mozygotes 3/3 and 4/4 at all temperature and *p*H conditions studied (*22*). The one kinetically effective homozygote, 2/2, is roughly equal in initial V_{max}/K_m ratio to 3/4, but much less stable to high body temperature than any other common genotype, so that it will not maintain its initial kinetic capacity with exposure to high body temperature (*22*). Thus, the genotypes considered to be kinetically favored are: 3/4, 2/3, 2/4, 2/5, and 3/5. The heterozygote 4/5 is kinetically disadvantaged.

29. We use the term "semispecies" in the sense of E. Mayr [*Animal Species and Evolution* (Harvard Univ. Press, Cambridge, MA, 1963)]: closely related allopatric populations that are not yet fully reproductively isolated, though they differ in many heritable characteristics.
30. Adjustment of solar absorptivity and insulating "fur" in relation to local thermal niche structure (*10, 23*) renders these populations very similar in their thermally dependent flight profiles through the day at the seasonal times in which sampling took place (see also *25*).
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Replacement of Liver Function in Rats by Transplantation of Microcarrier-Attached Hepatocytes

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Isolated hepatocytes, harvested from normal rat livers by portal vein collagenase perfusion, can be attached to collagen-coated dextran microcarriers and transplanted by intraperitoneal injection into rats. Survival and function of the transplanted hepatocytes have been demonstrated in mutant rats lacking bilirubin–uridine diphosphate glucuronosyltransferase activity (Gunn strain) and rats with inherited lack of plasma albumin (Nagase analbuminemia rat strain). This simple technique promises to be useful in the treatment of acute liver failure in humans.

ALTHOUGH VARIOUS THERAPIES have been developed for treating severe acute liver insufficiency (*1–5*), none but whole liver transplantation have proven clinically useful (*6, 7*). During the past few years, several investigators attempted to develop techniques for transplanting hepatocytes (*8–10*), which would be simpler and less expensive than transplanting whole organs, would allow the use of living related donors, permit the use of a single donor organ for multiple recipients, and make possible the storage of hepatocytes for future use. However, long-term survival and function of the transplanted hepatocytes in vivo have not been unequivocally demonstrated (*8–12*). Mutant rats (Gunn strain) with inherited deficiency of bilirubin–uridine diphosphate glucuronosyltransferase (UDPGT) activity lack conjugated bilirubin in bile and exhibit lifelong nonhemolytic unconjugated hyperbilirubinemia (*13*). Gunn rats have been used to evaluate the success of hepatocyte transplantation (*11, 12, 14*): after intrasplenic injection of normal hepatocytes, only transient biliary excretion of relatively minor amounts of conjugated bilirubin was observed (*14*). Thus, so far, the results of hepatocyte transplantation have been largely disappointing.

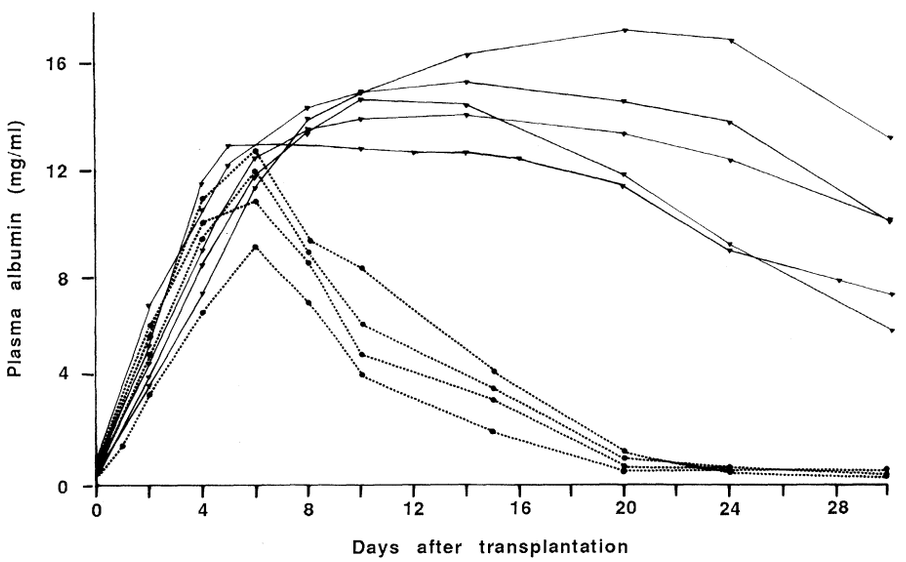

Fig. 1. Plasma albumin concentration in NAR rat recipients of transplanted allogeneic normal hepatocytes. Solid lines represent rats treated with cyclosporin A; interrupted lines represent untreated recipients.

We describe a technique in which isolated rat liver cells are attached to collagen-coated dextran beads and are intraperitoneally injected into rats. Two mutant rat strains, Gunn rats and Nagase analbuminemic rats (NAR rats), which have an inherited defect of albumin synthesis and only a trace of albumin in plasma (*15*), were used as recipients to evaluate the survival and function of the transplanted hepatocytes. The function of transplanted hepatocytes was demonstrated by the appearance of albumin in the plasma of analbuminemic recipients and conjugated bilirubin in the bile of recipient Gunn rats.

Rat liver cells were released by portal vein collagenase perfusion of donor rats (*16*); hepatocyte-enriched fractions were prepared by differential centrifugation (*17*). Between 80 and 95% of the harvested hepatocytes were viable, as assessed by trypan blue exclusion. Collagen-coated microcarriers (Cytodex 3, Pharmacia Fine Chemicals, Uppsala, Sweden) were hydrated in sterile phosphate-buffered saline (PBS) and incubated at 37°C for 90 minutes. The microcarriers were washed, resuspended into Dulbecco's minimal essential medium with 20% fetal calf serum, and transferred into 175-ml polystyrene flasks. Hepatocytes were inoculated into the flasks and incubated for 2.5 hours at 37°C in 5% CO_2 to allow cells to attach to the microcarriers. The number of viable cells was always greater than 90% in aliquots that had been treated with collagenase to release the hepatocytes. Subsequently, 1×10^7 cells

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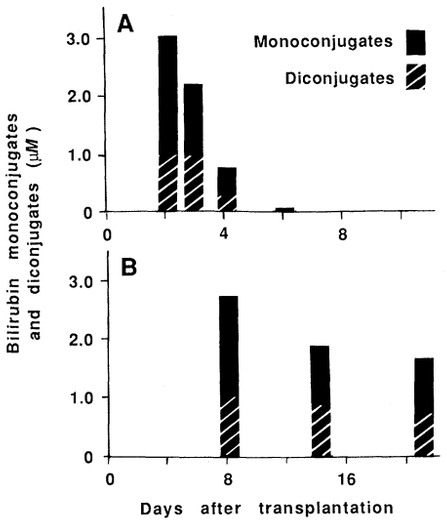

Fig. 2. Bilirubin conjugates in the bile of (A) allogeneic and (B) congeneic Gunn rats that had received transplanted normal rat hepatocytes. Each time point represents an individual animal.

attached to microcarriers were washed twice, resuspended in 8 ml of PBS, and injected intraperitoneally into recipient NAR or Gunn rats.

In selected recipients, the function of the transplanted hepatocytes was demonstrated by radionuclide (technetium-99m diisopropyl iminodiacetate) scanning (*18*) 5 to 6 days after hepatocyte transplantation. The radionuclide was taken up by the microcarrier-attached hepatocytes in the peritoneal cavity 5 minutes after intravenous injection; the liver was visualized 8 to 10 minutes after injection.

In NAR rats, the function of the transplanted hepatocytes was determined by SDS–10% polyacrylamide gel electrophoresis (*19*) of plasma; pure rat albumin was used as a reference. Plasma albumin was quantitated by an enzyme-linked immunosorbent assay (*20*) with a specific antiserum. After transplantation of allogeneic Wistar rat hepatocytes into NAR rats, plasma albumin concentration progressively increased from 0.5 mg/ml to a peak of 10 mg/ml 6 days after transplantation; this rise was followed by a decline (Fig. 1). When the immune systems of the recipient NAR rats were suppressed with cyclosporin A (25 mg per kilogram of body weight) given intragastrically daily for the first 5 days after transplantation, plasma albumin concentration reached a peak of 13 mg/ml 14 days after transplantation and was maintained close to that level until day 30, the last day of the study (Fig. 1). In normal Wistar rats, plasma albumin concentrations ranged from 35 to 40 mg/ml.

To determine the function of the transplanted hepatocytes in the recipient Gunn rats, a cannula was inserted into the bile duct under light ether anesthesia. Starting 1 hour after the rat's return to consciousness, bile was collected on ice in light-protected tubes for 5 hours. Bile pigments were analyzed as underivatized tetrapyrroles by high-pressure liquid chromatography (*21*), through the use of authentic biosynthesized standards; bilirubin monoglucuronide and diglucuronide were identified (*21*). Diconjugated and monoconjugated bilirubin were also independently determined by thin-layer chromatography after alkaline methanolysis of conjugated bile pigments (*22*). In addition, bile pigments were analyzed by thin-layer chromatography after being converted to azodipyrroles by reaction with diazotized ethylanthranilate (*23*) with or without prior treatment with β-glucuronidase (*23*).

When donor hepatocytes from outbred Wistar rats were injected into allogeneic Gunn rats, conjugated bilirubin was detected in the bile of the recipients for as long as 6 days after transplantation (Fig. 2); no conjugated bilirubin was detected in the bile of control Gunn rats. The greatest concentration of conjugated bilirubin was observed 2 to 4 days after hepatocyte transplantation (Fig. 2). To eliminate rejection of donor cells, hepatocytes were harvested from normal congeneic Wistar (RHA) rat donors that, because they are genetically identical to the recipient Gunn rats except for the bilirubin-UDPGT locus, are able to conjugate bilirubin. When hepatocytes from such normal congeneic Wistar rats were transplanted intraperitoneally into Gunn rat recipients, conjugated bilirubin persisted in the bile of recipients (Fig. 2) through day 21, the last day of the study. In congeneic recipients, 30 to 40% of the total bilirubin excreted in bile was conjugated; both mono- and diconjugates were found (Fig. 2). On treatment with diazotized ethylanthranilate, both unconjugated and glucuronidated azodipyrroles were produced; the absence of detectable glucuronidated azodipyrrole after β-glucuronidase hydrolysis indicates that the conjugates were normal 1-*O*-acylglucuronides of bilirubin. Maximum conjugated bilirubin concentration in bile was 3 μ*M*. In normal Wistar rats, conjugated bilirubin concentration in bile ranged from 150 to 175 μ*M*.

Serum bilirubin was markedly reduced in all Gunn rats receiving intraperitoneal microcarrier-attached hepatocyte transplants (Fig. 3); however, the reduction was both greater and more sustained when the recipient Gunn rats were congeneic (Fig. 3). That the decrease in serum bilirubin concentration was out of proportion to the excretion of conjugated bilirubin in bile suggests additional mechanisms of bilirubin elimination,

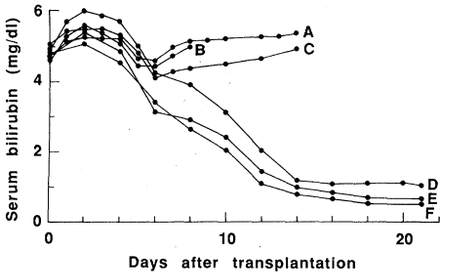

Fig. 3. Serum bilirubin concentrations after intraperitoneal hepatocyte transplantation into representative Gunn rats (A–C) with allogeneic normal hepatocytes and (D–F) with congeneic normal rat hepatocytes.

such as urinary excretion and degradation of bile pigments. Injection of Gunn rat hepatocytes into Gunn rats did not result in the excretion of bilirubin conjugates in the bile of the recipients, and serum bilirubin concentration did not decline.

Injection of collagen-coated microcarriers into the peritoneal cavity was not associated with any apparent toxicity or inflammatory reaction. When the abdominal cavity was explored under ether anesthesia 6 days after transplantation, the injected microcarriers had formed aggregates in the peritoneal cavity. Light and electron microscopic examination revealed hepatocytes attached to the collagen-coated surface of microcarriers or embedded in a network of fibroblasts and connective tissue. New blood vessels had begun to form within the conglomerates.

The long-term fate of intraperitoneally injected microcarriers is yet to be determined. Gross observation of the peritoneal cavity 8 weeks after intraperitoneal administration of microcarriers revealed some microcarrier aggregates with minimal surrounding inflammatory reaction and adhesion formation. The size of individual microcarrier beads was not obviously reduced.

When hepatocytes alone (1×10^7) were injected intraperitoneally into allogeneic or congeneic Gunn rats, conjugated bilirubin was not detectable in the bile of the recipient. Gross examination of the peritoneal cavity beginning 4 days after transplantation revealed no cell aggregates in the peritoneal cavity. These observations indicate the importance of microcarrier attachment in the survival and function of the transplanted hepatocytes.

We have used collagen-coated microcarrier-attached hepatocytes to develop a simple and effective method of intraperitoneal hepatocyte transplantation that allows the survival and function of transplanted cells. Successful intraperitoneal hepatocyte transplantation would provide a promising simple and cost-effective method of treating patients with acute hepatic insufficiency.

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Correction of Murine β-Thalassemia by Gene Transfer into the Germ Line

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A murine β-thalassemia was corrected by the transfer of cloned β-globin genes into the mouse germ line. The cloned mouse β^{maj}-globin gene or the cloned human β-globin gene was introduced into mice deficient in β-globin synthesis because of a deletion of the β^{maj}-globin gene. Both introduced genes produced functional β-globin chains, leading to a reduction in one case, and elimination in another case, of the anemia and associated abnormalities of the red blood cells.

HUMAN β-THALASSEMIA IS A HEREDITARY disease in which insufficient β-globin chains are produced in red blood cells, because of a variety of lesions in the β-globin gene. Although the severity of the resulting anemia varies according to the level of β-globin chains produced, many forms of β-thalassemia lead to childhood fatality. No satisfactory treatment has yet been devised (*1*). A murine model of human β-thalassemia, which has recently been described, may provide a valuable experimental system for the development of new treatments for human thalassemia. The mutation responsible for murine β-thalassemia arose spontaneously in a mouse of the β-globin haplotype Hbb^{d}, which includes the two adult β-globin genes β^{maj} and β^{min}. In the mutant haplotype, $Hbb^{th\text{-}1}$, the β^{maj} gene is entirely deleted but the β^{min} gene remains intact. Animals homozygous for this deletion produce a reduced amount of β-globin, and consequently suffer from a hemolytic anemia similar to a human β-thalassemia of intermediate severity (*2*).

It has been suggested that β-thalassemia might eventually be treated by the insertion of the normal β-globin gene into the hemopoietic stem cells and its consequent expression in red blood cells of affected individuals (*3*). While cloned genes have been successfully introduced into murine hemopoietic cells by means of retroviral vectors (*4*), the efficient expression of a globin gene introduced in this manner has not yet been achieved. In contrast, when introduced into the mouse germ line via the fertilized egg, cloned β-globin genes have been expressed specifically in erythroid cells, often at very high levels (*5, 6*). Although the correction of genetic deficiencies by transfer of genes into the germ line (*7*) is not a feasible strategy for human gene therapy (*3*), we thought it useful to attempt to correct the murine β-thalassemia by this procedure, if only as a preliminary step toward eventual somatic gene therapy for human thalassemia.

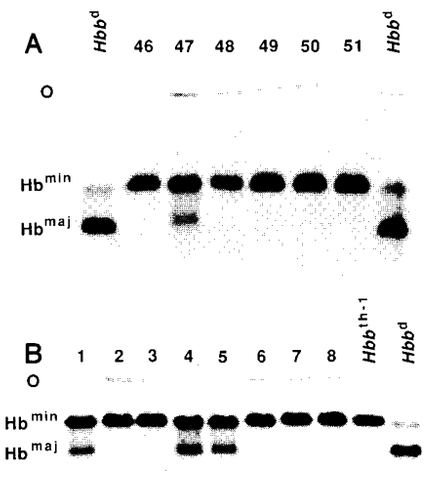

Fig. 1. Expression of the cloned β^{maj}-globin gene in transgenic mice. (A) Cellulose acetate electrophoresis of cystamine-modified hemolysates (*8*) from two normal mice (Hbb^{d}) and six mice (MB46–MB51) developed from homozygous $Hbb^{th\text{-}1}$ zygotes that were microinjected with β^{maj} gene. Mice MB47 and MB51 are transgenic. (B) A similar analysis of eight offspring (1–8) of mouse MB47 mated to a homozygous $Hbb^{th\text{-}1}$ female mouse.

Two different experiments were performed, one utilizing the cloned mouse β^{maj}-globin gene and the other the cloned human β-globin gene. In the first experiment, a 7.0-kb Eco RI fragment containing the mouse β^{maj}-globin gene (*8*) was microinjected into homozygous $Hbb^{th\text{-}1}$ mouse zygotes. A total of 129 eggs were transferred to foster mothers, ten mice were born, and two (mice MB47 and MB51) carried multiple copies of the microinjected gene, as determined by Southern blot analysis. When hemolysates of peripheral blood were analyzed by cellulose acetate electrophoresis (*9*), one of the two transgenic mice (MB47) was found to synthesize a significant amount of β^{maj}-type hemoglobin (Fig. 1A). This animal, a male, was mated to homozygous $Hbb^{th\text{-}1}$ females, and several of the progeny were found to synthesize β^{maj} hemoglobin, indicating that they inherited and expressed the microinjected gene (Fig. 1B). The mouse β^{maj} gene continued to be transmitted as a Mendelian trait and expressed at a similar level in subsequent generations.

While the ratio of β^{maj} to β^{min} hemoglobin is 4:1 in normal mice homozygous for the Hbb^{d} haplotype, and in $Hbb^{d}/Hbb^{th\text{-}1}$ heterozygotes it is 1:1 (*2*), in these transgenic animals the ratio was approximately 1:2. Thus, the introduced β^{maj}-globin genes in this transgenic line produced fewer globin chains than a single endogenous β^{maj} gene.

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