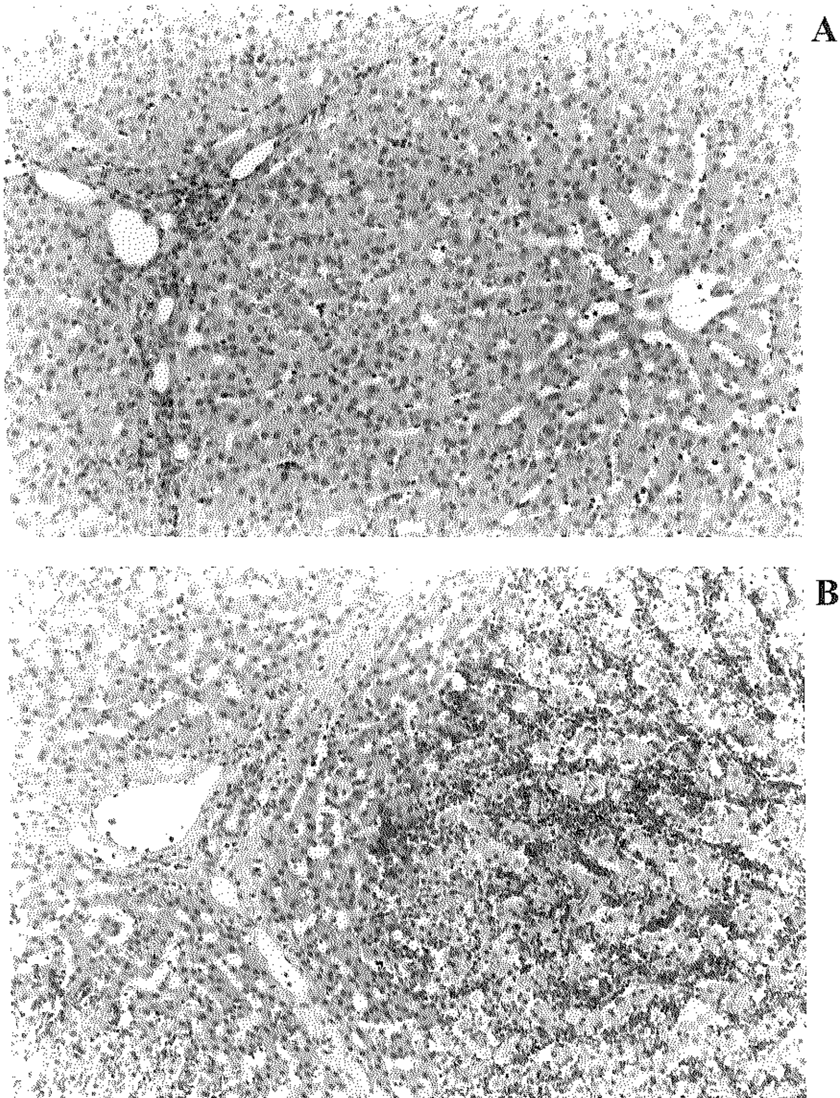

Figure 5a. HE-staining of a liver biopsy of the central part of the liver showing only slight neutrophil invasion in zone one and normal liver tissue towards the central vein (to the right). **Figure 5b.** HE-staining of a liver biopsy taken of the peripheral part of the liver evidently showing stasis of blood, enlarged sinusoids filled with erythrocytes towards the central vein.

Our experience indicates that extracorporeal liver perfusion is technically feasible and provides a rather simple and rational method of temporary hepatic support with an excellent function of the ex vivo liver. In our model we demonstrated the occurrence of metabolic acidosis, which should be taken into account when further development of this technique is considered, since this may limit the quality of extracorporeal liver perfusion. Future studies should adress the immunological implications of this method in a xenogeneic model, possibly with the use of transgenic animals, before it can be applied safely in patients with acute liver failure.

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Acute liver failure attenuates hyperacute xenograft rejection

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ABSTRACT

Pig livers, transgenic for complement regulatory proteins (CRP), are being used to study the feasibility of extracorporeal liver support in the management of acute liver failure. However, thus far, effective temporary ex vivo liver perfusion has been applied without immunological drawbacks. Complement is known to play a major role in hyperacute rejection in xenotransplantation and the level of complement is significantly decreased in patients with acute liver failure. We examined the possibility of prevention of the hyperacute rejection in a guinea pig heart to rat transplantation model.

Acute liver failure was induced in rats using Galactosamine (300-1000mg/kg). After 24 hours, a guinea pig heart was transplanted into the rat and survival of the transplanted heart was observed. C_3, CH_{50} and biochemistry of the liver were measured at 1, 6, 24 and 48 hours and deposition of complement in the transplanted heart was established. Animals treated with CVF (Cobra Venom Factor) and a group receiving no treatment were taken as controls.

All GALAC-treated animals showed severely disturbed liver function at the moment of transplantation. C_3 and CH_{50} levels at 24 hours were 50% decreased. Non-treated controls rejected in 14 to 36 min (median 22 min.). Hearts in CVF-treated animals were rejected after 1 day. There was no difference between animals that had received either 600 or 1000 mg/kg GALAC. Most animals of this group did not survive the transplantation procedure and died on the operating table with a functioning transplant (median survival time > 45 min.). Animals given 300 mg/kg GALAC sustained the operation well and rejected their transplants after 26 to 270 min. (median 57 min.). Immunohistochemical staining for C1q and C_9 was positive in all specimens but revealed no differences between hearts from nontreated controls and grafts from GALAC-treated animals.

These experiments show an increase in xenograft survival time in rats with acute liver failure, probably due to a significant decrease in complement levels and activity as can be measured in rats with CVF-induced complement depletion. Therefore, transgenesis for CRP may not be necessary for successful temporary xenogeneic perfusion in the treatment of acute liver failure.

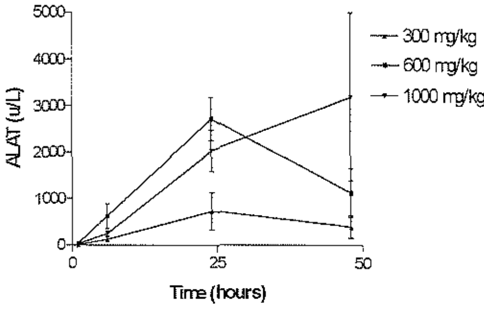
INTRODUCTION

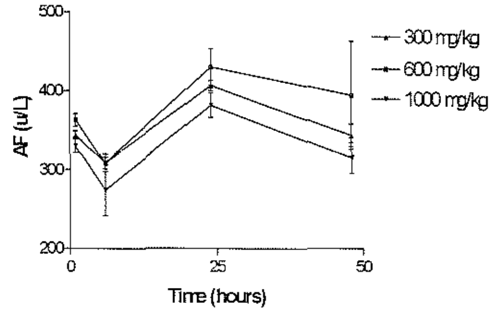
The lack of immediate availability of a donor liver implies that many patients with acute liver failure die before a liver transplantation can be performed. Extracorporeal pig liver perfusion

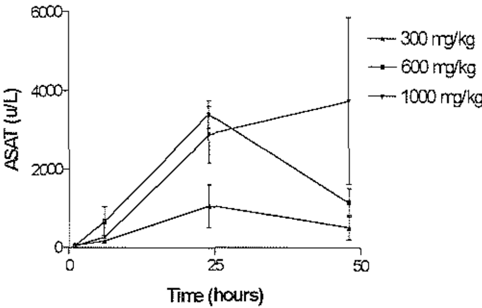
is one of the options for temporary liver support. The concept stems from the 1960s, but the improved success rate of liver transplantation for acute hepatic failure and progress in xenogeneic immunobiology has rekindled interest in this technique [1]. It is known that patients with acute liver failure become immunosuppressed, which is thought to be partly due to a defective synthesis and increased consumption of complement [2]. This potentially life-threatening complication may be beneficial if extracorporeal pig liver perfusion is considered; it may prevent the occurrence of hyperacute rejection (HAR). And indeed, in most clinical trials on extracorporeal liver perfusion utilizing normal pigs, classical HAR is not seen [3]. This could mean that the use of livers from pigs that are transgenic for human complement regulatory proteins is not essential to enable uncompromised short-term liver perfusion. The aim of the present study was to investigate whether acute liver failure might provide a window of opportunity for the acceptance of a discordant xenograft.

MATERIALS AND METHODS

The experiments were performed using male Wistar rats (Harlan CPB, Austerlitz, the Netherlands) weighing 200-250 g and female Dunkin Hartley guinea pigs (Harlan CPB, Austerlitz, the Netherlands) weighing 200-250 g. They were kept under controlled laboratory conditions and received food and tap water ad libitum. When treatment had started, the rats had free access to 10% glucose water. The experiments were approved by the Committee of Animal Research of the Erasmus University. D-Galactosamine (Sigma) was dissolved in phosphate buffered saline (PBS, Sigma P-4417), pH 7.4 at desired concentration. The animals were divided into three groups. In group 1, acute liver failure was induced by injecting 300 (n=6), 600 (n=3), or 1000mg/kg (n=8) galactosamine (GALAC) intraperitoneally. Blood samples were obtained by orbital punction at 1, 6, 24, and 48 hours after injection. Levels of CH_{50}, C_3, NH_3, ALAT, ASAT, AF, Bili, Urea and Creatinine were measured. After 24 hours a guinea pig heart was transplanted heterotopically as described elsewhere [4], and the duration of the heart beating was assessed by abdominal palpation. Group 2 (n=6) received no GALAC, but was treated 1 day before guinea pig heart transplantation with a single dose i.p. of 9000 units of cobra venom factor (CVF). Group 3 (n=5) did not receive any treatment before







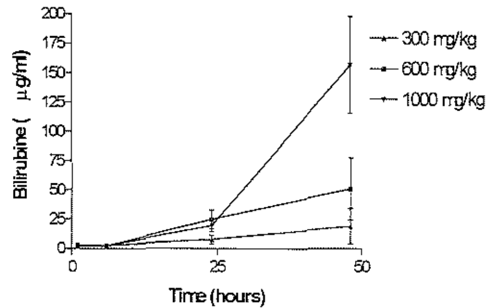


Figure 1. Concentration of ALAT and ASAT (mean ± sd)

Figure 2. Concentration of AF and bilirubin (mean ± sd)

xenotransplantation. Well-functioning hearts from the 300 and 600 mg/kg GALAC groups beating for longer than 30 min. and from the nontreated control group beating for about 10 min, were used for immunohistochemical analysis. Complement factors C1q and C9 were stained by rabbit anti-rat antibodies and secondary FITC-labelled swine anti-rabbit antibodies (Dako ITK Denmark) and analyzed by fluorescence microscopy.

RESULTS

In figure 1 the concentrations of the liver enzymes ALAT and ASAT are shown. There is a significant increase correlated to the concentration of GALAC used in all groups in the first 24 hours accounting for severe liver cell damage. At 48 hours, the levels of ASAT and ALAT decrease to one third of the level at 24 hours in the animals treated with 300 and 600 mg/kg GALAC, whereas in the animals treated with 1000 mg/kg GALAC the enzymes continue to increase to double the amount at 24 hours. Figure 2 shows the results for AF and bilirubin. In all groups there is a decreases of AF at 6 hours, an increase above the intitial value at 24 hours

and a decrease again at 48 hours. There is no significant difference amongst the groups or between the different time points in the concentration of AF. In the first 24 hours, bilirubin shows a steady increase to 7.5 ± 3.5 μg/mL in the GALAC-300 group, 24 ± 8 μg/mL in the GALAC-600 group and 19 ± 5 μg/mL in the GALAC-1000 group. At 48 hours, the level of bilirubin continues to rise for GALAC-300 to 19 ± 15 μg/mL, GALAC-600 to 51 ± 27 μg/mL and GALAC-1000 to 156 ± 41 μg/mL. The concentration of NH_3 is shown in figure 3. In all three groups, there is a steady increase to 380 ± 40 μg/mL at 48 hours. No difference amongst the groups is detected. Figure 4 shows the kidney-function in all groups represented by urea and creatinine concentration. There is no major decrease in kidney function during the experiment.

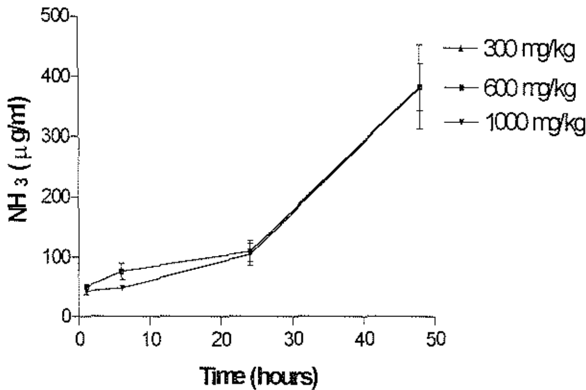
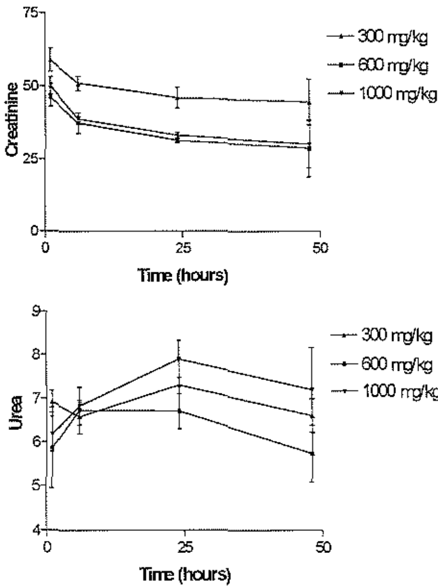


Figure 3. Concentration of NH_3 (mean ± sd)

The amount of total complement (CH_{50}) in the animals treated with 300 mg/kg GALAC and the amount of complement factor 3 (C_3) in all three groups is shown in figure 5. There is a significant decrease of CH_{50} in the GALAC-300 group at 24 hours showing again an increase at 48 hours. In the animals treated with 600-1000 mg/kg GALAC, the level of complement factor 3 decreases from 0.44 ± 0.07 g/L to 0.21 ± 0.01 g/L at 24 hours and then increases to 0.25 ± 0.05 g/L at 48 hours. In the animals treated with 300 mg/kg GALAC, the concentration of complement factor 3 decreases from 0.60 ± 0.03 g/L to 0.32 ± 0.07 g/L, and remains constant in the second 24 hours (0.30 ± 0.06 g/L at 48 hours).

Table 1 shows the actual survival times of the transplanted guinea pig hearts to rats with acute liver failure, treated with CVF or receiving no treatment at all. When no treatment was given



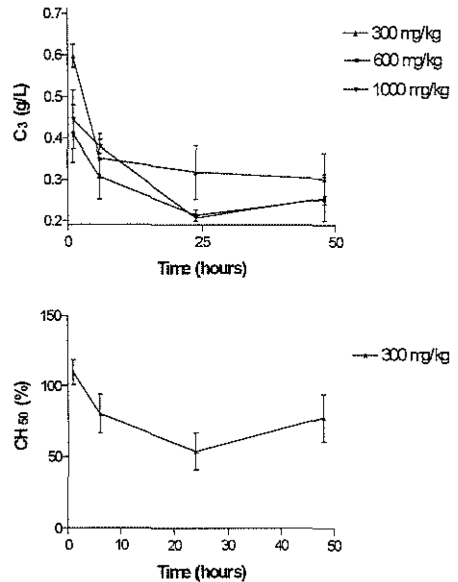


Figure 4. Concentration of Urea and Creatinine (mean ± sd).

Figure 5. Concentration of C_3 and CH_{50} (mean ± sd).

the survival times varied from 14 to 36 min (median 22 min.). With an injection of 9000 units of Cobra Venom Factor (CVF) 24 hours before transplantation, the survival of the graft was at least one day. There was no difference in survival of the heart graft between animals that had

Table 1. Survival of Guinea Pig hearts grafted in rats with acute liver failure

Treatment	Survival time (min.)
None	14 – 15 – 15 – 32 – 36
CVF	1 – 1 – 1 – 1 – 1 – 1
GALAC 600-1000 mg/kg	16* - 30 – 33 – 35* - 37 – 45*
	47* - 51* - 60* - 67* - 80*
GALAC 300 mg/kg	26 – 30 – 52 – 63 – 120 – 270

Liver failure was induced by a single injection of galactosamine (GALAC) 24 hours before heart transplantation. CVF: 9000 units on day -1.
*Animals died with functioning graft. Groups 3 and 4 vs 1: $p < 0.05$.

received either 600 or 1000 mg/kg GALAC, therefore they are presented as one group. Most animals died with a functioning transplant on the operating table due to acute liver failure with subsequent anaesthesia and surgical procedure. Mostly coagulation problems were encountered. The median survival time of the graft, however, was 45 min. (range 16-80 min.),

which was significantly longer than in the non-treated group. The animals treated with 300 mg/kg GALAC did survive the procedure and significant longer survival times were found (median survival time 57 min, range 26 to 270 min.).

The deposition of C1q and C9 on liver tissue sampled from well-functioning grafts for longer than 30 min. from the 300 and 600 mg GALAC groups and from the non-treated control group beating for 10 min. was established. The results for complement factor 9 are shown in figure 6. Although on first sight there seems to be more neutrophil invasion in the control group as compared to the GALAC-treated group, no difference in C9-deposition can be established between the two groups.

DISCUSSION

Patients with hepatic disease have an impaired antibacterial host defense possibly due to impaired hepatic synthetic function [2,5]. Knowing that the liver is the major site of production for the complement proteins C_3, C_6, C_8, and factor 12 [6], a severely compromised complement activity can be seen in these patients, which appears to be multifactorial in origin and involves all aspects of the complement system including the classical activation pathway, the alternative activation pathway, the terminal cascade, and critical regulatory proteins [7]. It is unclear, however, whether the complement deficiency is the result of the impaired synthesis of components alone, an ongoing activation of the complement system, or a combination of the two. Because of this deficient complement activity, systemic bacterial infection is one of the major complications in patients with chronic hepatic disease.

On the other hand, patients treated for acute liver failure could possibly benefit from this compromised condition. It is known that the complement system plays a major role in hyperacute rejection, e.g. complete inactivation of complement can fully prevent hyperacute rejection [8]. Also in our study, we demonstrated the absence of hyperacute rejection when the animals were treated with Cobra Venom Factor (CVF), which resulted in a one-day survival of the xenogeneic heart transplant, whereas in the untreated group hyperacute rejection was shown. Therefore, the compromised activity of the complement system may possibly explain the fact that thus far, classical HAR is not seen in most clinical trials on the treatment of patients suffering from acute liver failure with extracorporeal liver perfusion utilizing normal pigs[3].

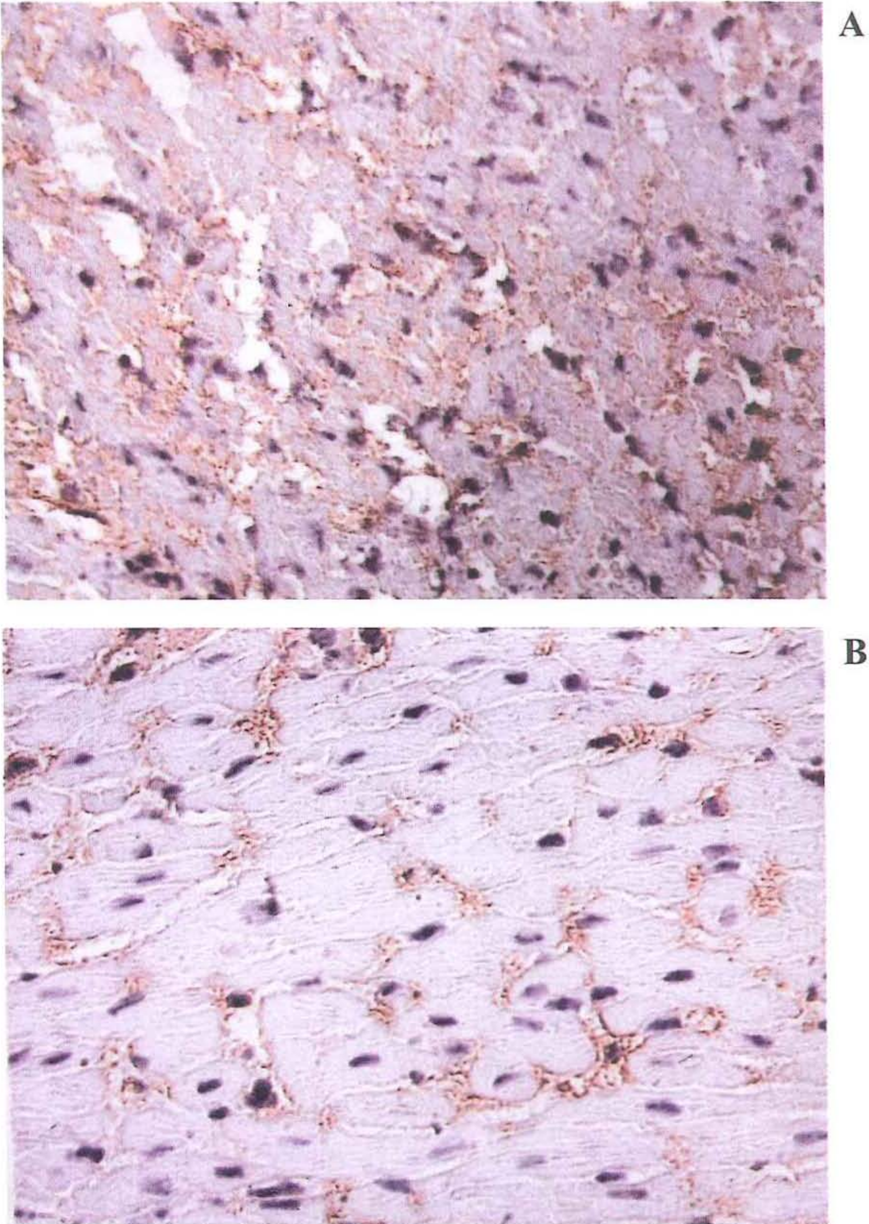


Figure 6. Immunohistochemical staining with rabbit anti-rat C_9 antibodies of well-functioning hearts from the non-treated control group beating for 10 min (fig. 6a) and the GALAC-600 group beating for 30 min (fig. 6b). The hearts from the control group show evidently more tissue damage, but no significant difference in the intensity of staining could be established between the two groups.

And indeed, in our animal model, all GALAC-treated animals showed severely disturbed liver function at the moment of transplantation and C_3 and CH_{50} levels were 50% decreased, which led to an increased survival of the xenograft. To some extent this may be due to observed reduced circulating levels of complement, although this was not substantiated by a reduced deposition of complement in nonrejecting grafts. On the other hand, it is likely that other factors, such as a diminished coagulability as observed during surgery and impaired antibody production also contributed to the attenuation of hyperacute rejection.

Our results suggest that the severity of liver failure and the subsequent amount of circulating complement is correlated to the extend of xenograft survival. In most clinical studies only patients with severe acute liver failure were treated with extracorporeal perfusion [9], possibly providing the least harmful xenogeneic circumstances, for which reason no hyperacute rejection was seen in these patients. Furthermore, although the increase in survival time induced by liver failure was only small, it can be argued that it might have been larger if a xenogeneic liver had been transplanted. It has been demonstrated frequently that the liver is less susceptible to the harmful effects of antibody and complement than other organs [10].

In conclusion, these results indicate that acute liver failure induced by galactosamine facilitates the acceptance of a discordant xenograft. The decreased susceptibility of the liver added to our current observation that recipients with acute liver failure are less prone to hyperacute rejection, suggests that short-term extracorporeal perfusion using non-transgenic xenogeneic pig livers is a relatively safe procedure.

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IgG, but not IgM, mediates hyperacute rejection in hepatic xenografting.

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Xenotransplantation 1999; 6: 110-116.

ABSTRACT

We reported previously that no classical features of hyperacute rejection (HAR) could be found in liver grafts in the guinea-pig (GP)-to-rat model and that recipients died shortly after transplantation, because of non-immunological causes. Thus, the GP-to-rat model is not suitable for studying the mechanisms of discordant liver xenograft rejection. In the hamster to rat model, long-term survival of a liver graft is possible, but extremely low levels of xenoreactive natural antibodies are present. To mimic a discordant situation with preformed IgM and IgG antibodies, we sensitised rats one or five weeks before grafting. Specific anti-hamster IgM antibodies were found in recipients sensitised at week -1, but not week -5. Anti-hamster IgG was present in all recipients, albeit considerably higher in animals sensitised five weeks before grafting. In these two models, we examined the mechanism of HAR of liver grafts and compared this with heart xenografts. Control heart and liver grafts rejected in 4 and 7 days after transplantation respectively. Liver grafts in recipients sensitised at week -5 showed venous congestion and bleeding after reperfusion, indicating HAR, however this was not observed following sensitisation at week -1. This surprising finding was confirmed by histology. Massive extravasation, edema, and acute liver cell degradation were noticed in grafts subjected to HAR. Liver grafts of recipients sensitised at week -1 showed only minimal changes. Heart grafts were rejected hyperacutely in both sensitisation models. IgG antibodies could be detected on liver grafts in the group sensitised at week -5 but not in the group sensitised at week -1. Minimal IgM depositions were found on liver grafts of animals sensitised one week before grafting. Rejected heart grafts of similar sensitisation groups showed identical antibody depositions; only IgM depositions were massive. Complement depositions were found in all groups. These results indicate that IgG, but not IgM, mediates HAR in hepatic xenografting. Such a predominance of IgG over IgM does not exist for heart grafts.

INTRODUCTION

Transplantation of transgenic organs or tissue between widely disparate species as the ultimate solution for donor shortage shows promising results [1]. However, many aspects of rejection are

still unknown, as evidenced by the muddle of immunosuppressive drugs that are needed to keep pig organs functional. Using small animals, the guinea-pig (GP)-to-rat rodent model provides an easy and fast way to study the basics of discordant transplantation. The recipient produces preformed xenoreactive natural antibodies (XNAs) against the GP, which are capable of rejecting heart grafts in a hyperacute manner [2]. XNAs are mainly IgM type [3], but some donor-specific IgG antibodies have been detected [4].

For allogeneic grafting, the relative insensitivity of liver grafts to antibody-mediated rejection has been described [5,6]. In a previous publication, we investigated whether this phenomenon also occurred in the GP-to-rat liver transplantation model [7]. We demonstrated that a 'rejected' GP liver was not characterised by the classical features of hyperacute rejection (HAR) [7]. However, recipients died within a few days, apparently because of non-immunological reasons. Therefore, no firm conclusions could be drawn regarding susceptibility of discordant liver graft toward antibody-mediated rejection [7]. Numerous studies have reported long-term survival of hamster grafts, indicating no interference of non-immunological problems in this model [8-10]. However, the rat has very low titres of preformed antibodies to hamster. To mimic a discordant situation with preformed IgM or IgG antibodies, we sensitised recipients with donor blood at one or five weeks before transplantation.

Hence, the aim of the current study was to analyse the mechanism of HAR of liver xenografts in the presence of preformed antibodies and to compare this with the rejection of heart xenografts.

MATERIALS AND METHODS

Animals. Female Syrian hamsters were used as donors and male Brown Norway rats as recipients. All animals were obtained from Harlan C.P.B. (Austerlitz, The Netherlands). They were kept under controlled laboratory conditions and received food and tap water ad libitum. Hamsters weighing over 120 grams and rats weighing between 250 and 300 grams were used. The experimental protocols adhered to the rules laid down in the "Dutch Animal Experimentation Act" (1977) and the published "Guidelines on the Protection of Experimental Animals" by the Council of the EC (1986). The specific protocol was approved by the "Committee on Animal Research" of the Erasmus University Rotterdam, The Netherlands.

Liver transplantation. Orthotopic liver transplantation (OLT) was performed according to a previously described method with some donor-related modifications [7,11]. The modifications were (1) donor pre-treatment with 0.1 mg/kg atropine (Centrafarm Services, Etten-Leur, The Netherlands), and (2) the outside diameters of the Teflon cuffs were 2.1 mm and 1.79 mm for the IVC and PV, respectively. Recipient death was taken as the endpoint of rejection.

Heart transplantation. Heterotopic abdominal heart transplantation (HTx) was performed as described by Ono and Lindsey [12]. Cessation of heartbeat as evidenced by abdominal palpation was taken as the endpoint of rejection.

Sensitisation. Hamster blood was obtained by orbital punction. One milliliter of heparinised blood was injected i.v. into the penile vein of the recipient one or five weeks before organ grafting.

Haemagglutination assay. Total antibody and IgG levels were measured using a haemagglutination assay. Plasma samples, taken immediately before grafting, were serially diluted. A suspension of freshly prepared hamster erythrocytes (4%) was added in equal amounts. After 1 h of incubation at 37°C, the wells were screened for agglutination. The dilution at which agglutination still occurred was considered the haemagglutination titre. For IgG level measurements, plasma samples were treated with 0.5 mM dithiotreitol for half an hour at 37°C to deplete IgM. Normal BN-serum was taken as negative control. Estimations of IgM titres were calculated by subtracting IgG titres from total antibody titres.

Histology and immunohistochemistry. After rejection of the grafts, necropsy was performed. The heart graft or upper liver lobe was removed and processed for histology and immunohistochemistry. Paraffin sections were stained with haematoxylin-eosine and examined with conventional light microscopy. The slides were examined for extravasation, edema, vessel damage, and infiltration. Changes were noted as compared to naive grafts and scored from – (no changes) to +++ (severe changes).

For antibody, complement depositions and natural killer (NK) cell infiltration, immunohistochemistry on frozen sections was performed. Macrophages were identified on paraffin sections. FITC-labelled mouse anti-rat antibodies to demonstrate IgM, IgG1, IgG2a and IgG2b were used (1:10; PharMingen, San Diego CA, USA). IgG2c and complement C3 were demonstrated by FITC-conjugated rabbit anti-sheep (1:100; DAKO A/S, Glostrup, Denmark) to sheep anti-rat antibodies (1:500; ANAWA Trading NA, Wangen Zürich, Switzerland). Complement factors C1q and C9 were stained by rabbit anti-rat IgG (1:25, 1:600, respectively, kindly provided by Dr. B.P. Morgan) and secondary FITC-labelled swine

anti-rabbit (DAKO A/S). Rat spleen was taken as positive control, whereas liver and heart from naive hamsters were used as negative controls. The slides were analysed by fluorescence microscopy. Location of the depositions was noted and the fluorescence intensity was scored from – (no depositions) to +++ (massive depositions). A 3-step indirect Ni-DAB immunoperoxidase staining was performed on paraffin slides to demonstrate macrophage infiltration. Mouse anti-rat macrophage was used as primary antibody (ED1, 1:800; Serotec Ltd., Oxford, UK). Staining was performed with Ni-DAB substrate after the application of rabbit anti-mouse PO and swine anti-rabbit PO antibodies (1:250; DAKO A/S). Applying the same protocol, NK cells were demonstrated on frozen sections using mouse anti-rat NK cells (NKR-P1, 1:400; Endogen, Woburn MA, USA). Conventional light microscopy was used to analyse the infiltration.

Experimental design. Heart and liver transplantations were carried out in different recipients. Sensitisation with 1 ml of hamster blood was performed 1 week before transplantation (1-week sensitized) in groups 2 and 5, and 5 weeks before transplantation (5-week sensitized), for groups 3 and 6. Groups 2 and 3 received heart grafts (n=5 for both); groups 5 and 6 obtained liver grafts (n=7 and n=5, respectively). Non-sensitized control groups were included for both heart (group 1, n=7) and liver transplantations (group 4, n=8). On day 0 the recipients obtained a heterotopic heart graft or orthotopic liver graft. Survival, as determined by abdominal palpation or death, was scored in minutes, hours or days, depending on the treatment. At necropsy the heart graft or upper liver lobe was removed and processed for histology and immunohistochemistry. Heart and liver grafts were semiquantitatively scored for type and quantity of antibody and histological changes.

Statistics. Statistical evaluation of the survival data was carried out for both heart and liver grafts. In cases of differences in variances as tested by Levene's test, mathematical transformation of the survival data was carried out. This was done to reduce the influence of outlying values. "One-way" analysis of variance (ANOVA) was performed on these data. If the ANOVA was significant on a 5% level, the Duncan's multiple comparison test or Games-Howell test was carried out for possible differences among the means. The tests were corrected for the fact that the comparisons were not statistically independent and for unequal group sizes.

Probability values lower than 0.05 were considered statistically significant. Survival of transplants with clear evidence of no rejection (liver enzymes, histology) was discarded from

statistical evaluation. All computing was done using the statistical software package SPSS for Windows, release 7.5.2.

RESULTS

Histology. Specimens of the transplanted organs were obtained for histological examination after killing the animals at the moment of rejection or death. Heart grafts rejected by non-sensitised rats showed vessel destruction, extravasation, edema and polymorphonuclear cell infiltrate, suggesting an antibody-mediated rejection. Also presence of mononuclear cells was noted. Groups 2 and 3 showed more severe extravasation of erythrocytes, fibrosis and edema. The overall architecture, including most vessels, was intact. In some cases, polymorphonuclear granulocytes were found.

Control liver grafts demonstrated polymorphonuclear infiltrate, destroyed vascular morphology with destroyed endothelial cell layers, edema, and a more predominant mononuclear cell infiltrate than heart grafts, consisting of lymphocytes, lymphoblasts, monocytes and macrophages. This indicates a mixture of cellular as well as antibody-mediated rejection. The infiltrate was situated around the portal areas.

Table 1. Histological changes in heart and liver grafts, rejected by sensitised recipients.

Group	Treatment	Extravasation	Oedema	Vessel damage	Infiltration
1	HTx, non-sens	+	++	++	+++
2	HTx, 1-wk sens	++	++	+	±
3	HTx, 5-wk sens	++	++	+	±
4	OLT, non-sens	+	++	++	+++
5	OLT, 1-wk sens	+	+	+	±
6	OLT, 5 wk-sens	+++	+	+++	+

Scores varied from - (no changes) to +++ (severe changes) compared to naive heart/liver.

Liver grafts of 1-week sensitized recipients showed extravasation, edema, signs of fibrosis, and vascular congestion, but little vessel damage. Focally, mononuclear granulocytes were found. Specimens of liver grafts in group 6 revealed extensive tissue damage, extravasation and acute liver cell damage. Semiquantitative histology scores are listed in Table 1.

Immunohistochemistry. Frozen sections of heart and liver grafts were stained for IgM, IgG subtypes, complement C1q, C3 and C9, and NK-cell infiltrates. Paraffin sections were used to stain for macrophages. All control stainings were positive on untreated rat spleen, whereas untreated hamster liver and heart sections were negative.

Control hearts showed major IgM depositions. Some IgG2a type antibodies and macrophages were noticed. Liver grafts from untreated rat recipients showed minor antibody depositions on cellular infiltrates or portal field, mainly being IgM. Macrophages, but not NK cells were deposited throughout the tissue.

Heart grafts from 1-week sensitized recipients demonstrated massive antibody depositions, whereas liver grafts demonstrated minor depositions. In group 3 and 6, moderate IgG1 depositions could be demonstrated. Additionally, depositions of IgG2a and IgG2b were detected on myocytes. For liver grafts depositions of IgG2b were found. Semi-quantitative antibody deposition scores are listed in Table 2.

Table 2. Antibody depositions in rejected heart and liver grafts by sensitised recipients.

Groups	Treatment	IgM	IgG1	IgG2a	IgG2b	IgG2c
1	HTx, non-sens	+++	-	+	-	-
2	HTx, 1-wk sens	+++	-	-	-	-
3	HTx, 5-wk sens	-	++	+	+	-
4	OLT, non-sens	+	±	±	±	±
5	OLT, 1-wk sens	±	-	-	-	-
6	OLT, 5-wk sens	-	++	±	+	-

Scores varied from - (no depositions) to +++ (massive depositions).

Liver grafts from untreated rat recipients showed some C1q depositions, but no C3 and C9, whereas all measured complement components could be detected on heart grafts. Sensitising rats one week before transplantation resulted in moderate C3 depositions in both heart and liver grafts. In addition, positive Kupffer-cells were found. C1q could only be detected in heart grafts, whereas little C9 was found in liver grafts. In 5-week sensitized recipients, massive C3 and moderate C9 depositions were demonstrated for heart grafts. Moderate C3 depositions and to a lesser extend C1q could be shown for liver grafts in group 6.

Graft survival. Heart and liver grafts showed a homogeneous reperfusion after releasing the clamps. After a few minutes, the heart grafts turned dark red. Liver grafts in group 6, but not

group 5, were purple and showed swelling 2 minutes after reperfusion. Most of the recipients of liver grafts died showing the clinical signs of shock.

Graft survival times are shown in Table 3. A highly significant difference was demonstrated between the group means in the heart transplanted groups ($p=0.000$, $s_{res}=35.730$) and between the group means in the liver transplanted groups ($p=0.000$, $s_{res}=0.567$). Non-sensitised heart graft recipients showed prolonged survival compared to both other heart graft recipients ($p<0.001$, both). No difference was found between 1-week sensitized and 5-week sensitized animals. The survival times of the non-sensitized group receiving liver grafts were significantly longer compared to groups 5 and 6 ($p=0.000$, both). Survival times of groups 5 and 6 were not significantly different, but a trend was noticed ($p=0.081$).

Table 3. Survival times following 'pseudo-discordant' heart and liver transplantation.[a]

Group	Surgery	Treatment	Survival times		Median survival
1	HTx	non-sens	3, 4, 4, 4, 4, 4, 4	days	4 days
2	HTx	1-wk sens	5, 6, 7, 120, 165	minutes	7 min.
3	HTx	5-wk sens	3, 3, 3, 9, 10	minutes	3 min.
4	OLT	non-sens	(3), (4), 6, 6, 7, 7, 8, 8	days	7 days
5	OLT	1-wk sens	1½, 1½, 2, 2½, 3, 21, 25	hours	2½ hours
6	OLT	5-wk sens	1, 1, 1¼, 1¼, 1½	hours	1¼ hours

[a]Hamster heart or liver was grafted in BN rat after sensitisation to hamster-blood at 1 week (group 2 and 5) or 5 weeks (group 3 and 6) before transplantation. In control groups 1 and 4, hamster heart or liver was grafted into naive BN rat. Both control groups were statistically different from their sensitisation groups. Survival times of animals that died with a functioning graft are put between parenthesis.

IgM and IgG agglutination titres. Haemagglutination tests revealed the presence of specific anti-hamster IgM antibodies in 1-week sensitized animals on day 0. The IgM titre was negligible in recipients sensitised 5 weeks before grafting. Anti-hamster IgG was present in all recipients, albeit considerably higher in recipients sensitised at week -5. Normal BN-serum revealed agglutinating titres of one-quarter or lower. No statistically significant correlation was found between pre-operative antibody titre and survival or histological changes in either sensitisation group.

DISCUSSION

The immuno-privileged position of liver grafts to antibody-mediated rejection has been a research subject for many years. Preformed antibodies in the circulation of patients before transplantation have been identified as the cause of HAR of allografts [13]. However, hyperacute antibody-mediated rejection is not necessarily observed [5,14]. Liver allografts seem to be less susceptible to antibody-mediated rejection. The phenomenon of reduced susceptibility has been demonstrated in previous studies concerning experimental liver allotransplantation in (sensitized) recipients [6,15]. In discordant liver grafting, we and others were unable to resolve this question, because the recipients died from non-immunological problems [7,16]. For that reason, we performed the current experiments. Similarly, as described by others, we found that non-sensitized controls rejected hearts significantly earlier than liver grafts [10,17]. Histologically, control heart grafts showed a prominent vascular rejection process, whereas rejected liver grafts (group 4) revealed a more predominant cellular rejection with mononuclear cell infiltrates. Moreover, antibody depositions of the IgM type were more pronounced on heart grafts than liver grafts. These findings indicate that in the untreated concordant hamster to rat combination the liver might be less affected by IgM.

It can be argued that liver grafts are less immunogenic compared to heart grafts, resulting in a diminished and sluggish antibody response resulting in less damage. However, this is not very plausible, because anti-donor antibody levels peak around day 5-7 for both liver and heart transplantation [18-20]. Moreover, Murase et al [21] reported that cytotoxic antibody titres after xenogeneic liver grafting are 10 times higher than after heart grafting. Infusion of hamster hepatocytes or non-parenchymal liver cells was able to induce high cytotoxic antibody titre within 1 week and splenic response to liver grafts is even higher then to cardiac grafts [22,23]. This indicates that liver grafts may be at least as immunogenic as heart grafts.

Another plausible explanation is that the liver graft protects itself, because the source of complement and the target organ are the same, leading to prolonged survival [24]. This is in accordance with the time required to transform the proteins to donor type profile [7,25]. Nevertheless, residual recipient complement components and neo-synthesis, by macrophages, monocytes, and fibroblasts, are still able to cause lysis [24].

In normal BN-serum, haemagglutinating antibodies were almost non-detectable. In the discordant situation, grafted organs encounter high titres of preformed antibodies.

Immunogenicity is, therefore, of less relevance than the affinity of antibodies for the different tissues. To mimic the preformed antibody situation, we sensitized rat recipients with hamster antigen in order to evoke an antibody response. This sensitisation, 1 or 5 weeks before transplantation, resulted in graft survival of minutes to hours for both liver and heart grafts. The differences found between liver and heart graft survival in the sensitised situation may be explained by the difference in survival readout for the grafts. It is known that rats can live for several hours after total hepatectomy with just a portal caval shunt [7].

At necropsy, massive antibody depositions could be detected in heart grafts from 1-week sensitized recipients. Extravasation of erythrocytes, edema, fibrosis and vascular congestion, suggesting complement-mediated endothelial damage, indicate an ongoing HAR. Liver grafts from recipients sensitised 1 week before transplantation, however, showed an overall intact morphology and only marginal signs of HAR. This corresponds to their phenotypic appearance after grafting. Yet, classical signs of HAR were noted in liver grafts from 5-week sensitized animals: acute liver cell degradation, extensive tissue damage, and extravasation of erythrocytes. Purple recolouration and swelling were noticed after reperfusion. Immunohistochemistry showed IgG depositions, whereas no graft depositions could be found in rats sensitised at week -1.

Circulating IgM, present one week after sensitization, is likely to deposit on the heart as well as liver grafts. However, the results indicate that liver grafts, in contrast to heart grafts, seem to be less affected by IgM as seen by immunohistochemisty and complement depositions. One possibility is that the liver is releasing blocking agents, preventing antibody deposition, which has been hypothesised by Kamada et al [26]. Another explanation is that graft size difference gives rise to difference in deposition density and therefore rejection. However, a more likely possibility is that Kupffer-cells absorb large amounts of lymphocytotoxic antibodies [27,28]. Moreover, Crafa et al [29] reported that Kupffer cell activation led to significant reduced circulation of anti-donor antibodies and a more intense IgM uptake after discordant liver grafting as demonstrated by immunohistochemistry. In the present study, however, liver grafts showed minor IgM depositions. Other authors have reported similar findings. Tuso et al [30] demonstrated that xenogeneic extracorporeal liver perfusion resulted in minimal immunohistochemical evidence of binding of human xenoantibodies. Nevertheless, reduced antibody binding to other organs was found after xenogeneic liver perfusion [30]. Even prolonged heart xenograft survival has been reported after preceding xenogeneic liver transplantation [31].

This indicates that liver grafts, unlike heart grafts, are less susceptible to antibody-mediated damage, probably because of absorption or non-binding of antibodies, possibly IgM.

Five weeks after sensitisation, mainly IgG type antibodies prevail. Histology of rejected livers showed a severe HAR with IgG and complement deposition, which were not detected in the grafts after recipient sensitisation at week -1. It seems, therefore, that IgG, specifically some IgG subtypes, may be responsible for the rejection process in liver grafts. In addition, several authors found that in experimental allogeneic liver transplantation after recipient sensitisation graft failure was dependent on the antibody class being IgG [32,33]. Moreover, IgG, in contrast to IgM, seems to be the most dangerous in clinical allotransplantation across positive crossmatches [34,35]. The minor damage seen in liver grafts of 1-week sensitized recipients might be caused by the already formed, relative low titres of IgG.

This could also explain why in discordant grafting no signs of HAR were seen, because most XNAs are of the IgM type [3,4,7,36].

In conclusion, this study suggests a more dominant role for IgG over IgM antibodies in the rejection of liver xenografts. Such predominance does not exist for heart grafts.

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7

No functional benefit for hDAF-transgenic rat livers despite protection from tissue damage following perfusion with human serum.

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ABSTRACT

Introduction. Currently, xenogeneic extracorporeal liver perfusion is used in the treatment of acute liver failure. In order to determine whether transgeneity for human regulatory proteins could improve the functional outcome of the ex vivo liver in relation to the histopathological changes, we studied the effect of the humoral mechanism in xenogeneic isolated rat liver perfusion in normal and transgenic rat livers.

Materials and Methods. Isolated rat liver perfusion was performed for 2 hours in normal rat livers with Krebs Henseleit (KH) and human serum (HS), and in livers transgenic for human DAF (Tg HS). Function of the liver was established through measurement of liver enzymes, bile production and clearance of bromosulphophtalleine (BSP). Tissue specimens taken after perfusion were analyzed using routine histology and immunofluorescent staining for C3c-deposition.

Results. No change in release of liver enzymes could be established throughout the perfusion period. In the second hour, a higher level of bile production was seen for the transgenic group, compared to the HS group. The transgenic rat livers outperformed the normal livers perfused with human serum, when BSP concentration in the bile was measured, however, clearance of BSP from the perfusate was not significantly different. HE-staining of the liver tissue showed evidence of hyperacute rejection in the HS group. There was only mild tissue injury in the transgenic liver. High intensity fluorescent staining for C3c-deposition was seen in the normal livers perfused with HS and significantly less in the transgenic livers.

Conclusion. Although histologically less tissue damage and less C3c-deposition was shown, no significant improved function of the livers transgenic for human DAF was established. These results suggest that for short term extracorporeal liver perfusion transgenesis offers no functional benefit.

INTRODUCTION

Less than 20% of the patients with fulminant hepatic failure survive when treated with conservative medical measures [1]. Liver transplantation, at present the only therapeutical option with a survival rate of 80-90%, is limited because of the amount of –on time- available

donor organs. Therefore, a system capable of temporary supporting patients with liver failure is needed to bridge either to liver regeneration or to liver transplantation.

In the early 1970s, as well as in recent years, ex vivo whole liver perfusion was clinically applied showing temporary improvement of the patient's clinical condition [2-4]. In these xenogeneic perfusions, evidence of tissue damage to the ex vivo liver was established, however, the pathogenesis of injury was not consistent with hyperacute rejection [5]. It seems that the liver is less susceptible to humoral injury, however, the effect of the humoral mechanism on liver perfusion remains to be determined.

Because of the critical role of complement in hyperacute rejection, the development of transgenic animals expressing species-specific complement inhibitors could provide a strategy for overcoming damage by the humoral mechanism. For instance, the human complement inhibitor human decay accelerating factor (hDAF) prevents the assembly of C3 and C5 convertases, and was shown to be variably expressed in different cell types of transfected pigs [6]. And indeed, hearts [7,8] and kidneys [9,10] transgenic for hDAF have successfully been tested without hyperacute rejection in xenoperfusion and xenotransplantation. In isolated liver perfusion, Adham et al showed a significant decrease of complement levels without significant alteration in liver function [11] and only mild histological evidence of hyperacute rejection [12] during xenogeneic perfusion of normal pig livers with human whole blood. However, in another study, Pascher et al [13] showed a marked complement activation and morphological damage in normal pig livers perfused with human whole blood, confirmed by an increase of the concentration of liver enzymes, as compared to livers transgenic for hDAF. Although reduction in activation of human complement and hepatocellular damage in organs transgenic for hDAF in this study were shown, no evidence of changes in liver function was reported. Also, it is not clear whether these effects may be solely due to humoral mediators or to other components present in human whole blood.

Therefore, in our study, isolated rat livers were perfused with human serum using organs from normal animals and animals transgenic for hDAF to determine whether this transgeneity could improve the functional outcome of the ex vivo liver related to the histopathological changes.

MATERIALS AND METHODS

Animals.

Experiments were performed using male Wistar rats weighing 350-450 g and Sprague Dawley rats transgenic for human DAF weighing 400-500 g. hDAF transgenic rats were generated as described by Charreau et al [14]. Briefly, a construct containing the hDAF cDNA was used under the transcriptional control of the endothelial cell-specific human ICAM-2 promotor. DAF expression was evaluated by immunohistology in several organs including heart, lung, kidney, liver and lymphoid organs, and by FACS-analysis of purified endothelial cells of heart and lung. Consistent levels of DAF were detected and showed uniform expression on the endothelium of all blood vessels. The rats were allowed free acces to water and rat chow before the isolation of the liver. The animals were divided into 3 groups: group 1 (n=6), perfusion of normal rat livers with Krebs Henseleit (KH), group 2 (n=6), perfusion of normal rat livers with 15% human serum (HS), and group 3 (n=6), perfusion with 15% human serum of livers from Sprague Dawley rats transgenic for hDAF (Tg HS). During the perfusions bile production, BSP-clearance and biochemical parameters of the liver (AF, ALAT,ASAT, and LDH) were measured. Immunohistochemistry was performed to asses the deposition of complement in the rat liver.

Surgical Protocol.

The animals were anaesthetized with ether and a midline laparotomy was performed. The intestines were placed in a warm saline-moistened swab to the left of the animal. The median and left lateral lobes of the liver were mobilized by division of the suspensory ligaments, and the entire liver everted onto another warm saline-moistened swab on the thoracic cavity. The procedure exposed the portal vein and bile duct for cannulation. A 5/0 silk ligature was placed around the bile duct, and the duct was cannulated with a 2FG cannula filled with PBS (Phosphate Buffered Saline, Sigma P-4417). The subsequent capillary pressure enabled a good bile flow. The vena porta was ligated and cannulated and perfusion with oxygen saturated Krebs Henseleit medium started immediately. The infrahepatic caval vein was cut to improve outflow of the medium and a homogenous, pinkish-brown colour of the liver was established. The suprahepatic caval vein was cannulated after opening the thoracic cavity and the infrahepatic caval vein was ligated. The hepatic artery was left open to ensure moistening of the liver during perfusion. The liver was left in situ and the whole animal is transported into the perfusion system.

Perfusate Preparation.

Perfusion was performed with Krebs Henseleit (KH) with 0.1 mM Bromosulfophtalein (BSP, Fluka Chemika, Riedel-de-Haën, 19360) and 0.6% Bovine Serum Albumin to bind the BSP in solution (BSA, Boseral, Organon Technika). Human serum (HS) is collected from coagulated blood from human donors. It is centrifuged at 2000 rpm (MSE Mistral, Beun-de Ronde B.V.), after which the serum is filtered through a 220 μm filter (Stericup Millipore Bedford, 0.22μm GP Expres Membrane P25531). It is added to KH at a 15% dilution at which concentration hyperacute reaction can still be studied [14] and less serum is needed.

Perfusion Technique.

The perfusion system consisted of an oxygenator and a liver reperfusion circuit connected in parallel to a common reservoir. The circuit tubing was composed entirely of Silastic silicone rubber tubing with an internal diameter of 2.0 mm and a wall thickness of 0.5 mm. The livers were perfused with a peristaltic pump at a constant pressure of 22 cm H_2O keeping a constant flow of 30-50ml/min depending on the size of the liver. The oxygenator existed of two parts passing a mixture of 95% oxygen and 5% carbon dioxide through the module, keeping the oxygen pressure at approximately 500 mmHg, necessary for sufficient oxygen diffusion to the liver tissue in the absence of oxygen carriers. The liver was perfused with KH for 20 min without reperfusion untill a uniform pale beige appearance of the liver is obtained, followed by a continuous antegrade reperfusion for 120 min at room temperature with a total volume of 150 ml perfusate.

Sample Collection and Immunohistochemical Analysis.

Bile is collected every 10 min. directly form the bile-cannula into an Eppendorf cup. The actual amount of bile produced is calculated by measuring the weight of the cup before and after bile collection using a density of bilirubin of 1 g/ml. Flow was monitored constantly throughout the perfusion and 0.5 ml samples of the perfusate were obtained from a seperate port directly after the liver every 10 min. About 100 μl is immediately stored in the refrigerator for BSP measurement, the rest is stored in the −20°C freezer for the biochemical analysis. At the end of the experiment samples of liver tissue were stored in formaldehyde and at −80°C. Formalin fixed tissue was paraffin embedded, cut and stained with haematoxylin-eosine for examination of morphological changes by conventional light microscopy. FITC-

conjugated rabbit anti-human C3c antibodies (Ra-αHuC3c/FITC, Dako ITK Denmark, Code No. F0201) were used to demonstrate complement deposition by fluorescence microscopy (Leica, DMRXA, No. 183235). Quantification of the C3c staining was performed by assessing the amount and intensity of the fluorescent spots in 2 slides from every experiment at 5 at random chosen fields of view. It was graded from 0 for no fluorescence to 5 for maximal fluorescence.

Statistical analysis.

The two-sample students T-test with a 95% confidence interval was used for statistical analysis.

RESULTS

Function of the liver

No increase in concentration of ASAT, ALAT, LDH and AF could be established during perfusion of normal rat livers with Kreb's Henseleit, human serum, or the livers transgenic for hDAF perfused with human serum, and no difference in concentration was found between the experimental groups (data not shown).

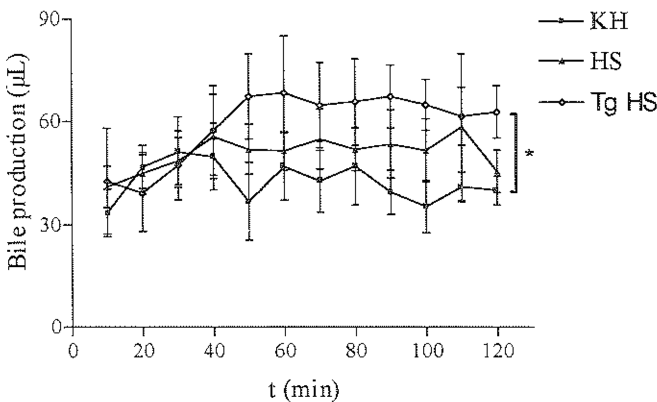

Figure 1. Bile production (mean with se); * significant difference Tg HS versus KH ($p<0.05$).

Figure 1 shows the mean bileproduction during the perfusion. There was a constant bile production throughout the perfusion, when the liver was perfused with KH (43 ± 5.7 μl/10min) and HS (50 ± 4.7 μl/10min). An initial increase in the first 40 min. in bileproduction from 43 ± 4 μl/10min to 64 ± 3.4 μl/10min was shown, when perfusion was performed with human serum in the transgenic liver. In the second hour of perfusion, the bile production of the transgenic livers is statistically significant higher than the bile production in the KH-group ($p<0.05$), but no significant difference was established between the Tg HS-group and the HS-group.

The concentration of BSP was measured in the bile and figure 2 shows the cumulative release of BSP into the bile. A steady increase for all groups is shown. In the last 20 min of perfusion, a significant difference ($p<0.05$) in cumulative BSP concentration in the bile was measured between the transgenic group and the HS group. No significant difference was established from the KH group with either other group.

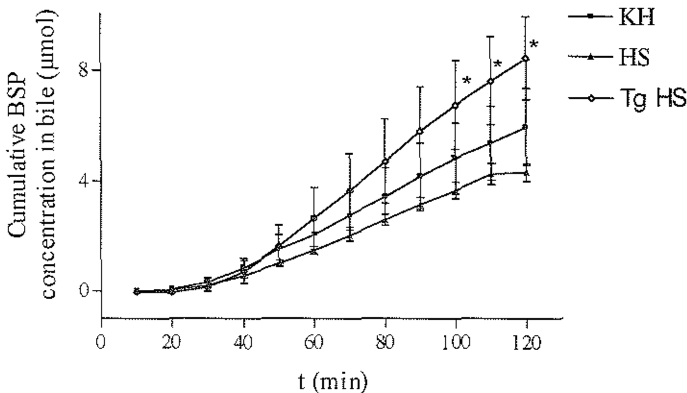

Figure 2. Cumulative concentration of BSP in bile (mean with se); * significant difference Tg HS versus HS and KH ($p<0.05$).

The mean decrease of the concentration of BSP in the perfusate as a percentage of the concentration at the start of the perfusion is shown in figure 3. When the perfusion was performed with human serum in the normal liver as well as in the transgenic liver, significantly more BSP was removed from the perfusate as compared to the perfusion with KH ($p<0.05$).

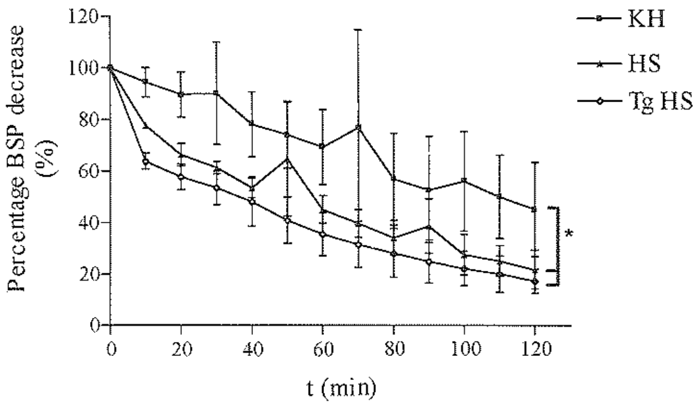

Figure 3. Concentration of BSP in the perfusate as a percentage of the concentration at the start (mean with se); * significant difference KH versus HS and Tg HS.

Immunohistochemistry of the liver

Figure 4 shows the parafin embedded slides of the livers with a HE-staining 2 hours after perfusion with KH (fig 4a), perfusion with human serum in the normal rat liver (fig 4b) and the transgenic rat liver (fig 4c). After perfusion with KH for 2 hours a normal hepatic structure can be seen. Perfusion with human serum of the normal rat liver shows a disintegration of hepatocyte plate architecture, with damaged endothelium and endovascular fibrin deposition. Histological examination of the transgenic livers shows a mostly intact hepatic structure with no damage to the vascular endothelium.

TABLE I

Quantification of C3c staining by assessing the amount and intensity of the flusorescent spots in 2 slides from every experiment at 5 at random chosen fields of view (0 for no fluorescence to 5 for maximal fluorescence).

Experimental groups	**Assessment of fluorescence intensity**
KH (negative control)	0.13 ± 0.021
HS	2.1 ± 0.25
Tg HS	0.93 ± 0.16

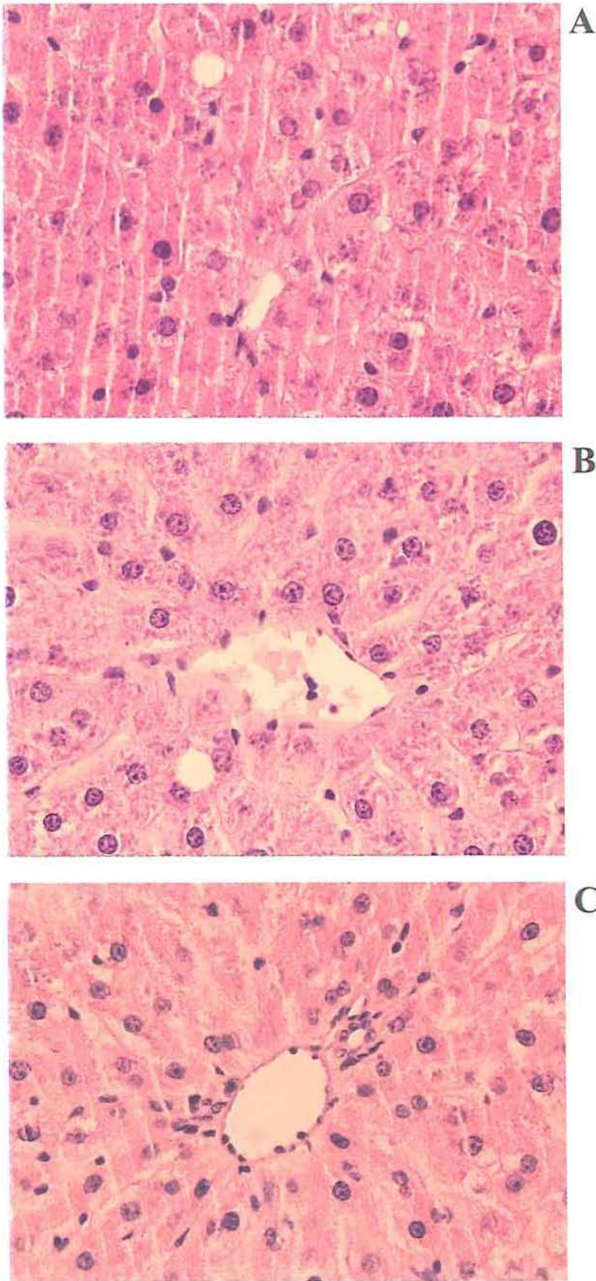

Figure 4. HE-staining of parafin embedded normal rat liver after 2 hours of perfusion with KH (A), human serum (B) and rat liver transgenic for hDAF with human serum (C).

In figure 5, immunofluorescent studies of the livers perfused with KH (fig 5a), human serum in normal (fig 5b) and transgenic livers (fig 5c) are shown. Evidently, there is more staining in the non-transgenic liver perfused with human serum as compared to the livers transgenic for human DAF, whereas the livers perfused with KH were taken as a negative control. Semi-quantification of the amount and intensity of fluorescence is presented in table 1. Significant more C3c-deposition is shown in normal rat livers perfused with human serum compared to transgenic livers as well as compared to the livers perfused with KH ($p<0.05$).

DISCUSSION

In our model, the isolated liver perfused with human serum or Kreb's Henseleit showed good function throughout the perfusion period, as has been demonstrated by bile production, concentration of BSP in the bile and decrease of BSP from the perfusate. Perfusion with human serum accounted for a significant higher bile production as compared to perfusion with KH suggesting an advantage in the xenogeneic situation, possibly due to the content of the human serum versus KH. Overall, although no significant differences could be established, perfusion with human serum has a tendency to benefit the function of the isolated perfused rat liver.

The livers transgenic for hDAF possibly showed a better function than the normal rat livers. After an initial increase, the bile production is significantly higher in the transgenic livers, there is more release of BSP into the bile and, most importantly, the decrease of concentration of BSP from the perfusate was higher. The initial increase of bile production in the transgenic liver may be due to the adjustment of the liver in the first 20 min., in which period it has been shown that complement and preformed natural antibodies are removed from the serum [11]. This removal seems to be more efficient in the transgenic livers as compared to the normal rat livers since bile production remains at a lower level in these organs.

Evidently more tisue damage was found in the normal rat liver compared to the transgenic rat liver on histopathological examination. Damage to the vascular endothelium and fibrin deposition in the normal rat liver suggests complement mediated tissue damage, indicating hyperacute rejection. On immunofluorescent examination, these findings were confirmed, showing significantly more C3c-deposition in the normal rat liver compared to the transgenic rat liver, indicating more humoral damage to the isolated nontransgenic rat liver. The extend of tissue damage was not sustained in release of liver enzymes into the perfusate. Very low

levels of transaminases, lactate dehydrogenase and alkalic phosphatase could be detected in the perfusate and no difference could be established between the experimental groups. Either our detection methods were not sensitive enough or the extend of tissue damage was not of major importance. However, the histopathological prove of tissue damage is convincing, parallel with a possible improved function of the perfused liver, and we therefore believe that a protective role of transgenesis has been established.

The exceptional position of the liver in the xenogeneic situation complicates the interpretation of the possible beneficial role of transgenesis for complement regulatory proteins in ex vivo liver perfusion. Xenogeneic perfusion of the heart causes hyperacute rejection and immediate dysfunction of the heart within 10 min., whereas transgenic hearts function for several hours [14]. In xenogeneic perfusion of the isolated normal liver with human whole blood [12] and xenotransplantation of the normal liver in a discordant model [15] no hyperacute rejection has been shown. An immediate decrease of xenoreactive natural antibodies and complement in xenogeneic liver perfusion has been established in several reports [16,17], the effect of which was not always seen on histological examination [12], as we showed in this study. However, it is known, that pig livers expressing hDAF on the endothelial cell surface exhibit less tissue damage, down-regulate complement activation and therewith the release of some cytokines during the humoral reaction [13,18]. Thus, although the normal liver seems to be capable of resisting the hyperacute rejection for several hours, the additional down-regulation may prove to be beneficial for isolated perfusion of the liver in terms of tissue damage and cytokine release possibly enabling a more effective use of the ex vivo liver for a longer period of time.Next to the use of organs transgenic for complement regulatory proteins on the endothelial cell membrane, other means to possibly reduce humoral damage in xenogeneic liver perfusion are under investigation. A reduction of the humoral effect was shown perfusing pig livers with human whole blood using continuous administration in the perfusion circuit of soluble complement receptor type 1 (300 μg/ml) [19]. An additional study showed a remarkable increase in bile production and prolonged survival of a dog with ischaemic induced liver failure, which was extracorporeally assisted using pig liver perfusion with continuous adiministration of soluble receptor type 1 [20]. Using immunoapheresis with an Ig-thereasorb 100 column, consisting of sheep-anti-human IgG antibodies covalently coupled to Sepharose CL-4B, Pascher et al significantly reduced the amount of circulating antibodies and complement, and revealed a significant reduction of cellular damage and functional restrictions of the ex vivo liver [21,22]. However, immunoadsorption alone was not able to prevent organ damage or loss of function completely [23]. In subsequent autologous reperfusion,

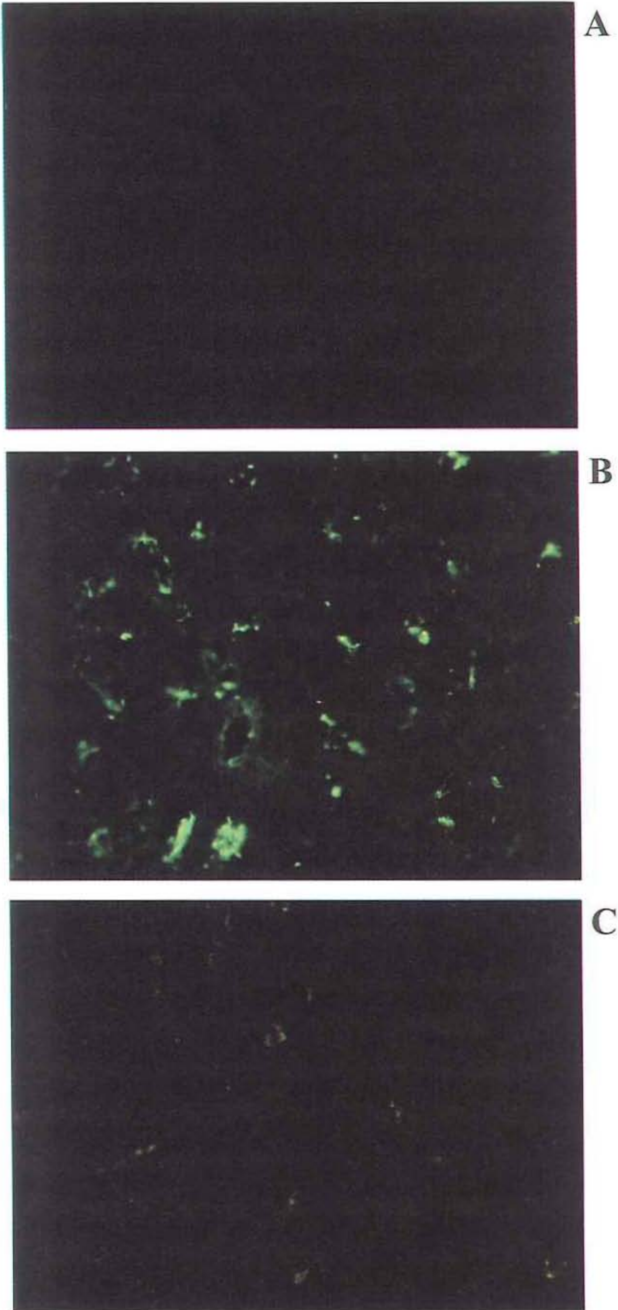

Figure 5. C3c-deposition in a normal rat liver after two hours of perfusion with KH (A), human serum (B) and in a rat liver transgenic for hDAF perfused with human serum (C).

the liver did not regenerate to it's initial level of performance, therewith pleading for the possible benefit of multiple short term ex vivo liver perfusions as opposed to a single long term perfusion, and the use of combinations of humoral damage reducing therapies. Despite the exceptional position of the liver in the xenogeneic situation, these studies confirm that a reduction of the humoral reaction clearly improves the outcome of xenogeneic ex vivo liver perfusion.

In conclusion, in our study, rat livers transgenic for hDAF are protected from tissue damage following perfusion with human serum, which does not result in a significant improved function of the isolated liver. However, the beneficial effect of transgenesis in xenogeneic perfusion of the isolated liver on tissue damage and the consequent release of cytokines and other cell products may provide better functional results in long term extracorporeal liver perfusion and may provide a safer microenvironment for the treatment of patients with acute liver failure.

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8

General discussion and conclusion

Future prospectives in the temporary treatment of acute liver failure

Stockmann HBAC, IJzermans JNM

Submitted.

Introduction

In the past 40 years numerous therapies have been developed and evaluated for the treatment of acute liver failure (ALF) [1,2], the most successful of which has been the orthotopic liver transplantation (OLT) from cadaveric donors [3], since it is known that only 20% of the patients with ALF survive when treated with conservative medical measures. Recent programs introduced the living related liver transplantation as an alternative source for cadaveric donor transplantation, which shows very encouraging results with survival rates of up to 90% in 5 years [4].However, this potential large group of donors is limited for several reasons, e.g. blood group mismatch, size discrepancy, use of medication, age or preexistent other disease, providing a donor organ in only 30% of cases [5]. Therefore, mortality rates in liver failure remain high. The shortage of donor organs for OLT and the lack of satisfactory means for temporary treatment to bridge to either liver regeneration or to liver transplantation, has renewed the interest in old and new methods for an extracorporeal approach to the treatment of ALF [26,31]. The current opinion is that the multiple and complex functions of the liver can only be replaced using the biologic substrate (i.e., hepatocytes), whether in a whole liver or in combination with artifical material. By using xenogeneic biologic material in the treatment modalities of patients with liver failure, several health hazards are introduced. In an already immunocompromized patient, short- and long-term immunological problems can be expected, and transmission of viral material to the patient is possible. In this review, the problems encountered in the interaction between the xenogeneic device and the patients with liver failure are discussed and directions for future research are given.

Existing temporary liver support techniques

The lack of successful temporary assistance may be due, in part, to an incomplete understanding of the underlying mechanisms causing acute liver failure. Several lower and middle molecular weight substances have been blamed for playing a role in hepatic failure, e.g. bilirubin, bile acids, digoxin-like immunoreactive substances, indoles, phenols, mercaptans, endogenous benzodiazepines, aromatic amino acids, ammonia and lactate [6-9]. They are thought to rapidly accumulate and cause multiple organ dysfunction and hepatic encephalopathy, eventually leading to hepatic coma as a life threatening complication of liver failure. Some of these endogenous toxins are water soluble, but most of them have a high binding affinity to albumin, a molecule overloaded with toxins in hepatic failure due to the

reduced detoxification capacity of the liver. For example, the limited binding capacity of circulating albumin for nitric oxide could be one mechanism of the decreased blood pressure and the need for pressors in hepatic failure when albumin bound toxins reach critical blood levels [10,11]. Furthermore, accumulated free toxins cause direct damage to the liver itself therewith creating a vicious circle of hepatic deterioration [12]. Therefore, many devices have been developed concentrating on the detoxification function alone without having the negative side effects that arise from bioincompatability and lack of selectivity [13,14]. Unfortunately, to date, these systems have not been able to improve the clinical status of the patient with liver failure because of low efficacy or because of the additional removal of factors that are essential for liver regeneration.

A second method providing possible treatment for patients with acute liver failure involves the use of isolated human hepatocytes transplanted into the patient's liver or spleen [15]. The additional integration of cellular components might complete the wide spectrum of functions that are necessary for successful treatment of hepatic failure [16]. Highly differentiated function and significant prolonged survival has been shown when a clonal human hepatocyte cell line was transplanted into the spleen of 90% hepatectomized rats [17]. In a single case human study [18], 96% viable human hepatocytes were intraportally infused under cyclosporin immunosuppression, showing a complete recovery of the patient after regeneration of the patient's liver. In another study, five patients with grade III to IV hepatic encephalopathy were treated with hepatocyte transplantation either intrasplenic, intrahepatic or a combination of both under cyclosporin immunosuppression and temporarily showed subsequent substantial improvement in encephalopathy scores [19]. However, in these reports, no sufficient control group was monitored. Moreover, first, the large scale isolation, culture and cryopreservation of human hepatocytes showing long term stable highly differentiated function needs optimization, and second, the shortage of donororgans necessary for the isolation of human hepatocytes limits the use of this procedure.

Besides the above mentioned non-xenogeneic techniques, ex vivo liver perfusion, in which a whole organ is connected in parallel to the patient's circulation, has been developed and optimized for clinical use in the 1960s and early 1970s [20-23]. This method has been applied with various results, but because of the successful development of the OLT in the 1970s, it was abandoned. During the last 10 to 15 years, a renewed interest in this temporary liver support technique has evolved. After 20 years, the disadvantages of OLT become clear: it is a major and costly procedure resulting in lifelong immunosuppressive therapy for the patient, and in 20% of cases a liver does not become available for transplantation in time [24].

Therefore, recently, studies were performed, which again showed promising results using extracorporeal liver perfusion in the treatment of patients with liver failure [25,26].

Recent advances in tissue engineering have encouraged the development of a fourth method for temporary assisting the failing liver: the bioartificial liver device [27-31]. In these systems, the biologic material, now consisting of hepatocytes only, incorporated in a synthetic framework, may provide a practical and save system to treat patients with liver failure. From the early studies of hepatocyte culture [32,33], there has been an increasing effort to create a culture system, large enough to replace the function of a normal human liver, but small enough to provide an extracorporeal perfusion circuit without haemodynamic disadvantages for the patient. Using primary hepatocytes, a stable highly differentiated function for a longer period of time has not yet been established, as has been difficult in the smaller scale hepatocyte culture systems [34]. Hepatoma cell lines (e.g. Hep G2 cells) have been used therewith avoiding this problem, but their function is inferior to primary hepatocytes as the source of hepatic function [35]. Moreover, Hep G2 cells were detected in the extracapillary compartment of the bioartificial liver, analogous to the patient's compartment during clinical application, which strongly suggests the possibility of tumor transmigration into an already immunocompromised patient [35]. Therefore, the development of the bioartificial liver is focused on short term stable function of primary hepatocytes within an artificial system, e.g. for 6 to 8 hours, after which a new batch of hepatocytes is needed for effective extracorporeal treatment. Applying this technique, encouraging results have been obtained [31], but further clinical studies need to be undertaken, not only to improve the current available systems, but also to achieve a better understanding of the many facets of liver failure so as to best care for patients affected by it.

The potential hazards

With the introduction of the biologic material in the treatment modalities of patients with acute liver failure, several health hazards are introduced into the treatment. First, a patient with hepatic failure has a decreased coagulation capacity and a decreased immunological defense, possibly due to a decreased synthetic function of the liver. The patient is hooked up to a tubing system, which, whatever biocompatible material is used, may cause coagulation and infectious problems of its own. Second, depending on the volume of the extracorporeal perfusion circuit, an already haemodynamically instable patient may experience even more haemodynamic and metabolic changes during ex vivo treatment. Third, although

immunocompromised, the patient may be sensitized to the animal material used in the ex vivo system, possibly causing health hazards in the patient's future. Fourth, the release of animal material, e.g. porcine endogenous retrovirus (PERV), into the circulation of the patient may in the long run endanger the patient and its environment. At present, these issues play a predominant role in the development of xenogeneic liver support systems as well as their acceptence in society, for which reason they will be discussed next.

Host versus Graft

The humoral response plays a predominant role in the ex vivo treatment of patients with liver failure, because the treatment is intended to last for several hours. In xenotransplantation, the hyperacute rejection (HAR), e.g. complement mediated cytotoxicity, pathological cellular reactions and humoral mechanisms by preformed naturally occurring antibodies (pNAbs), has been observed within a few hours [36]. These pNAbs are directed against the GAL-alpha membrane epitope, the most important target antigen of the human humoral defense against porcine organs [37]. There are two major factors when the temporary treatment of patients with liver failure is considered from an immmunological point of view.
First, the patient suffers from liver failure, resulting in a decreased synthetic function, accounting for a decrease of the components of the immunological reaction. It has been found that the amount of complement in patients with ALF is significantly reduced [38], whereas no decreased levels of pNAbs were observed. Also, when ALF is induced in rats, a decreased level of complement correlated to a significant increase in xenograft survival is noticed, although a decrease in complement deposition was not established [39]. Second, the liver has an exceptional capacity to remove antibodies from a perfusion circuit without being harmed, as compared with other organs. Immunohistochemical analysis of kidneys perfused with human blood demonstrates the binding of both IgM and IgG xenoantibodies and complement (C3) to kidney vascular endothelium, whereas staining was minimal for liver vascular endothelium, although levels of antibodies for both organs were significantly decreased after perfusion [40]. It was suggested that the liver is capable of removing antibody and complement without cell lysis, possibly by endocytosis, compared with aortic or other endothelium [41]. And indeed, in a recent clinical study using extracorporeal perfusion for the treatment of patients with acute liver failure, the disappearance of complement and antibodies was demonstrated without affecting the function of the ex vivo liver [42]. Recently, an experimental study described the deviant role in hepatic xenografting of the antibodies, indicating a predominant role of IgG

over IgM [43]. These results suggest that for short term use the function of the ex vivo liver is not affected by the patients immunological system.

However, evidence of tissue damage in ex vivo liver perfusion has been established, which may cause a suboptimal function of the ex vivo organ and in return induce a hazard for the patient when breakdown products of the xenogeneic organ are released into the patient's circulation [44,45]. In patients with liver failure treated with ex vivo perfusion, the level of pNAbs increased with the number of treatments per patient, indicating a possible cumulative effect of the xenogeneic perfusion [46], and in one patient an anaphylactic reaction was observed after 16 extracorporeal perfusions in 2.5 months [47]. However innocent for short term use, long term or repetetive use of an extracorporeal system may indeed affect the function of the ex vivo organ and endanger the patient.

In bioartificial liver support systems, a plasma separation unit is used and the communication between plasma and the isolated hepatocytes, or between the patient and the xenogeneic material, depends on the pore size used in the membranes of the bioreactor. The ideal nominal molecular weight cutoff (NMWCO) of the membranes may be between 50 and 100 kDa allowing breakdown products and proteins (molecular weight 0.6 to 70 kDa), essential for the health and recovery of the patient, to pass through, whereas the cytokines and immunoglobulins (molecular weight 100 to 900 kDa) preferably do not make contact with the liver cells [48]. Theoretically, a humoral as well as a cellular immune response should be prevented in these systems. However, either through contact of the patient's plasma with the hepatocytes during perfusion or because of pig hepatocyte material entering the human circulation, it has been shown that sensitization has occurred in patients receiving several treatments with a BAL [49]. In an experimental study, isolated porcine hepatocytes were exposed to 103 different human sera in the presence and absence of complement and tested for cytotoxicity [50]. A range of 10 to 100% cytoxicity was found in the presence of complement, whereas no cell lysis could be established in the absence of complement. No membranes, however, were part of these experiments and therefore, these results may not be extrapolated to the actual situation in a hybrid BAL.

Another factor to consider is whether xenogeneic bridging may sensitize the patient to subsequent allogeneic transplantation. Ye et al [51] showed no hyperacute, antibody-mediated or accelerated rejection in baboons, which received xenografts from pigs and African green monkeys, followed by allotransplantation. This mechanism seems to be unjustified, since the GAL-epitope is not prevalent in humans, and thus, the anti-pig-antibodies produced after xenoperfusion, are not directed against human cell surface proteins.

Graft versus host

The risk to patients, posed by the transfer of (parts of) hepatocytes or other porcine cells from the ex vivo system to the patient, should be investigated. In extracorporeal whole liver perfusion, damage to the pig liver endothelium, as has been shown in the mild immunologic reaction in ex vivo perfusion, may cause transmission of animal material to the patient. As early as 1965, it was shown that C^{14}-tagged glycine and lysine were incorporated into protein by the ex vivo pig liver, suggesting exchange of biologic material between graft and host [20]. Also, pig serum proteins have been detected in the host circulation after whole-liver xenoperfusion [46]. On the other hand, in a baboon-to-human liver transplantation, no baboon antibodies were detected in the human serum [52]. Whether direct damage to the ex vivo liver may or may not affect it's long term function and therewith the effectivity of the treatment of the patients with liver failure remains to be determined. Alternative strategies are developed to minimize the immunological effects in extracorporeal perfusion, for instance transgenesis [53], the addition of soluble receptor type 1 [54], and immunoapheresis [55]. Improvement of the function of the ex vivo organ has been established, but the wished for effect on minimizing the amount of material that enters the patient's circulation and therewith the possibility of causing health hazards in the long run is still uncertain.

Obviously, because of the absence of a blood-endothelium contact, the hazards of xenogeneic interaction in bioartificial systems are less severe. However, the nature of the methods used to isolate hepatocytes for a bioreactor mean that non-parenchymal cells from the donor liver may get into the bioreactor, and therefore affect the patient. These include sinusoidal endothelial cells and also immune cells such as Kuppfer, PIT- and ITO- (Stellate) cells together with other cells circulating in the blood stream of the donor. With a NMWCO of 100 kDa, transfer of any kind of cell through the membrane of a bioreactor should not be possible. However, contact of the patients plasma to xenogeneic cells causes metabolic products of pig hepatocytes to enter the patients circulation and become immunogenic. Most products of the porcine liver would have to be taken into account since few of the macromolecules produced in the liver exceed the membrane nominal molecular weight cutoff. Lanza et al [56] seeded islets of porcine pancreas into diffusion chambers from permselective acrylic membranes with a NMWCO of 80 kDa, implanted intraperitoneally. Within 2 to 6 weeks, the recipients sera showed strong immunoreactivity, however, even after additional injection of "naked" islet

tissue into the peritoneal cavity, no anaphylactic or other immediate-type hypersensitivity was observed in the recipient.

Table 1. Experiments performed to detect the infection of PERV in human cells *in vitro*.

Reference	Experiments	Tissues tested	Type test	PERV infection
Patience et al. [60] Nat Med 1997	Supernatant from porcine kidney (PK15) cell-line culture	Human kidney-, lung-, muscle-, Bcell-, and Tcell culture	Perv DNA Perv mRNA	positive positive
	Co-culture of PK15 cells with	human embryonal kidney cell line (HEK 293 cells)	Perv DNA Perv mRNA	positive positive
Martin et al. [61] Lancet 1998	Primary porcine endothelial cells, hepatocytes, lung-, and skin cells		Perv DNA and Perv mRNA positive: capable of Perv-infection	
	Co-culture of porcine aortic endothelial cells	with HEK 293 cells	Perv DNA Perv mRNA	positive positive
Wilson et al. [62] J Virol 1998	Peripheral blood mononuclear cells (PBMC) mitogenic activated with phytohemagglutinin (PHA) and phorbol myristate acetate (PMA)		Perv DNA and Perv mRNA positive: capable of Perv-infection	
	Co-culture of activated PBMC's	with HEK 293 cells	Perv DNA Perv mRNA	positive positive
Nyberg et al [63] Transplantation 1999	Possibility of Perv transmission from a hollow fiber cartridge. Intraluminal culture with PK15 cells	Intraluminal supernatant	Perv DNA Perv mRNA	positive positive
	Supernatant of the extraluminal space from above cultured on	HEK 293 cells after pore size 200 nm pore size 10 nm pore size 5 nm	 DNA/mRNA DNA/mRNA DNA/mRNA	 positive negative until day 3 negative until day 7
Nyberg et al. [59] Liver Transpl. 2000	Porcine hepatocytes with normal medium and serum from patients with fulminant hepatic failure		Perv DNA and Perv mRNA positive: capable of Perv-infection	
	Supernatant of the above cultures on	HEK 293 cells, supernatant of this culture tested for	Perv DNA Perv mRNA	negative negative
Martin et al. [64] Xenotranspl. 2000	Supernatant of PK15 cells on	Human endothelial cells and fibroblasts	Perv DNA Perv mRNA	positive positive
		Bone marrow stromal cells and mesangial cells	Perv DNA Perv mRNA	positive negative

One of the major concerns with the use of porcine organs in this manner is the threat of pig-to-human transmission of activated endogenous retrovirus present in the pig genome [57]. Porcine endogenous retrovirus (PERV) was described in 1973, but at that time was shown to be unable to infect human cells [58]. However, the observation that PERV DNA and PERV RNA are detected in the supernatant of pig hepatocyte culture systems [59] and such viruses can infect human cell lines in vitro [60], has led to intense scrutiny of such research by regulatory agencies worldwide. Table 1 shows an outline of the six studies that have been performed thus far, in which the transmission and infection of PERV was examined *in vitro* [59-64]. Productive PERV infection, detected with PCR (PERV DNA) and RT-PCR (PERV mRNA) has been shown from most porcine tissues into a human cell line (HEK 293), human endothelial cells and fibroblasts in culture. It is as yet unknown whether these findings represent in vitro artifacts or whether infection can occur in the in vivo situation.

The experiments performed *in vivo* are summarized in table 2, representing two non-human and three human *in vivo* studies [65-69]. PERV transmission was tested by PCR, RT-PCR and the detection of anti-PERV antibodies in the serum of the acceptant. Thus far, *in vivo* PERV transmission has been established in non-obese diabetic, severe combined immunodeficient mice [66], but not in humans treated with porcine tissue, as has been shown by Paradis et al [69] in a retrospective study of 160 patients. Infection only occurs if first, the provirus sequence in porcine tissue is activated to produce and release intact virus; second, in the case of a bioreactor, the virus must cross the semipermeable membrane and third, once in the patient's circulation, the virus must be capable of productively infecting human cells. In the BAL, the second step may be the limiting factor, as has been shown by Nyberg et al., since the MWCO of most bioreactors is between 50 and 100 kDA (pore size ± 5 nm), which prevented PERV infection in human cells for 7 days, whereas the therapy is intended to last for only a day at a time. Moreoever, the transmission of PERV to normal, healthy humans might be prevented by natural alpha-GAL binding antibodies and complement, as has been shown for other retroviruses [70-72].

If infection is possible, one needs to determine whether the virus is transmitted by blood contact and/or by food, and why considerable segments of the human population are not PERV-positive. In xenogeneic treatment, the heavily immunosuppressed patient potentially has a lower titer of natural antibodies, including the anti alpha-GAL antibodies. This could result in a diminished virolysis of PERV particles. Furthermore, genetic modifications, such as the elimination of porcine GAL epitopes or the introduction of complement regulators, proposed to overcome hyperacute rejection, could promote the infection of human cells with

Table 2. Experiments peformed to detect the infection of PERV in humans *in vivo*.

NON-HUMAN				
Reference	**Experimental setup**	**Tissues tested**	**Time tested**	**PERV**
Martin et al. [65] Transpl. Int. 1998	Porcine aortic endothelial cells transplanted into baboons with high dose cyclophosphamide for 0-4 days	Baboon skin, liver, leucocytes, lymphnodes for DNA and mRNA	2 years after transplantation	negative
Van der Laan et al. [66] Nature 2000	Purified pig islets transplantation under kidney capsule of non-obese diabetic, severe combined immunodeficient mice	Transplantation site, spleen, liver, salivary gland, skin, small bowel, lung, kidney for DNA and mRNA	18 to 56 days	positive

HUMAN				
Reference	**Experimental setup**	**Tissues tested**	**Time tested**	**PERV**
Heneine et al. [67] Lancet 1998	Porcine fetal pancreatic islets transplanted into 10 diabetic patients under immunosuppression (cyclosporin, prednisolone, azathioprine)	Blood lymphocytes, sera for DNA, mRNA and anti-PERV antibodies	4,5 to 7,5 years	negative
Patience et al. [68] Lancet 1998	Extracorporeal pig kidney perfusion in 2 patients	Peripheral blood mononuclear cells	6 hrs to 36 months after perfusion	negative
Paradis et al. [69] Science 1999	160 patients with: Extracorporeal spleen, liver and kidney-perfusion, extracoproreal perfusion with a bioartificial liver, pig skin grafts, porcine pancreatic islet cell transplants	Peripheral blood mononuclear cells and serum for DNA, mRNA, and anti-PERV antibodies	Up to 12 years	negative

PERV [73]. Scientists, physicians, and ethicists are currently discussing whether clinical trials should be postponed due to the risks of xenozoonosis [73-76]. In our country, a public debate has been initiated by the minister of health in december 1999, which to date has put a complete hold on clinical trials using xenogeneic material in the treatment of patients. Thus far, no transmission of pig endogenous virus was detected from porcine endothelial cells transplanted to baboons or humans. However, because of the variety of potentially dire consequences after clinical xenogeneic treatment in immunosuppressed patients, including the risk of patient disease and epidemic transmission of humanized viruses, preclinical models are needed to further evaluate this issue.

New possibilities in liver support therapy

Although the knowledge of immunology and xenobiology has improved, the possible impact of xenogeneic treatment is still uncertain. Temporary treatment of patients with liver failure with conservative therapy or passive detoxification techniques is safe, but success rates are limited. The short term treatment with extracorporeal whole liver perfusion has a reasonable succes, with and without genetic modification of the ex vivo liver. It seems that genetic modification could potentially benefit the treatment modality, because evidently less damage is found in these livers after perfusion, suggesting a possible longer effective use of the organ. The impact of the xenogeneic interaction in the treatment of patients with liver failure with bioartifical liver systems seems to be minimal. Therefore, it is my strong opinion, that xenogeneic non-transgenic tissue or cells could be used in the short term treatment of patients with liver failure.

In the mean time more permanent solutions to the problem of treating patients with liver failure should be pursued, which could eventually completely replace the above mentioned systems including their disadvantages. Several alternative sources of liver cells are under investigation. Newly developed immortalized human cell lines, e.g. NKNT-3 cells and OUMS-29 cells, have been shown to express highly differentiated liver specific function [17,77]. Another study shows the successful use of human foetal hepatocytes, harvasted from a liver obtained from mid-trimester abortions [78]. Also, the function of freshly isolated porcine hepatocytes in a bioartificial liver may be improved, if the cells are pre-cultured for a longer period of time before inoculation [79].

A possible indefinite source of hepatocytes for bioartificial means may be the use of stem cells [80]. A stem cell can be defined as a cell that has the capacity both to produce daughter cells –'self-renewal'- and to produce daughters that are fated to differentiate –'commitment'- (fig 1). Self-renewal plays a key role in these developments: first, because it enables the long term maintenance of grafts of renewing tissues, and second, because it provides a large scale production. Stable differentiation or commitment is necessary to eventually enable the repetetively culture of the cell type of interest. Efforts to isolate, expand and genetically manipulate stem cells from adult tissues have to date met with only partial success [81]. An alternative to the primary derivation of tissue-specific stem cells would be the development of an in vitro system in which lineage-restricted stem cells could be generated from a founder stem cell population of human pluripotent stem cells (HPCs). These cells, with the capacity to generate all foetal and adult cell types, exist only in the early embryo and in a particular type

of germ cell tumour, teratocarcinoma. HPCs from teratomacarcinomas have been established in culture, but their tumour origin makes them unsuitable for therapeutic exploitation. In mice, however, the isolation of teratomacarcinoma stem cells was followed by the derivation of non-transformed pluripotent stem cells directly from embryos [82]. These cells proved to be pluripotent, immortal and phenotypically stable in vitro and in vivo [83-85]. The isolation of HPCs analogous to mouse embryonic stem cells would thus create the possibillity of generating human cells for transplantation. Recently, Thomson et al [86] have been able to derive pluripotent embryonic stem cell lines from human blastocysts, that have normal karyotypes, express high levels of telomerase activity, which is known to play an important role in the replicative life-span, and express cell surface markers that characterize primate embryonic stem cells but do not characterize other early lineages.

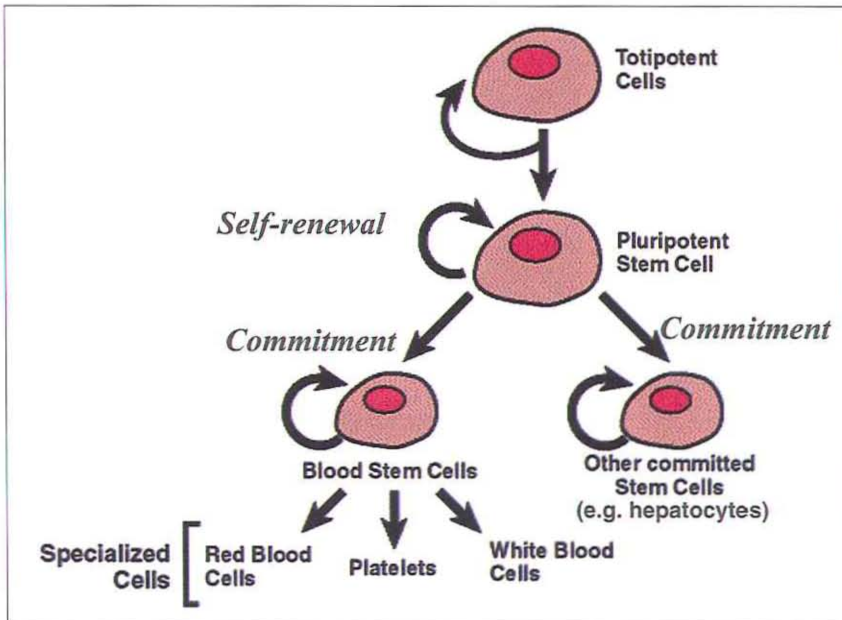

Figure 1 In this figure, the development of the totipotent cell into the pluripotent stem cell is given, at which stage it is harvasted from the blastocyst. The further development should reassure the self-renewal of the stem cell population in order to have an unlimited source of pluripotent cells and to provide large scale production of a specific cell type; commitment should ensure the stable development of the cell type of interest, e.g. into different type of blood cells (arrow to the left) or other cell types, for instance hepatocytes (arrow to the right).

Although these results are encouraging, the overall availablity of human blastocysts is limited due to the fact that in many countries, research using pre-implantation stages of human development is not permitted. Eggs may be donated for research by couples receiving infertility treatment, or by couples who have completed such treatment and have eggs remaining frozen in storage, however, in many cases they turn out to be abnormal and fail to develop the blastocyst stage. An alternative route is to attempt to derive HPCs at later stages using aborted foetal tissue, as has been shown in mouse [87], pig [88], and recently also in humans [89]. However, the molecular basis of the pluripotent phenotype and the mechanism by which germ cells convert to embryonic stem cells are stil unknown.

At present, stable pluripotent stem cell lines are being established, which show a heterogenous, disorganized differentiation, producing a mixture of cells, whereas the generation of a pure population of defined cell types is wanted. The characterization of inductive pathways that mediate lineage and cell-type-specific differentiation complemented by techniques for purifying specific cell types, e.g. by applying a genetic selection for expression of a marker gene, holds the key to progress [85,90]. Although quality and differentiated function of stem cells are stil unknown, these cells may provide a readily available source without immunological drawbacks, host versus graft and vice versa, and therefore may hold the future of safe and effective cellular systems in the treatment of patients with liver failure.

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9

Summary
Samenvatting

SUMMARY

In the past 40 years, various approaches have been used to treat patients with acute liver failure. Thus far, orthotopic liver transplantation has had the best results, with survival rates ranging from 80 to 90 %. However, mortality rates in liver failure remain high, because of the shortage of donor organs and the lack of satisfactory means for temporary treatment of patients with liver failure to bridge to either liver regeneration or to liver transplantation. Therefore, in this dissertation we further investigated the applications used to treat patients with a failing liver and studied in more detail the xenogeneic environment of the ex vivo hepatic support system.

The general introduction and the outline to the dissertation are presented in *chapter 1*.

Renewed interest has developed in old and new methods for an extracorporeal approach to the treatment of acute liver failure. Although temporary clinical improvement has been established, further research is needed to achieve a successful long-term clinical outcome. In *chapter 2*, a review is given in which the various approaches to temporary liver support as well as the immunologic and metabolic developments towards a solution for this problem are assessed. Their shortcomings are discussed and directions for future research are suggested.

The successful development of a bioartificial liver relies, in part, on the ability to maintain viability and differentiated function of a large number of hepatocytes in vitro for extended periods of time. Microcarriers have been widely used to scale up anchorage-dependant mammalian cell cultures. In *chapter 3*, an attempt to create optimal culture conditions for hepatocytes attached to microcarriers is described. Hepatocytes cultured on gelatin-coated plastic microcarriers and embedded in type I collagen showed albumin and urea secretion rates comparable to that seen in small-scale long term culture systems. In addition, the specific albumin secretion rate obtained was similar to that found in vivo. Thus, long term liver-specific function can be induced by surrounding hepatocytes seeded on microcarriers with a collagen gel. This suggests that microcarriers might be useful in the development of a

large scale hepatocyte culture system expressing high and stable levels of liver-specific function.

Transgenic pig livers are being used to study the feasibility of extracorporeal liver perfusion in the treatment of patients with acute liver failure. However, it remains to be determined whether non-immunological aspects may limit the quality of extracorporeal liver perfusion. In *chapter 4*, an autogenous pig liver perfusion model was used to study the haemodynamic and metabolic changes in an ex vivo perfusion system. Microscopic examination of the liver demonstrated venous stasis mainly in the peripheral parts of the liver and although a good function of the liver was established, an increasing lactate-acidosis was measured, correlating with haemodynamic instability of the pig after approximately 3 hours of ex vivo perfusion. Thus, although the ex vivo liver may give adequate hepatic support, this autogenous model demonstrates that irrespective of immunological responses, physiological parameters limit the period of effective use of extracorporeal liver perfusion.

Thus far, effective temporary ex vivo liver perfusion has been applied without immunological drawbacks. Complement is known to play a major role in hyperacute rejection in xenotransplantation and the level of complement is significantly decreased in patients with acute liver failure. In *chapter 5*, we investigated the possibility of acceptance of a discordant xenograft in rats with acute liver failure induced by galactosamine. And indeed, an increase in xenograft survival time in rats with acute liver failure is shown, probably due to a significant decrease in complement levels and activity as can be measured in complement depleted rats induced by pre-treatment with cobra venom factor. This could mean that the use of livers from pigs that are transgenic for human complement regulatory proteins is not essential to enable uncompromised short term liver perfusion.

The liver has been found to be less susceptible to humoral injury. For instance, no classical features of hyperacute rejection are found in liver grafts in the discordant guinea-pig-to-rat model. To further enlighten the underlying mechanisms involved in xenogeneic applications of the liver, discordancy is mimicked in the sensitized hamster-to-rat liver transplantation model. By using different immunisation protocols, predominance of either IgG or IgM

antidonor antibodies was created. Transplantation of hamster heart or liver in both sensitisation models revealed a difference in rejection pattern between both organs. As described in *chapter 6*, a predominance of IgG over IgM was found regarding the hyperacute rejection of liver grafts. This difference in rejection was not found for heart grafts.

The role of the hyperacute rejection remains obscure in xenogeneic extracorporeal liver perfusion. In order to achieve a better understanding of the humoral aspects of liver perfusion, and to study the possible benefit of the use of a transgenic liver, we determined the function and the immunopathological features of the isolated normal and transgenic rat liver perfused with human serum. It was found, as described in *chapter 7*, that although histologically less tissue damage and less C3c-deposition was shown in the livers transgenic for human DAF, there is no significant functional difference between the transgenic and non-transgenic liver perfusion. These results do suggest that the beneficial effect of transgenesis in xenogeneic perfusion of the isolated liver on tissue damage and the possible consequent release of cytokines may ensure a more effective long term use of the extracorporeal liver.

At present, a number of major hurdles are to be taken in order to safely apply the hepatic extracorporeal perfusion modalities. These include the immunological implications of these techniques, e.g. the host versus graft reaction and vice versa, and the possible health hazards for the patient with liver failure and it's environment treated with a xenogeneic device. In *chapter 8* these difficulties are described and the possible solutions for short term and long term treatment are discussed.

SAMENVATTING

In de laatse 40 jaar zijn vele manieren toegepast om patiënten met acuut leverfalen te behandelen. De beste resultaten worden tot op heden bereikt met de orthotope levertransplantatie, met overlevingspercentages variërend van 80 tot 90%. Echter, de mortaliteit van leverfalen blijft hoog door het tekort aan donororganen en het ontbreken van adequate methoden om patiënten tijdelijk te behandelen ter overbrugging naar

leverregeneratie of naar levertransplantatie. Om die reden wordt in dit proefschrift verder ingegaan op de bestaande methoden voor de behandeling van patiënten met een falende lever en de xenogene omgeving van de lever wordt nader bestudeerd.

In *hoofdstuk 1* wordt een algemene introductie gegeven tot het onderwerp en wordt de inhoud van het proefschrift in grote lijnen uiteengezet.

Er is hernieuwde belangstelling voor oude en nieuwe methoden in de ex vivo behandeling van patiënten met leverfalen. Hoewel tijdelijke klinische verbetering is aangetoond, is verder onderzoek nodig voor succesvolle resultaten op de lange termijn. In *hoofdstuk 2*, wordt een overzicht gegeven van de verschillende manieren van extracorporele behandeling van leverfalen. De immunologische en metabole ontwikkelingen worden uiteengezet, de tekortkomingen worden besproken en mogelijkheden voor de toekomst worden gesuggereerd.

De succesvolle ontwikkeling van een kunstlever is deels afhankelijk van de mogelijkheid om een groot aantal hepatocyten met een goed gedifferentiëerde functie voor een langere periode in leven te houden. Microcarriers zijn veelvuldig gebruikt om de hoeveelheid menselijke cellen, die voor een goede functie afhankelijk zijn van aanhechting, per volume te vergroten. In *hoofdstuk 3* wordt een manier beschreven om optimale kweekomstandigheden voor hepatocyten aangehecht aan microcarriers te creëeren. Hepatocyten, gekweekt op met gelatine bedekte microcarriers en omgeven met type I collageen, lieten een albumine en ureum productie zien, die vergelijkbaar is met de productie in de kleinere kweeksystemen. Bovendien was de albumine productie vergelijkbaar met de in vivo albumine productie. Derhalve is gebleken, dat een goede lever-specifieke functie verkregen kan worden door hepatocyten te kweken op microcarriers in type I collageen. Dit suggereert, dat het gebruik van microcarriers een mogelijkheid biedt, om een hepatocyten-kweek systeem op grote schaal te creëeren met een hoog en stabiel niveau van lever-specifieke functie.

Transgene varkenslevers worden gebruikt om de haalbaarheid van extracorporele leverperfusie bij de behandeling van patiënten met acuut leverfalen te bestuderen. Echter, het is onduidelijk of niet-immunologische factoren de kwaliteit van de ex vivo perfusie beperken. In *hoofdstuk 4* wordt een autogeen varkensleverperfusiemodel beschreven, waarin de haemodynamische en metabole aspecten van het ex vivo systeem worden bestudeerd. Microscopisch onderzoek laat veneuze stase zien voornamelijk in de periferie van de lever; hoewel een goede functie van de lever verkregen werd, werd een toenemende concentratie van lactaat gemeten, correlerend met haemodynamische instabiliteit van het varken na ongeveer 3 uur perfusie. Dus, hoewel de ex vivo lever adequate functionele ondersteuning biedt, toont dit autogene model aan dat onafhankelijk van de immunologische reacties, physiologische parameters de periode van effectief gebruik van de ex vivo lever kunnen beperken.

Tot nu toe is effectieve tijdelijke ex vivo leverperfusie toegepast zonder immunologische problemen. Van xenotransplantatie is bekend, dat complement een belangrijke rol speelt in de hyperacute afstoting, en ook weten we, dat patiënten met acuut lever falen een significant verlaagd hoeveelheid circulerend complement hebben. In *hoofdstuk 5* hebben we de mogelijkheid van de acceptatie van een discordant xenotranplantaat bestudeerd in ratten met leverfalen geïnduceerd door galactosamine. En inderdaad, een verlengde xenotransplantaat overleving wordt gevonden in deze ratten, waarschijnlijk door een significante daling van het circulerend complement zoals bekend bij complement gedepleteerde ratten door cobra venom factor. Dit betekent, dat het gebruik van levers van varkens transgeen voor complement regulerende eiwitten wellicht niet noodzakelijk is om ongecompliceerde korte termijn extracorporele leverperfusie mogelijk te maken.

De lever is minder gevoelig voor antilichaamgemedieerde schade. Bijvoorbeeld worden geen verschijnselen van klassieke hyperacute afstoting gezien in het discordante cavia-naar-rat transplantatie model. Discordantie werd daarom nagebootst in een gesensitiseerd hamster naar rat model. Predominantie van of IgG of IgM antidonor antilichamen werd gecreëerd, gebruik makend van verschillende immunisatie protocollen. Transplantatie van hamsterhart en –lever in beide sensitisatie modellen onthulde een verschil in afstotingspatroon tussen beide organen. Zoals beschreven in *hoofdstuk 6* werd er een predominantie gevonden van IgG ten opzichte

van IgM wat betreft hyperacute afstoting van de lever. Dit verschil in rejectie werd niet gevonden bij harttransplantaten.

Het werkingsmechanisme van hyperacute afstoting in xenogene extracorporele leverperfusie is onduidelijk. Om de aspecten van de humorale reactie beter te besturen en het mogelijke voordeel van een transgene lever boven een normale lever te analyseren hebben we de functie en immunopathologische kenmerken van de geïsoleerde normale en transgene rattenlever geperfundeerd met humaan serum bestudeerd. In *hoofdstuk 7* wordt beschreven, dat hoewel histologisch minder weefselschade en minder C3c-neerslag werd gevonden in de levers transgeen voor humaan DAF, geen significant verschil in functie werd gemeten. Deze resultaten suggereren dat de verminderde weefselschade en de mogelijk daaraan verbonden verminderde productie van cytokines veroorzaakt door transgenese wellicht een langer effectief gebruik van de extracorporele lever mogelijk maken.

Voor de veilige toepassing van extracorporele lever ondersteunende systemen moeten nog vele horden genomen worden, o.a. de immunologische implicaties van deze technieken, bijvoorbeeld de host versus graft reactie en vice versa, en de mogelijke gevaren voor de gezondheid van de patient met leverfalen behandeld met een xenogeen systeem. In *hoofdstuk 8* worden deze problemen besproken en mogelijke oplossingen voor korte en lange termijn ter discussie gesteld.

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CURRICULUM VITAE AUCTORIS

Hein Stockmann was born on May 25th, 1966 in Rotterdam, the Netherlands. After finishing secondary school (Gymnasium at the Citycollege Emmaus-Franciscus, Rotterdam) in 1984, he went to Medical School at the University of Amsterdam, Amsterdam, the Netherlands. As part of the graduation program he spent a period at the department of History of Medicine at the Free University of Amsterdam (History of Medicine, Prof. dr. M.J. van Lieburg). During graduation he studied History of Art and Archeology at the University of Amstrdam for one year and worked for 4 months at the Neurology Department and the Clinical Research Center of the Hospital of the University of Pennsylvania, Philadelphia, U.S.A. (Neurology, Prof. dr. K.H. Fischbeck).
He graduated in 1992 from medical School and subsequently served in the Dutch Royal Army as a medical doctor and instructor at the Dutch Education Center of Military Medicine in Hilversum, the Netherlands. From August 1993 until January 1996, he worked as a Surgical Research Fellow at the Shriner Burns Institute and the Massachusetts General Hospital, Harvard Medical School, Boston, U.S.A. (Surgery, Prof. dr. R.G. Tompkins and Prof dr. M.L. Yarmush).
In March 1996, he started his residency in General Surgery at respectively the Surgical Departments of the University Hospital Rotterdam, Rotterdam, the Netherlands (Prof dr. J. Jeekel, head of department, Prof dr. H.A. Bruining and Prof dr. H.J. Bonjer) and the Sint Franciscus Gasthuis, Rotterdam, the Netherlands (dr. J.C.J. Wereldsma and dr. C.H.A. Wittens). In this period he worked at the Laboratory of Experimental Surgery and the Erasmus University of the University Hospital Dijkzigt, Rotterdam, the Netherlands (dr. R.L. Marquet and dr. J.N.M IJzermans) to finish this dissertation.