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Internal Bioartificial Liver With Xenogeneic Hepatocytes Prevents Death From Acute Liver Failure

An Experimental Study

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Objective
To demonstrate that a bioartificial liver, using allogeneic or xenogeneic hepatocytes protected from rejection by a semipermeable membrane, could prevent death from acute liver failure.

Summary Background Data
An implantable bioartificial liver using isolated hepatocytes could be an alternative to orthotopic liver transplantation to treat patients with acute liver failure. It could serve either as a bridge until liver transplantation or as the main treatment until recovery of the native liver. However, allogeneic or xenogeneic hepatocytes that could be used in clinical applications are spontaneously rejected.

Methods
Acute liver failure was induced in rats by 95% liver resection. Twenty-five million hepatocytes harvested in rats (allogeneic) or guinea pigs (xenogeneic) were encapsulated in a semipermeable membrane to protect them from rejection. The hollow fibers containing hepatocytes were transplanted into the peritoneum of recipient rats. Survival rates were compared between rats transplanted or not with hepatocytes.

Results
In groups not transplanted with viable hepatocytes, 73% to 93% of rats died after 95% liver resection. The mortality rate was reduced to 39% in rats transplanted with allogeneic hepatocytes and 36% in rats transplanted with xenogeneic hepatocytes. The bioartificial liver could be removed 1 month after transplantation, when regeneration of the native liver was complete. Allogeneic and xenogeneic hepatocytes remained viable.

Conclusions
The implantable bioartificial liver was able to prevent death in this model of acute liver failure. This could be an important step toward clinical application of the method.

Spontaneous survival of patients with fulminant hepatic failure is 15% to 25%.[1] Although orthotopic liver transplantation has now become the treatment of choice for acute liver failure,[2–4] with a survival rate of 65% to 70% after 1 year, it remains limited by the shortage of donors and requires lifelong immunosuppression, with its own side effects. However, when the condition of the patient with acute liver failure deteriorates rapidly, ABO-incompatible or poor-quality livers could be used, leading to a higher risk of primary nonfunction and retransplantation.[5,6] Furthermore, orthotopic liver transplantation leaves no chance of spontaneous recovery of the native liver function, which occurs in some cases.[7]

It would be important to find alternative methods that would either replace orthotopic liver transplantation or would allow the patient to wait for a suitable donor while preventing the occurrence of irreversible neurologic damage. Encouraging results have been reported with auxiliary

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transplantation of a partial liver.[8,9] A bioartificial liver using isolated hepatocytes is another alternative. The main advantages of the bioartificial liver are that it is immediately available when needed and does not compromise the chances of the native liver to regenerate; moreover, orthotopic liver transplantation remains a possibility if needed. The extracorporeal bioartificial liver, in which hepatocytes are inserted in an external device, has been more extensively studied both in animals[10,11] and in humans[12–14] than the implantable bioartificial liver. The implantable bioartificial liver offers the theoretical advantage of greater technical simplicity,[15,16] and has been used recently in humans.[17]

The concept of an auxiliary liver using isolated hepatocytes has been validated in animals with syngeneic hepatocytes. However, allogeneic or xenogeneic hepatocytes are invariably rejected,[18,19] even in the presence of immunosuppressive therapy.[20,21]

Microencapsulation of cells with a semipermeable membrane, allowing metabolic exchanges but protecting the cells from the immune response of the host,[22] was first tested for transplantation of pancreatic islets.[23] Success has been obtained in humans with type I diabetes.[24,25] Alginate–polylysine membranes[26–29] have been used for encapsulation of hepatocytes. A good biocompatibility of alginate with microencapsulated hepatocytes and surrounding tissues was observed. However, immunoprotection was partial, and fibrosis developed around the capsules.[29]

We have developed a new semipermeable membrane derived from a copolymer of a dialysis membrane AN 69 (Hospal, France) that was transformed into a hydrogel.[30] We have previously reported that allogeneic hepatocytes inserted in hollow fibers made of this material could survive at least 3 months in the peritoneum of recipient rats. At that time, the cells remained viable and functional.[31]

The aim of the present study was to demonstrate that such an implantable bioartificial liver can prevent death of animals in a model of acute liver failure, and that xenogeneic hepatocytes were as efficient as allogeneic hepatocytes in improving survival.

MATERIALS AND METHODS

Animals

Inbred male Wistar-Furth ($RT1^u$) rats (Iffa Credo, l'Arbresle, France), and Lewis ($RT1^e$) rats weighing 280 to 320 g were used as recipients. Inbred male DA ($RT1^a$) rats weighing 250 g were used as allogeneic donors. Guinea pigs (Hartley Dunkin/BP) weighing 350 g were used as xenogeneic donors of hepatocytes. All animals were fed a standard pellet diet and given water *ad libitum* and were kept in the animal unit for at least 2 days before the experiments. All the experiments were conducted according to local institutional guidelines for the care and use of laboratory animals.

Hepatocyte Isolation

Hepatocytes were isolated by *in situ* liver perfusion and enzymatic collagenase digestion using a method described by Berry and Friend,[32] modified by Seglen.[33] Under light ether anesthesia, a median celiotomy and cannulation of the portal vein were performed. The inferior vena cava was ligated just above the renal vein and then was cannulated close to the heart. The liver was perfused at 37°C and pH 7.6 with 400 ml calcium-free phosphate-buffered saline (Merck Laboratories, Darmstadt, Germany) through the portal vein. Then the liver was perfused with 300 ml collagenase H (Boehringer, Meylan, France) solution at a constant flow of 15 ml/minute. The softened liver was then excised and hepatocytes were separated from the connective liver tissue by gentle agitation. The resulting cell suspension was filtered through a layer of sterile gauze. The cells were washed twice, suspended in HAM F12 culture medium (Gibco, Mannheim, Germany) and counted. Cell viability was determined by the erythrosine exclusion test. Only suspensions with cell viability of $\geqslant 85\%$ were used for encapsulation and transplantation. After hepatocyte isolation, the final pellet was resuspended in HAM F12 culture medium supplemented with glucose (3 g/l), bovine insulin (260 IU/l), dexamethasone (1.6 μg/ml), penicillin (100 IU/ml), and streptomycin (100 μg/ml) at a concentration of 10^7 cells/ml. Dead hepatocytes to be used in control group 4 were obtained by heating the cell suspension at 50°C for 50 minutes, and the absence of viable cells was controlled by the erythrosine exclusion test.

Encapsulation

The semipermeable membrane used for macroencapsulation was a hydrogel produced in our laboratory[30] from a copolymer of acrylonitrile-methallyl-sodium sulfonate (AN 69). The hydrogel contains 83% water and has a molecular cutoff between 150 and 165 kD. Hollow fibers (HFs) were constructed with this polymer with an internal diameter of 0.8 mm and a wall thickness of 0.1 mm.[30] HFs decontaminated with peracetic acid and washed with sterile saline were filled with 0.5 ml suspension containing 5×10^6 hepatocytes per meter. Extremities of the HFs were closed with surgical clips (9.75; Merlin Medical, Lyon, France).

Surgical Procedure in Recipient Rats

Under light ether anesthesia, a median celiotomy was performed and 5 meters of HF containing 25×10^6 viable hepatocytes was implanted intraperitoneally in rats in groups 5, 6, and 7. Rats did not receive any immunosuppression, even after transplantation of allogeneic or xenogeneic cells. Ninety-five percent liver resection were performed as described previously (Fig. 1).[34] The left lateral, median, and right lateral lobes and the anterior part of the caudate lobe were removed. The percentage of liver resec-

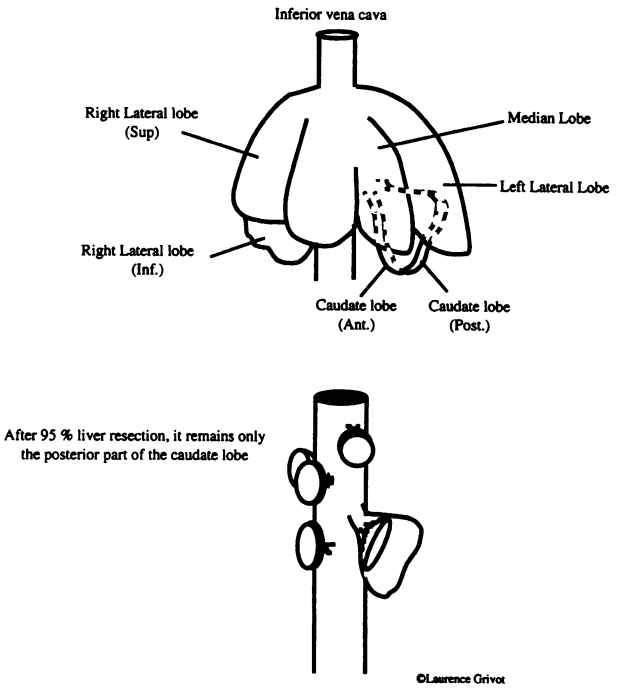

Figure 1. Model of acute liver failure: 95% liver resection in rat. Resection of the right, median, and left lobes and the anterior part of the caudate lobe.

tion was controlled in 16 rats who died during the 24 hours after surgery, before liver regeneration (94.1 ± 1.35%, mean ± standard deviation). Liver resection was performed 7 days after transplantation of hepatocytes. Hypoglycemia was corrected by giving 20% glucose in the drinking water, coupled with repeated intraperitoneal injections of 5% glucose adapted to glycemia (2 ml for glycemia <2.8 mmol/l, 1 ml for glycemia 2.8 to 5.6 mmol/l).

Metabolic Assays

Glycemia was controlled on blood samples taken from the tail with a Glucometer (Bayer Diagnostic, Puteaux, France) 0, 2, 4, 6, 8, 10, 14, 18, and 24 hours after 95% liver resection. Before and 24 hours after subtotal liver resection, in groups 1, 3, and 5, prothrombin time (PT) and factor VII activity were measured on citrated plasma using Thromborel S (Hoechst-Behring) as thromboplastin reagent; the PT is expressed in seconds. Ratios of PT and factor VII activity between time 0 and time 24 hours (PT_{24}/PT_0 and VII_{24}/VII_0) were calculated.

Experimental Design

Control Groups

Two groups of rats, groups 1 and 2, received 95% liver resection only. Group 1 comprised 13 Wistar-Furth rats and served as a control group for group 5 (in which Wistar-Furth hepatocytes were transplanted to Wistar-Furth recipients [syngeneic transplantation]) and for group 7 (in which guinea pig hepatocytes were transplanted to Wistar-Furth recipients [xenogeneic transplantation]). Group 2 comprised 15 Lewis rats and served as a control group for group 6 (in which DA hepatocytes were transplanted to Lewis rat recipients [allogeneic transplantation]).

Two other control groups were used. Group 3, 15 Wistar-Furth rats, received 95% liver resection 7 days after intraperitoneal implantation of 5 meters of HF filled with culture medium without hepatocytes. Group 3 rats served as a control group for groups 5 and 7, in whom syngeneic and xenogeneic hepatocytes were transplanted, and were used to determine if any effect on survival could be observed after administration of culture medium without hepatocytes. Group 4, 15 Lewis rats, received 95% liver resection 7 days after transplantation of 25×10^6 encapsulated DA hepatocytes heated at 50 °C for 50 minutes. Group 4 rats served as a control group for group 6, in whom allogeneic hepatocytes were transplanted, and were used to determine if any effect on survival could be observed with dead hepatocytes.

Groups With Transplantation of Viable Hepatocytes

Group 5 comprised 16 Wistar-Furth rats that received 95% liver resection 7 days after transplantation of 25×10^6 viable encapsulated Wistar-Furth hepatocytes (syngeneic transplantation). Group 6 comprised 23 Lewis rats that received 95% liver resection 7 days after transplantation of 25×10^6 viable DA encapsulated hepatocytes (allogeneic transplantation). Group 7 comprised 14 Wistar-Furth rats that received 95% liver resection 7 days after transplantation of 25×10^6 viable encapsulated guinea pig hepatocytes (xenogeneic transplantation).

In all surviving animals, HFs were explanted 1 month after liver resection under light ether anesthesia for histologic study of transplanted cells. The abdomen was carefully closed and the rats were kept alive for 1 more month before being killed. Autopsies were performed to evaluate regeneration of the native liver.

Morphologic Studies

HFs removed from the peritoneum were divided into small fragments and fixed in Carson solution. Light microscopy was performed on paraffin-embedded sections stained with hematoxylin and eosin or Masson trichrome. Semithin (0.5 μm) and ultrathin (600 Å) sections on Carson-fixed, epon-embedded HFs were done for ultrastructural examination. Semithin sections were stained with toluidine blue and examined with a light microscope. Ultrathin sections were contrasted with uranyl acetate and lead citrate and examined with a transmission electron microscope (Zeiss EM 10).

Statistical Analysis

Survival in the different groups was computed by the Kaplan–Meier method; groups were compared using the

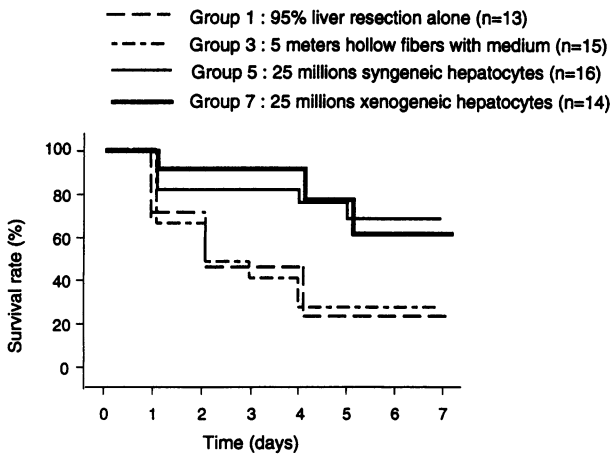

Figure 2. Survival rate after 95% liver resection of Wistar-Furth rats transplanted or not transplanted with syngeneic or xenogeneic hepatocytes. Group 5 rats underwent syngeneic transplantation with 25 × 10^6 encapsulated Wistar-Furth hepatocytes. Group 7 rats underwent xenogeneic transplantation with 25 × 10^6 encapsulated guinea pig hepatocytes. Group 1 rats (control group) underwent 95% liver resection. Group 3 rats (control group) underwent 95% liver resection and were transplanted with empty HFs. $p < 0.05$ for group 5 or 7 *vs.* group 1 or 3.

log-rank test. Survival at day 7 after liver resection were also compared using the chi square test. The differences were considered significant at $p < 0.05$.

RESULTS

Survival

Control Groups

After 95% liver resection, <30% of rats survived, and the rate was not improved by transplantation of empty fibers or fibers filled with dead hepatocytes. After 7 days, the survival rates in groups 1 and 2 (Wistar-Furth and Lewis rats with a 95% liver resection only) were respectively 23% (3/13) and 7% (1/15). The survival rate was 27% (4/15) after 7 days in group 3 (Wistar-Furth rats transplanted with HFs containing culture medium), and 20% (3/15) in group 4 (Lewis rats transplanted with heated allogeneic hepatocytes). Differences in the rates of survival between these groups were not statistically significant.

Groups Transplanted With Viable Hepatocytes

After 7 days, the survival rates were 69% (11/16) in group 5 (Wistar-Furth rats transplanted with encapsulated syngeneic hepatocytes), 61% (14/23) in group 6 (Lewis rats transplanted with encapsulated DA hepatocytes), and 64% (9/14) in group 7 (Wistar-Furth rats transplanted with encapsulated guinea pig hepatocytes).

In groups 5 and 7 (Wistar-Furth rats that had received syngeneic and xenogeneic hepatocytes), the survival rate after 7 days was significantly improved when compared with control Wistar-Furth rats who had received 95% liver resection alone (group 1) or transplantation of HFs filled with culture medium only (group 3)—69% and 64% *versus* 23% and 27% (chi square = 10.2; ddl = 3; $p = 0.01$; $p < 0.05$ for group 5 or 7 *vs.* group 1 or 3, using the log-rank method; Fig. 2).

In group 6 (Lewis rats that had received allogeneic hepatocytes), the survival rate after 7 days was significantly improved when compared with group 2 (control Lewis rats with 95% liver resection alone) or group 4 (rats transplanted with dead allogeneic hepatocytes—61% *versus* 7% and 20% (chi square = 13.7; ddl = 2; $p = 0.001$; $p < 0.01$ for group 6 *vs.* group 2 or 4, using the log-rank method; Fig. 3).

Hemostasis

PT_{24}/PT_0 ratios were 2.9 ± 1.5 in group 1, 2.8 ± 1.1 in group 3, and 3.6 ± 1.9 in group 5. There were no statistical differences between groups ($p = 0.7$). VII_{24}/VII_0 ratios were similar, at 1.6 ± 0.2 in groups 1, 3, and 5.

Morphologic Studies

In surviving animals transplanted with syngeneic, allogeneic, or xenogeneic encapsulated hepatocytes, explantation of HFs was easy because they were free of adhesions to adjacent organs in the peritoneal cavity. In surviving animals, the regeneration of the native liver was complete: the posterior part of the caudate lobe had increased to match the volume and the weight of a normal liver (9 to 10 g for a rat weighing 300 g). All rats survived after explantation of the HFs.

Light microscopy revealed that most tubes were intact. Hepatocytes looked well preserved. The nucleus had a normal aspect, with some cells binucleated. The cytoplasm was

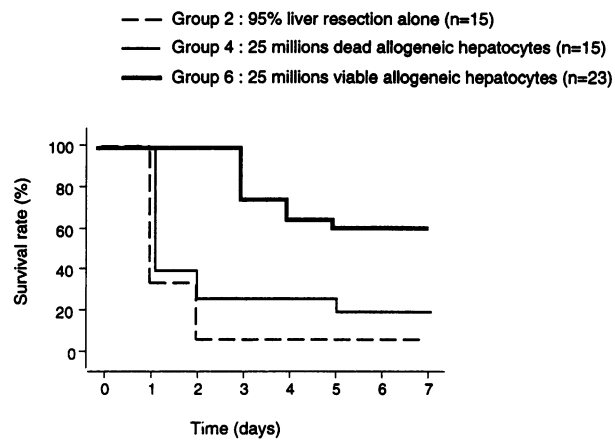

Figure 3. Survival rate after 95% liver resection in Lewis rats transplanted or not with allogeneic encapsulated hepatocytes. Group 6 rats underwent allogeneic transplantation with 95% liver resection with 25 × 10^6 encapsulated DA hepatocytes. Group 2 rats (control group) underwent 95% liver resection. Group 4 rats (control group) underwent 95% liver resection and were transplanted with heated allogeneic hepatocytes. $p < 0.01$ for group 6 *vs.* group 2 or 4.

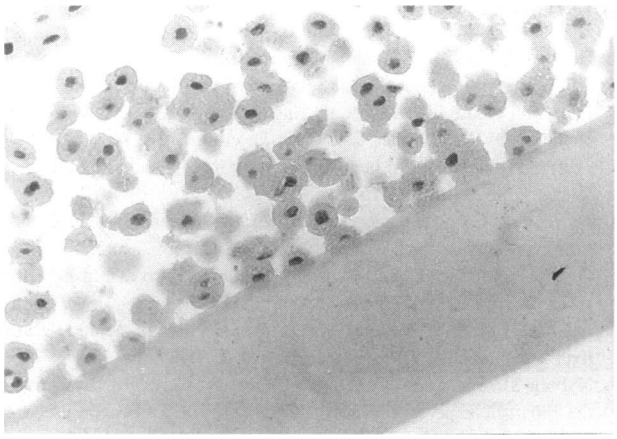

Figure 4. HFs containing xenogeneic hepatocytes, removed from the peritoneum of a surviving recipient rat 1 month after liver resection. Light microscopy on paraffin-embedded sections. Viable hepatocytes are present in the HFs. Some of them are binucleated.

well preserved, with the presence of organelles. Hepatocytes were adherent to the hydrogel or to other hepatocytes. The histologic appearance of the hepatocytes was the same whatever type of hepatocytes had been transplanted. No inflammatory cells were seen inside the HFs (Fig. 4).

DISCUSSION

This study demonstrates that the temporary use of an implantable bioartificial liver can markedly improve survival in a reliable model of acute liver failure. Results were similar whether encapsulated hepatocytes were syngeneic, allogeneic, or xenogeneic. Cells were well immunoprotected by the semipermeable membrane and remained viable despite the absence of any immunosuppression. After spontaneous regeneration of the host liver, the bioartificial liver could be explanted, allowing prolonged survival of the animals.

Although orthotopic transplantation has become the recognized treatment for acute liver failure,[2,3,35] the rationale for developing a bioartificial liver is based on the shortage of organ donors and the need for lifelong immunosuppression in recipients. It is likely that xenogeneic hepatocytes will be available in larger quantities and with shorter delays than human hepatocytes, because harvested livers are used in priority for organ transplantation and it may be difficult to harvest a sufficient amount of cells from a living donor.

Little is known about the immunogenicity of xenogeneic hepatocytes. Xenogeneic transplantation of whole organs is followed by hyperacute rejection in a few minutes or hours. After isolated xenogeneic hepatocytes are transplanted into the spleen[19] or peritoneal cavity,[36] they are rejected within a few days. The recipient's natural antibodies and complement are involved in the rejection of isolated xenogeneic cells, but the effect is slower than with whole organs because there is no vascular thrombosis. A cellular response involving helper and cytotoxic T cells could also be involved. In a pig-into-rabbit combination, cyclosporine provided adequate immunosuppression, with survival times >60 days, whereas hepatocytes were rejected in the absence of immunosuppression. This success could be related to the low target antigen expression by hepatocytes, to the secretion of immunosuppressive substances by hepatocytes, or to the low level of natural antibodies in this combination.[37] However, one of the goals of cellular transplantation is to avoid the complications of long-term immunosuppression.

The concept of encapsulation of hepatocytes in a semipermeable membrane is more appealing. The principle, originally developed for islet cells and applied to hepatocytes by Chang,[38] is to surround the cells with a membrane that allows their functional activity but protects them from the attack of immunocompetent cells and proteins. Several types of materials have been tested for encapsulation, but most of them induce inflammation and a fibrotic reaction around the cells because of insufficient immunoprotection. We have developed a semipermeable membrane for encapsulation of hepatocytes from a dialysis membrane, AN 69.[30] Previous studies have shown that this membrane, a hydrogel with 83% water content, was compatible both with the peritoneum and the cultured hepatocytes.[30] The cutoff of the pores is 150 Kd, thus preventing lymphocytes and large proteins such as immunoglobulins or complement proteins, which are involved in the rejection of xenogeneic cells, from entering the capsule and coming in direct contact with hepatocytes. Allogeneic hepatocytes, protected by this membrane and transplanted in recipient rats for 3 months, remained viable and retained functional activity.[31] Once in the peritoneum, HFs are easy to localize and can be explanted when they are no longer useful.

A surgical model of acute liver failure was preferred to a toxic model because it is more reproducible and induces only liver damage. Subtotal hepatectomy with 95% liver resection is associated with a mortality rate of 80% to 90%,[34] similar to the mortality rate of patients with fulminant or subfulminant hepatitis.[1,2] It induces hepatic coma, the PT drops to <20%, and the animals die of liver failure. In less extensive liver resections in rats, death is mainly caused by hypoglycemia and can be prevented by the administration of glucose, titrated to the level of glycemia.[39–41] These models thus do not appear well adapted to the evaluation of a method of temporary liver support. After 95% liver resection, glucose must be administrated, but it is not sufficient to prevent death from liver failure in most animals.[34] The potential reversibility of this model is another similarity with the clinical situation. If the animals can be kept alive during the acute phase of hepatic failure, spontaneous regeneration of the liver can occur, allowing indefinite survival. In humans with fulminant hepatic failure, complete recovery can also be observed. A temporary hepatic support has prevented severe neurologic disorders or other irreversible complications during the acute phase of liver failure.

Prevention of death was observed only after transplantation of viable hepatocytes. Injection of encapsulated dead hepatocytes or empty tubes had no effect. It is difficult to know whether transplanted hepatocytes acted directly as an auxiliary liver support or by stimulating native liver regeneration. The number of hepatocytes necessary to prevent death suggests a direct effect. Twenty-five millions hepatocytes were inserted into the HFs and transplanted into the rats. Preliminary studies have shown that the transplantation of more cells did not improve the effect. This amount corresponds to approximately 5% of the total amount of hepatocytes that can be isolated from the liver of a 300-g rat. Because 5% of the cells were left in place after 95% liver resection, 7% to 10% of the total liver mass was sufficient to allow survival of the animals. This is in accordance with the observation that liver resections leaving 10% of the liver mass are compatible with life when hypoglycemia is prevented. In humans, it is generally considered that 20 to 50 billion viable hepatocytes would be necessary to provide sufficient liver support.

Other observations suggest a direct effect of hepatocytes. Hepatocytes transplanted in HFs remain differentiated and can synthesize albumin 3 months after transplantation.[31] We have previously shown that hepatocytes transplanted into the spleen of rats could reverse the neurologic disorders associated with hepatic encephalopathy.[42] However, intraperitoneal hepatocytes cannot replace all liver functions because of the absence of biliary output in this model. Moreover, proteins with a molecular weight larger than the size of the pores of the membrane, such as some proteins of coagulation, cannot be released, whereas smaller growth factors can. This probably explains the rapid regeneration of 95% of the liver volume within 1 month.[43–47] Thus, it is likely that both mechanisms—direct action of transplanted hepatocytes and indirect stimulation of native liver—are involved.

Although encapsulated liver cells could survive and function for at least 1 month, the pore diameter is likely to allow the release of secreted proteins, as well as proteins shed by the cells, into the recipient's circulation. The long-term consequences of the possible immunization against those proteins, in terms of inactivation of their biologic properties, formation of deleterious complexes, or progressive destruction of the graft, remain to be demonstrated.

In conclusion, this study demonstrated that an internal bioartificial liver with 25×10^6 viable hepatocytes immunoprotected by encapsulation in semipermeable HFs provided a metabolic support that was able to improve survival after 95% liver resection. Encapsulation allowed us to use allogeneic or xenogeneic hepatocytes without immunosuppression, with results similar to those obtained by syngeneic transplantation. This could be an important step toward clinical application of the method in humans with fulminant liver failure.

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