Survival, organization, and function of microcarrier-attached hepatocytes transplanted in rats
(hepatocyte transplantation/microcarriers/Gunn rats/analbuminemic rats/cyclosporin)

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ABSTRACT Hepatocytes harvested by collagenase perfusion of rat liver were attached to collagen-coated microcarriers and injected intraperitoneally into congenic or allogeneic Gunn rat (UDP-glucuronosyltransferase EC 2.4.1.17)-deficient Gunn rats or allogeneic analbuminemic rats. Five days later, the microcarriers were observed to have formed conglomerates chiefly on the anterior surface of the pancreas. Scanning electron microscopy showed hepatocytes attached to the granular collagen-coated surface of the microcarriers and newly formed connective tissue. Light microscopy revealed that the microcarriers formed a lattice with the collagen tissue; hepatocytes were seen within this lattice or on the surface of the microcarriers. Hepatocyte plasma membranes were nucleoside-diphosphatase (NDPase)-positive. Newly formed blood islands, blood vessels containing erythrocytes and leukocytes and NDPase-positive endothelium were observed in close proximity to the hepatocytes and fibroblasts. Transmission electron microscopic examination showed hepatocytes with microvilli and nucleoid-containing peroxisomes with catalase activity. Hepatocytes were present for up to 2 months in congenic recipients, the longest period of observation after transplantation. After normal microcarrier-attached hepatocytes were transplanted into allogeneic Gunn rats, bilirubin glucuronides were present in bile for 6 days. When congenic Gunn rat recipients were used, bilirubin glucuronides were present in bile throughout the study (28 days); this was accompanied by reduction of serum bilirubin concentrations to nearly normal levels. After injection of normal hepatocytes into allogeneic NAR rats, plasma albumin concentration progressively increased for 6 days and then declined. In NAR recipients which were immunosuppressed with cyclosporin A, peak plasma albumin levels were reached in 14 days and persisted nearly at that level throughout the study (28 days).

Severe acute liver failure is associated with dismal prognosis. Among currently available therapeutic procedures, only liver transplantation has been clinically useful. Liver transplantation is limited because of the scarcity of immediately available donors and the requirement for sophisticated technology and support teams. Many of these difficulties would be obviated by a technically easy, successful technique of hepatocyte transplantation. Transportation of hepatocytes (1–3), injection of hepatocyte extracts (1), and hepatocyte culture supernatants have been reported to prolong survival of rats with D-α1,6-galactosamine-induced liver injury (4) and animals with acute liver ischemia (5). Because homozygous Gunn rats lack UDP-glucuronosyltransferase activity for bilirubin, they cannot efficiently excrete bilirubin and, thus, exhibit lifelong nonhemolytic unconjugated hyperbilirubinemia (6–8). Hepatocyte transplantation has been used to impart the transferase activity in Gunn rats (9, 10). However, up to now, long-term function of transplanted hepatocytes was not demonstrated unequivocally.

We describe a technique in which isolated rat hepatocytes, attached to collagen-coated dextran microcarriers, were injected i.p. in homozygous Gunn rats and genetically analbuminemic (NAR) rats. The injected microcarrier-attached hepatocytes form conglomerates in the peritoneal cavity. Light and electron microscopic examination of the conglomerates showed differentiated hepatocytes, newly formed blood vessels, and fibroblasts. Function of the transplanted hepatocytes was shown in Gunn rats by biliary excretion of conjugated bilirubin and reduction of serum bilirubin concentration and in NAR rats by the appearance of albumin in the plasma.

MATERIALS AND METHODS

Male Wistar rats (200–250 g) were purchased from Charles River Breeding Laboratories. Syngeneic Wistar (RHA) rats and congenic Gunn rats, which have identical genetic make-up with the Wistar (RHA) rats except for the bilirubin conjugation locus, were developed by C. Hansen of the National Institutes of Health and maintained as congenic strains at the Albert Einstein College of Medicine (New York, NY). Analbuminemic (NAR) rats, mutants of Sprague–Dawley rats, were provided by S. Nagase (Sasaki Institute, Tokyo, Japan) and maintained at the Albert Einstein College of Medicine. All rats were maintained on a standard laboratory chow (Rodent Chow 5001, Purina) and tap water ad lib in a 12-hr light/dark cycle.

Dextran microcarriers coated with cross-linked type I collagen (Cytodex 3) were obtained from Pharmacia. Collagenase (type 1), chemicals used for perfusion, and bacterial β-glucuronidase were purchased from Sigma. Tissue culture media was from GIBCO. All other chemicals were of the purest available grade.

Preparation of Hepatocytes and Attachment to Microcarriers. Aseptic methods were used for all procedures. Isolation of rat liver cells (10, 11), preparation of hepatocyte-enriched cell population, and attachment to Cytodex 3 were carried out as described elsewhere (12). Aliquots of the microcarriers were treated with collagenase to release the cells, and the viable cells were counted. Microcarrier-attached liver cells, including hepatocytes (1 × 10^8 viable cells), in Dulbecco’s modified Eagle’s medium without fetal calf serum were injected i.p. into recipient allogeneic NAR or Gunn rats or congenic Gunn rats. Several allogeneic NAR recipient rats were treated with cyclosporin A (25 mg/kg of body weight daily intra gastrically for 7 days).

Abbreviation: NDPase, nucleoside-diphosphatase.
Morphological Characterization of Transplanted Hepatocytes. Three to 60 days after transplantation, the peritoneal cavity was examined. The injected microcarriers formed tan conglomerates attached chiefly to the anterior surface of the pancreas. For light microscopy, portions of these conglomerates were excised and fixed in 2.5% gluteraldehyde/0.1 M sodium phosphate, pH 7.4/7.5% sucrose. Approximately 30-μm-thick "Vibratome" sections were prepared and placed in 7.5% sucrose. After methyl green pyronine staining (13) or after incubation for nucleoside-diphosphatase (NDPase) activity (14), the sections were mounted in glycerol gelatin and examined by light microscopy.

Scanning electron microscopy was performed as described (12, 15).

For transmission electron microscopy, the Vibratome sections were embedded in Epon, and sections were examined with en bloc uranyl stain and a Philips electron microscope 300 after fixation and embedding as described elsewhere (16). In some experiments, the electron microscopic examination was preceded by incubation of the Vibratome

![Image 1]
sections with dianminobenzidine for demonstration of catalase activity (17) or for NDPase activity (14).

Bile Pigment Analysis. Bile was collected from recipient Gunn rats through bile duct fistulae, and bile pigments were analyzed by reverse-phase HPLC (18) as described elsewhere (12). For characterization of the conjugated bilirubin excreted in bile, aliquots of the bile were treated with bacterial  )-glucuronidase (12) before bile pigment analysis (19, 20).

**Plasma Albumin Determination.** Plasma albumin was quantitated by densitometry of Coomassie blue-stained NaDodSO4/10% polyacrylamide slab gels after electrophoretic separation of plasma proteins (21) with pure rat serum albumin as the standard. Specificity of the assay was confirmed by immunotransblot experiments (22) with an anti-rat serum albumin (rabbit) antiserum.

**RESULTS**

**Organization and Morphology of Transplanted Microcarrier-Attached Hepatocytes.** *Macroscopic appearance.* Tan conglomerates of microcarrier-attached liver cells were found attached primarily to the anterior surface of the pancreas and surrounded by loops of bowel (Fig. 1 *Upper Left*).

*Light microscopy.* The microcarriers were observed to have formed a lattice with newly formed collagen tissue (Fig. 1 *Upper Right*). Hepatocytes were either attached to the collagen-coated microcarrier beads or were embedded within the connective tissue lattice. Blood islands were observed. New endothelium-lined vascular spaces containing erythrocytes and leukocytes were in close proximity to the hepatocytes (Fig. 1 *Upper Right and Lower*). The new blood vessels appeared within five days after injection of microcarrier-attached hepatocytes and persisted throughout this study (60 days). The plasma membranes of the hepatocytes were NDPase-positive and had a "chicken-wire" appearance, suggesting intercellular contact among the hepatocytes (Fig. 1 *Lower Inset*). The vascular epithelium also showed positive NDPase staining.

*Scanning electron microscopy.* Microcarriers with a granular surface and collagen-coated surface with attached cells and newly formed fibrillar collagen were observed (Fig. 2).

*Transmission electron microscopy.* Hepatocytes with nuclei, smooth and rough endoplasmic reticulum, mitochondria, and lysosomes were observed as in hepatocytes of rat liver (Fig. 3). Bile canaliculi with NDPase activity were observed (Fig. 3). Peroxisomes with nucleoids, characteristic of rat hepatocytes, were present (Fig. 3 *Lower Inset*); these showed catalase activity (Fig. 3 *Upper Inset*). Typical mitochondria, nuclear membranes, rough and smooth endoplasmic reticulum, and Golgi apparatus were also seen. In allogeneic recipients, morphologically intact hepatocytes were observed for only 5 days; after this time these cells showed signs of degeneration and soon could not be identified readily as hepatocytes. In contrast, when hepatocyte rejection was eliminated by using congenic Gunn rat recipients, hepatocytes remained intact for up to 2 months after transplantation (duration of this study).

**Bilirubin Conjugates in the Bile of Gunn Rats Transplanted with Microcarrier-Attached Wistar Rat Hepatocytes.** Bilirubin diglucuronide and monoglucuronide were present in the bile of both congenic and allogeneic Gunn rats after injection of microcarrier-attached normal hepatocytes but not Gunn rat hepatocytes. Bilirubin glucuronides were quantitatively hydrolyzed after incubation with  )-glucuronidase, indicating that they were 1-O-acyl glucuronides. In allogeneic Gunn rats, bilirubin conjugates were present in bile for only 6 days after transplantation; however, in congenic Gunn rat recip-

![Fig. 2. Scanning electron micrograph showing a microcarrier with hepatocytes (arrows). G, granular collagen coating on the microcarrier; C, newly formed collagen fibers.](image-url)
vessels may explain the prolonged survival and function. Rejection of the transplanted hepatocytes apparently affected their longevity in nonimmunosuppressed allogeneic rats. Five days after transplantation in allogeneic recipients, the transplanted cells exhibited signs of degeneration. In contrast, when hepatocyte rejection was eliminated by transplanting in congenic recipients, hepatocytes with well-preserved surface membranes and intracellular organelles were observed for 60 days, the duration of the study.

Mutant rats with specific inborn errors of liver function offer an excellent opportunity to evaluate the function and longevity of transplanted hepatocytes. Transplantation of hepatocytes by intrasplenic injection in Gunn rats has been shown to decrease serum bilirubin concentrations (8, 23). However, bilirubin levels also decreased after intrasplenic injection of Gunn rat hepatocytes, suggesting that decrease in bilirubin levels might have been due to interference with splenic bilirubin production (9). In a recent study, only minor amounts of conjugated bilirubin were found in the bile of Gunn rats after intrasplenic injection of normal hepatocytes; no bilirubin conjugates were present in bile beyond day 6 after hepatocyte transplantation (10). In the present study, using microcarrier-attached hepatocytes, we observed a much higher concentration of bilirubin conjugates in the bile of Gunn rats transplanted with normal hepatocytes; in congenic recipients conjugated bilirubin persisted for at least 4 weeks. The decrease in serum bilirubin concentrations cannot be fully accounted for by the amount of conjugated bilirubin excreted in bile, suggesting excretion of bilirubin through other routes, such as urine, and possibly increased breakdown of bilirubin in Gunn rats, which received microcarrier-attached hepatocyte transplantation. As reported elsewhere, i.p. injection of hepatocytes in Gunn rats without attachment to the microcarriers did not result in the excretion...
of conjugated bilirubin in bile (12). Prolonged survival and function of hepatocytes observed in this study suggests that attachment of hepatocytes to the collagen-coated surface of the microcarriers may improve survival and differentiated function of the transplanted hepatocytes by providing a matrix for cell attachment.

NAR rats have not been used previously for evaluation of the function of transplanted liver cells. The rapid increase of plasma albumin concentration in NAR rats after transplantation of microcarrier-attached hepatocytes may be explained by the finding of newly formed blood vessels in close proximity to the transplanted hepatocytes. Prolonged duration of increased plasma albumin levels in cyclosporin A-treated NAR recipients suggests that cyclosporin A is efficacious in inhibiting rejection of the transplanted hepatocytes.

In experiments to be reported elsewhere, we found that microcarrier-attached rat hepatocytes stored at −80°C for up to 2 months can be transplanted successfully into NAR and Gunn rats. Cryopreservation will allow the storage of microcarrier-attached hepatocytes for use at a future date.

Fig. 4. NaDodSO4/polyacrylamide gel electrophoresis of plasma proteins of a cyclosporin A-treated NAR rat at various intervals after hepatocyte transplantation; rat serum albumin is marked with an arrow. Lanes: A, rat serum albumin; 1-5, sera at days 0, 7, 14, 21, and 28 after transplantation, respectively. Molecular mass is shown ×10^{-3}.

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