New Method of Hepatocyte Transplantation and Extracorporeal Liver Support

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A technique has been developed by the authors that allows hepatocyte attachment on collagen-coated microcarriers resulting in prolonged hepatocyte viability and function both *in vivo* and *in vitro*. Rat hepatocytes were obtained by portal vein collagenase perfusion. Intraperitoneally transplanted microcarrier-attached normal hepatocytes into congeneic Gunn rats were functioning 3–4 weeks later, as shown by the presence and persistence of conjugated bilirubin in recipient bile, sustained decrease in serum bilirubin, uptake of Tc99m-DESIDA, and morphologic criteria. Intraperitoneal transplantation of normal microcarrier-attached hepatocytes into genetically albumin deficient rats (NAR) resulted in marked increase in plasma albumin levels (6 days without and 21 days with Cyclosporin A immunosuppression). Microcarrier-attached hepatocytes transplanted after 2 weeks of storage at −80 C into congeneic Gunn rats were viable and functional as assessed by criteria outlined above. An extracorporeal liver perfusion system was developed using the microcarrier-attached hepatocytes that was capable of synthesizing and conjugating bilirubin and synthesizing liver-specific proteins.

ALTHOUGH VARIOUS THERAPEUTIC MODALITIES have been described for treating severe liver insufficiency, with the exception of whole liver organ transplantation, none has proven to be clinically useful. Liver transplantation is not used widely because of the difficulty in obtaining cadaver donor organs, the technical difficulty and need for sophisticated technology and support teams, and the high cost. Several investigators developed various techniques of hepatocyte transplantation.[1–3] However, up to now, none has shown unequivocally long-term survival and retention of specific hepatocyte functions of the donor cells. Were this to be accomplished, it could serve as the basis for successful clinical application of hepatocyte transplantation. This would be important because it could allow living related donor use and use of a single donor for multiple recipients, and it would be simple and cost-effective, especially if hepatocytes could be stored for later use. We have developed a technique that allows hepatocyte attachment on collagen-coated dextran microcarriers that results in prolonged hepatocyte viability and function both *in vivo* and *in vitro*. We hypothesized that this technique can be used to prolong viability and function of transplanted cells, to construct an extracorporeal liver support system, and to develop a new method of storage of hepatocytes for long periods of time. A series of experiments were carried out to test our hypotheses.

Materials and Methods

Materials

Cytodex 3 collagen-coated dextran microcarriers, peristaltic pump, and tubing were purchased from Pharmacia (Pharmacia Inc., Piscataway, NJ). Collagenase (Type I), beta-glucuronidase, and all chemicals used for perfusion were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Tissue culture media were purchased from Grand Island Biological Company (Grand Island, NY) and Millipore filters were obtained from Nalgene (Nalge Co., Rochester, NY).

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Rat Liver Perfusion with Collagenase

Aseptic methods were used throughout all procedures. Hepatocytes were harvested using the portal vein collagenase perfusion method of Berry and Friend,[4] as modified by Seglen.[5] Two perfusate solutions were prepared from Leffert's buffer.[6] A "pre-wash" consisted of Leffert's buffer, pH-adjusted to 7.4 and filtered through a 20 μm millipore filter. The "after-wash" solution, which was used for cell washing, consisted of Leffert's buffer, pH-adjusted to 7.4 and 1 ml/100 ml 2.7% $CaCl_2$–$2H_2O$. A 0.05% collagenase solution was prepared using Leffert's buffer, pH 7.4 and 1 ml/100 ml 2.7% $CaCl_2$–$2H_2O$. This was filtered through a 45 μm and then a 20 μm Millipore filter.

The perfusion system consisted of a perfusate reservoir connected to a pump through an oxygen-permeable Silastic® tubing. The tubing was run through a water bath maintained at 37 C through which 100% O_2 was vigorously bubbled, using a gas bubbling tube. After running through the bath, the perfusate passed through a standard IVAC air trap and then into the animal. The total volume of the system was 50 ml. The system was sterilized by perfusion with 70% ethanol and washing with sterile Leffert's buffer prior to use.

An adult rat weighing 300–325 g was anesthetized with pentobarbital (6.5 mg/100 g body weight, intraperitoneally), the chest and abdominal hair was clipped, and the skin was cleansed with 70% ethanol. The abdomen was opened through a midline incision, and the portal vein was catheterized using a #18 angiocath (Deseret Medical Inc., Sandy, UT), which was secured with a 3-0 silk tie. The perfusion system, which had been primed with pre-wash, was then connected to the catheter and the pump set at a flow rate of 20 ml/min. The abdominal inferior vena cava was then ligated above the level of the renal veins. The chest was opened in the midline, and the thoracic inferior vena cava was catheterized with a #16 angiocath through the right atrium; the catheter was secured with a 3-0 silk tie. Tubing was connected to the thoracic catheter and the outflow was collected. Great care was taken throughout the procedure to avoid air embolization to the liver. The liver was perfused with pre-wash until the effluent became clear and the liver assumed a tan color. At that time the perfusate was changed to the collagenase solution, which was perfused through the system at the same rate until the liver begun to ooze profusely through its capsule and it visibly started to break up. The perfusate was then discontinued, and the liver was gently excised and transferred to a sterile plastic petri dish.

Liver Cell Harvesting

The perfused liver was cut into small fragments with iris scissors and raked in 10 ml of collagenase solution. At removal, the liver was soft and friable. About 15 minutes later, the tissue suspension was passed through a polypropylene mesh (Spectramesh, 75 μ, Spectramesh Medical Industries Inc., Los Angeles, CA) with the aid of a rubber policeman. Throughout this process, ice-cold after-wash was constantly added to the cell suspension. The total volume of the cell suspension was about 150 ml. This was then centrifuged at 500 g for 2 minutes and the cell pellet was resuspended in cold after-wash. The wash was repeated three times and the final pellet suspended in 60 ml of cold after-wash.

Microcarrier Preparation and Cell Attachment

Cytodex 3 microcarriers consist of a surface layer of type I collagen covalently bound to a matrix of cross-linked dextran. The amount of collagen bound to the microcarrier matrix is approximately 60 $\mu g/cm^2$ and results in maximum cell yields.[7] The collagen (MW 60,000–200,000) is derived from pig skin type I collagen that has been extracted and denatured by acid treatment, concentrated and purified by an ion exchange step, and steam sterilized before being coupled to the microcarrier matrix. Cytodex 3 microcarriers (1.6 g) were hydrated in 125 ml of phosphate buffered saline (25 mM phosphate, without calcium and magnesium) and incubated for 90 minutes at 37 C. All glassware was siliconized (Sigmacote, Sigma Chemical Co., St. Louis, MO), air-dried, and washed to prevent adherence of the microcarriers. The phosphate buffered saline (PBS) was decanted and the microcarriers washed with 50 ml of Dulbeccos' minimal essential medium (DMEM) and 10% fetal calf serum (FCS). The microcarriers were then allowed to settle, and the total volume was brought to 125 ml. The microcarrier suspension was then transferred to 175 cm tissue culture flasks and incubated at 37 C in a 5% CO_2 atmosphere for approximately 60 minutes.

The liver suspension was centrifuged gently at 500 g for 30 seconds, the supernatant discarded, the cell pellet suspended in a small volume of DMEM and 10% FCS, and inoculated into the microcarrier culture flasks. The microcarriers and cells were allowed to stay in the incubator for at least 2½ hours before use in any of the experiments. At the end of the incubation period, the microcarrier-attached cells were washed with PBS and resuspended in DMEM (1×10^7 cells in 5–7 ml) for intraperitoneal transplantation.

Determination of viability of microcarrier-attached cells. Five ml of the microcarrier/cell suspension was washed twice with PBS. A collagenase solution was prepared by adding 2.5 mg of type I collagenase to 25 ml of Leffert's buffer and 0.25 ml of 2.7% $CaCl_2$–$2H_2O$. The washed microcarrier/cells were added to the enzyme solution at 37 C and shaken gently for five minutes. The suspension was then filtered through a 75 μ mesh and the filtrate

centrifuged at 400 g for 1 minute. The supernatant was discarded and the pellet consisting of liver cells was resuspended in cold PBS for trypan blue exclusion testing.[8]

Hepatocyte Transplantation

Male Wistar rats and male Sprague–Dawley rats weighing 200–350 g were purchased from Charles River Breeding Laboratories (Wilmington, MA). Syngeneic Wistar (RHA) rats and cogeneic Gunn rats, which have identical genetic make-up with the Wistar (RHA) rats, except for the bilirubin UDPglucuronosyltransferase locus, were developed by Dr. Carl Hansen of the National Institutes of Health (Bethesda, MD) and maintained as congeneic strains by inbreeding and backcrossing at the Albert Einstein College of Medicine (Bronx, NY). Nagase analbuminemic rats (NAR), which are mutants of Sprague–Dawley rats, were described and propagated by Dr. S. Nagase (Sasaki Institute, Tokyo, Japan) and were housed individually in No. 2 mesh stainless-steel cages at constant temperature (24–26 C) and relative humidity (40%). The cages were kept in an inside room with 12 hour day/night cycle. The animals were acclimatized to our laboratory conditions for 1 week prior to use in the experiments. All rats were maintained on standard laboratory rodent chow (#5001, Ralston Purina, St. Louis, MO) and tap water *ad libitum*. All animals were monitored closely, weighed periodically, and their food and water intakes were recorded.

Experimental design. In all transplantation experiments, 1×10^7 microcarrier-attached cells were injected intraperitoneally into recipient rats. Three series of experiments were carried out: (1) Hepatocyte transplantation into Gunn rats. The Gunn rat[9] is unable to conjugate bilirubin because of a lack of the enzyme UDPglucuronosyltransferase for bilirubin, and it thus has nonhemolytic unconjugated hyperbilirubinemia. (2) Hepatocyte transplantation into analbuminemic (NAR) rats, which have genetically deficient albumin synthesis resulting in the presence of only trace levels of plasma/albumin. (3) Morphologic, light microscopic, and scanning electron microscopic examination of transplanted hepatocytes.

Gunn rats. Hepatocytes were harvested from normal Wistar rats, attached to microcarriers, and injected intraperitoneally into allogeneic Gunn rats (N = 20) or congeneic Gunn rats (N = 18; these are genetically identical to the Wistar donors except for lack of the bilirubin UDPglucuronosyltransferase locus). In another experiment, hepatocytes were harvested from Gunn rat donors, attached to microcarriers, and injected into syngeneic Gunn rat recipients (N = 5).

In the Gunn rat recipients, normal donor hepatocyte function was assessed by anesthetizing the rats with ether at various time intervals following transplantation, exposing and cannulating the bile duct with fine polyethylene tubing (PE-10, Clay Adams, Sandy, UT), which was then brought to the skin surface through a separate wound. The animals were kept in restraining cages, and, starting 1 hour after return to consciousness, bile was collected for 4–5 hours in light-protected tubes kept on ice.

Bile pigments were analyzed as derivatized tetrapyrroles by high pressure liquid chromatography,[10] using authentic biosynthesized standards; bilirubin mono-glucuronide and di-glucuronide were identified as previously described.[10] Di- and mono-conjugated bilirubin was also independently determined by thin layer chromatography after alkaline methanolysis of conjugated pigaments.[11] In addition, bile pigments were analyzed by thin layer chromatography after conversion to azodipyrroles by reaction with diazotized ethylanthranilate[12] with or without treatment with beta-glucuronidase.[12]

In another experiment, serum bilirubin levels of transplanted allogeneic Gunn rats (N = 6) and congeneic Gunn rats (N = 6) were determined[13] at various time intervals following transplantation with normal microcarrier-attached hepatocytes. Serum bilirubin levels at various time intervals following transplantation were also measured in Gunn rats (N = 5) injected with microcarrier-attached Gunn rat hepatocytes.

In selected allogeneic Gunn rat recipients (N = 6), function of the transplanted microcarrier-attached normal hepatocytes was demonstrated by radionuclide (Tc99m-DISIDA) scanning[14] 4 days after hepatocyte transplantation. The radionuclide was administered intravenously (500 μCi in 100 μl), and uptake was measured for 30 minutes.

Nagase analbuminemic rats (NAR). In analbuminemic rats (N = 20), function of the transplanted normal microcarrier-attached hepatocytes was determined by $NaDodSO_4$/10% polyacrylamide gel electrophoresis[15] of plasma; pure rat albumin was used as reference. Plasma albumin was quantitated by an enzyme linked immunosorbent assay[16] using specific antiserum.[17] Plasma albumin was determined at various time intervals following transplantation. To prolong transplanted hepatocyte survival and function, several recipient rats (N = 12) were treated with Cyclosporin A (Sandimmune oral solution, Sandoz Inc., East Hanover, NJ) 25 mg/kg body weight administered intragastrically, daily for 5 days following transplantation. Plasma albumin was determined as above.

Morphologic studies. Rats transplanted intraperitoneally with microcarrier-attached cells were killed with ether at various time intervals following transplantation, the peritoneal cavity was examined, and the cell/microcarrier aggregate was excised and submitted for histologic examination. For light microscopic examination, tissue was fixed in 10% buffered formalin and stained with Harris' hematoxylin.[18] Tissue was fixed in Karnovsky's[18] fix-

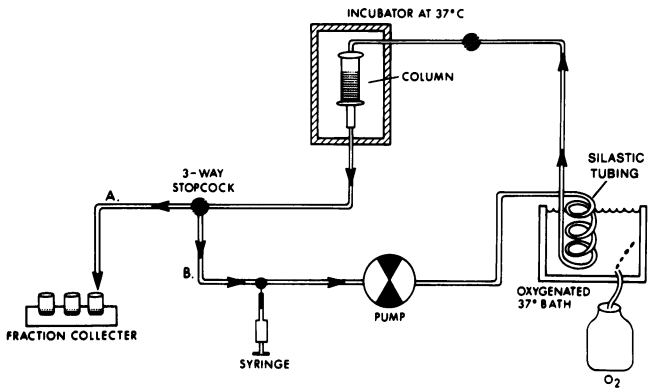


FIG. 1. Schematic representation of the extracorporeal liver assist system (Superfusion Column). The system could be connected for a single pass through the column and collection of fractions (A) or continuous reperfusion through the column (B). Proximal and distal three-way stopcocks allowed inflow and outflow sampling.

ative and processed for scanning electron microscopic examination as previously described.[18]

Extracorporeal Liver Support System (Superfusion Column)

The column superfusion system was assembled by placing a standard siliconized (I.D. 2.5 cm, 10 ml volume) chromatography column (Biorad, Rockville Center, NY) in an incubator at 37 C. The perfusion medium was injected into the polyethylene tubing, which ran through a peristaltic pump and was then connected to 30 cm of oxygen-permeable Silastic tubing placed in a water bath at 37 C, vigorously bubbled with 100% O_2. From the bath, the perfusion medium tubing was run through a flow adapter to the column. The eluent from the column was collected into tubes in a fraction collector. The system is shown diagrammatically in Figure 1. Microcarrier-attached hepatocytes were loaded on the column (1.6×10^8 viable cells in a 10 ml volume of Leffert's buffer with 10% FCS). The flow rate was set at 1 ml/min. Leffert's buffer and 10% FCS were run through the system for 10 minutes for equilibration. Serial pH measurements of the effluent from the column were carried out, and the pH was maintained above 7.2. Materials to be tested were injected into the system through a port proximal to the column. Cell viability was determined at the beginning and the end of the perfusion by trypan blue exclusion.[8]

Bilirubin conjugation. This was determined by two techniques: (1) Unconjugated bilirubin was dissolved in 0.05 N NaOH and added to Leffert's buffer containing 10% FCS at 150 μmol. Solutions containing bilirubin were protected from light and stored at −80 C. The column was arranged for continuous reperfusion (Fig. 1), and the effuent was recirculated for 90 minutes before starting fraction collection. Effluent fractions were assayed for bilirubin conjugates by thin layer chromatography after conversion to azodipyrroles by reaction with diazotized ethylanthranilate,[12] both with and without prior treatment with beta-glucuronidase or NaOH.[12] Eluent bile pigments were also analyzed as underivatized tetraphyrroles by reverse-phase high pressure liquid chromatography.[10]

(2) [^{3}H]Bilirubin with a specific activity of 30 μCi/μmol was isolated and purified from rat bile[19] after injection of delta-[2,3-^{3}H]aminolevulinic acid (7mCi/mmol, Schwarz/Mann, Orangeburg, NY). The column was prepared and loaded as above but arranged for direct collection without reperfusion (Fig. 1). Five hundred nmol of [^{3}H]bilirubin (15 μCi) was dissolved in 0.05 N NaOH and diluted in 3 ml of Leffert's buffer with 10% FCS, loaded on the column, and fractions were collected. After 1 hour, the perfusate was changed to 0.05% collagenase, and fractions were collected for 45 minutes. Di- and mono-methylesters of bilirubin were separated by thin layer chromatography after alkaline methanolysis[11] of conjugated bilirubin eluent pigments. Silica gel strips from the appropriate bands were scraped, pigments extracted in methanol, and the radioactivity determined in a Packard scintillation counter (United Technologies Packard, Sterling, VA) for 10 minutes.

Bilirubin synthesis. Delta-[2,3-^{3}H]aminolevulinic acid (15 μmol, 400 μCi) was loaded onto a column prepared and run as described above, and the eluent was concentrated by affinity chromatography on an albumin–agarose column and eluted with methanol.[20] The microcarrier-attached hepatocytes were removed from the column and the cells released from the microcarriers by treatment with collagenase. Subsequently, the hepatocytes were detergent-solubilized, centrifuged, and the supernatant analyzed.[20] Di- and mono-methylesters of bilirubin were separated by thin layer chromatography after alkaline methanolysis. The silica was extracted in methanol and counted as above.

Protein Synthesis

One mCi of [^{35}S]methionine (1.8 nmol, New England Nuclear, Boston, MA) was loaded onto a column prepared and run as described above (with the exception of using methionine-free media). After a 15-minute pulse followed by perfusion with "cold" methionine, the microcarrier-attached hepatocytes were removed and the hepatocytes released by collagenase treatment. The hepatocytes were detergent-solubilized, and proteins in the lysate were analyzed by $NaDodSO_4$/10% polyacrylamide gel electrophoresis,[15] immune absorption,[16] and autoradiography using [^{14}C]-labeled proteins as standards.[16]

Hepatocyte Storage

Storage medium. The storage medium contains 5% dimethyl sulfoxide (DMSO) (v/v) and 10% (v/v) heat-

inactivated FCS in DMEM. The pH was adjusted to 7.2–7.4.

Cells were attached to microcarriers as described above. The microcarriers/cells were then rinsed in cold (4 C) storage medium containing one half the final concentration of DMSO, the cryoprotectant used in this series of experiments. After 5 minutes, the supernatant was removed by gentle centrifugation and replaced by cold storage medium containing the final concentration of cryoprotectant. After another 4 minutes, the supernatant was removed, and the microcarriers were suspended in storage medium at a concentration of 5–10 × 10^6 cells/ml. One ml aliquots of cells attached to the microcarriers were then transferred to chilled sterile ampules that were cooled to −80 C at −1 C/min. This rate of cooling was achieved by using a 5-cm-thick expanded polystyrene box.

Recovery of frozen cells was achieved by rapidly thawing the ampules in a 37 C water bath and transferring the contents to a centrifuge tube. The cells and microcarriers were washed once in five volumes of medium and allowed to sediment, and the supernatant was discarded. The cells were then resuspended into fresh medium containing 10% FCS and were ready for use. Microcarrier-attached hepatocytes (1.5 × 10^7) were injected intraperitoneally into allogeneic (N = 7) and congeneic (N = 8) Gunn rats. Transplanted normal microcarrier-attached hepatocyte function was assessed by assaying for bilirubin conjugates in the recipient rat bile by thin layer chromatography as described above.[12]

Results

Hepatocyte Transplantation

Gunn rats. No conjugated bilirubin was detected in the bile of control Gunn rats transplanted with microcarriers alone or microcarrier-attached hepatocytes from Gunn rat donors. When donor microcarrier-attached hepatocytes from normal outbred Wistar rats were transplanted into allogeneic Gunn rats, conjugated bilirubin (both mono- and di-glucuronides) was detected in the bile of the recipient rats up to 6 days following transplantation (Fig. 2); the greatest concentration of conjugated bilirubin was observed 2–4 days after transplantation (Fig. 2). To prolong transplanted hepatocyte survival and function, hepatocytes were harvested from normal congeneic Wistar (RHA) rat donors, which are genetically identical with the recipient Gunn rats except that the Wistar (RHA) rats have the bilirubin UDPgluronosyltransferase locus and are thus able to conjugate bilirubin. When microcarrier-attached hepatocytes from such donors were transplanted into Gunn rat recipients, conjugated bilirubin was detected in the bile for up to 21 days after transplantation (Fig. 2), the last day of the study. In congeneic recipient Gunn rats, 30–40% of the total bilirubin secreted in the

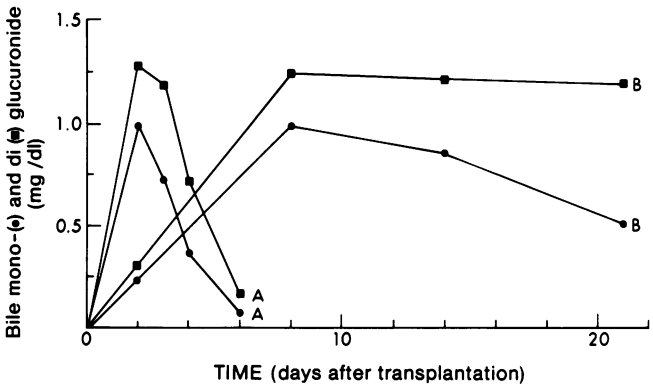


FIG. 2. Bilirubin conjugates in the bile of allogeneic (A, N = 5) and congeneic (B, N = 5) Gunn rats tranplanted with normal microcarrier-attached rat hepatocytes. Bilirubin mono-conjugates (●) and di-conjugates (■) were quantitated by thin layer chromatography after conversion to mono- and di-methylesters by alkaline methanolysis. Each time point represents an individual animal. Results of a typical experiment are shown.

bile was conjugated; both mono- and di-glucuronide bilirubin conjugates were detected (Fig. 2). On treatment with diazotized ethylanthranilate, both unconjugated and glucuronidated azodipyrroles were produced; no glucuronidated azodipyrrole was detected after beta-glucuronidase hydrolysis, indicating that the conjugates were normal 1-0-acylglucuronides of bilirubin. Figure 3 shows a typical high pressure liquid chromatograph of the bile of an allogeneic Gunn rat recipient 4 days following trans-

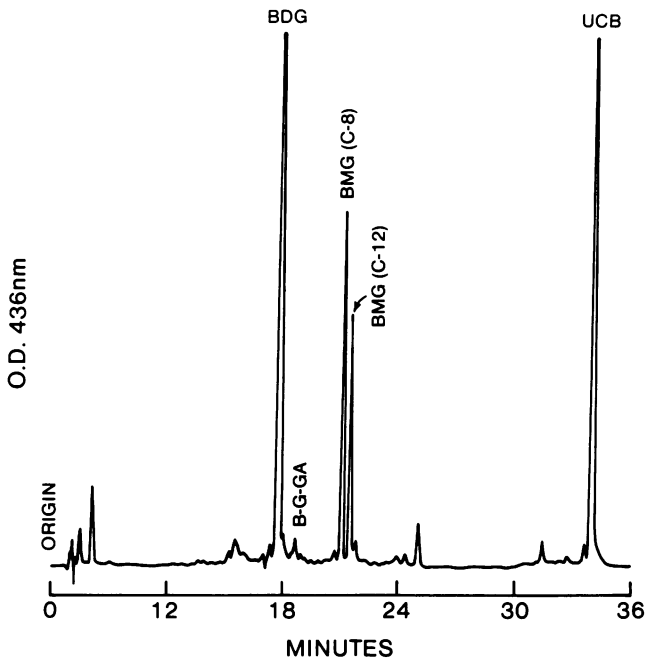


FIG. 3. Reverse phase high pressure liquid chromatograhy of bile obtained from an allogeneic Gunn rat four days following transplantation with normal microcarrier-attached hepatocytes. BDG: di-conjugated bilirubin, BMG: bilirubin mono-conjugates, UCB: unconjugated bilirubin.

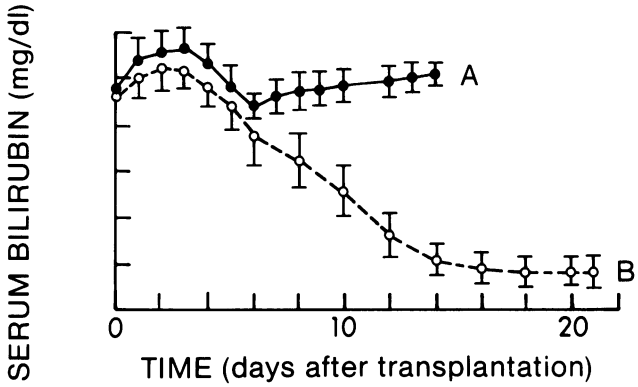


FIG. 4. Serum bilirubin levels following intraperitoneal normal Wistar (RHA) microcarrier-attached hepatocyte transplantation into Gunn rats. Data from six Gunn rats transplanted with allogeneic hepatocytes (A, mean + S.E.M.) and six Gunn rats transplanted with congeneic hepatocytes (B, mean + S.E.M.) are shown.

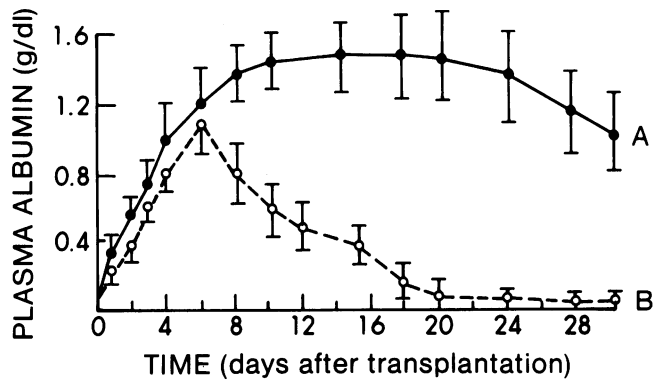
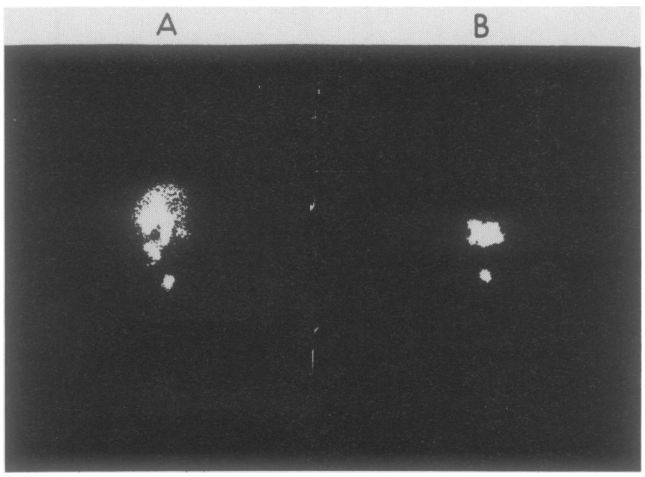


FIG. 6. Plasma albumin levels in analbuminemic (NAR) rats transplanted with microcarrier-attached allogeneic normal hepatocytes. Results of a representative experiment are presented. A = Cyclosporin A-treated recipients (N = 5, mean + S.E.M.); B = untreated recipients (N = 5, mean + S.E.M.).

plantation with normal microcarrier-attached hepatocytes. There was a marked reduction in serum bilirubin levels in Gunn rats transplanted with microcarrier-attached normal hepatocytes; the reduction was more sustained in congeneic Gunn rat recipients (Fig. 4). There was an early increase in recipient serum bilirubin levels (Fig. 4), which may be due in part to breakdown of some of the transplanted hepatocytes. When Gunn rat microcarrier-attached hepatocytes or microcarriers alone were injected into Gunn rat recipients, there was no reduction of serum bilirubin levels.

In selected allogeneic Gunn rat recipients, function of transplanted normal hepatocytes was demonstrated by radionuclide (Tc99m-DISIDA) scanning 4 days following transplantation. There was early (4 minutes) diffuse uptake of the radioisotope by the microcarrier-attached cells (Fig. 5).



FIGS. 5A and B. Radionuclide (Tc99m-DESIDA) uptake by the liver of a Gunn rat and by intraperitoneally transplanted normal microcarrier-attached hepatocytes four days after transplantation (*A*). *B* shows uptake in a nontransplanted Gunn rat. Uptake in both animals was measured 4 minutes following intravenous administration of the radionuclide.

Nagase analbuminemic rats (NAR). When microcarrier-attached hepatocytes from normal allogeneic rats were transplanted into analbuminemic rats, there was a progressive increase in plasma albumin concentration from 0.05 g/dl to a peak of 1.0 g/dl 6 days after transplantation; this was followed by a gradual decline in plasma albumin concentration (Fig. 6). Following recipient immunosuppression with Cyclosporin A, there was a more sustained increase in plasma albumin concentration (Fig. 6) with a peak level at 6 days (1.3 g/dl), which was maintained close to that level for up to 30 days (last day of the study).

Morphologic studies.

Gross findings. When the abdominal cavity was explored at various times following intraperitoneal administration of microcarrier-attached hepatocytes, most of the cells and microcarriers formed an aggregate over the anterior surface of the pancreas. The aggregate (Fig. 7) was surrounded by adherent loops of bowel. The reaction around the aggregate was intense in the early posttransplantation period (3–4 days), but by the tenth day (in syngeneic rats) the inflammatory reaction had subsided with minimal adhesions remaining around the aggregate. By day 20, there was only minimal reaction around the hepatocyte/microcarrier aggregate. In allogeneic transplanted rats, the early appearance (3–4 days) was indistinguishable from that of the syngeneic rats. In the allogeneic group, however, by 1 week after transplantation the tissue aggregate had disappeared almost completely. There was no evidence of infection (peritonitis or abscess), hemorrhage, or intestinal obstruction in any of the animals examined.

Light microscopy. Light microscopic examination of the microcarrier-attached hepatocytes retrieved from the peritoneal cavity 1 week following intraperitoneal trans-

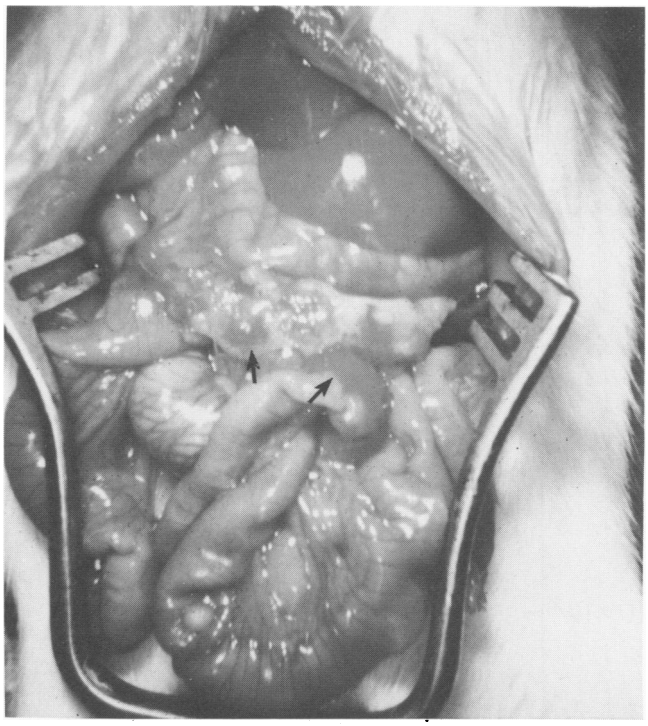


FIG. 7. Gross appearance of the peritoneal cavity of a Gunn rat 4 days following intraperitoneal transplantation with 1×10^7 allogeneic microcarrier-attached normal hepatocytes. The recipient animal liver appears normal. There are several aggregates of tissue grossly resembling "liver" scattered throughout the peritoneal cavity (↑).

plantation into syngeneic rats revealed evidence of epithelial cells attached to the microcarriers and surrounded by fibroblasts and connective tissue; there was evidence of neovascularization in the interstices between the microcarriers (Fig. 8).

Scanning electron microscopy. Scanning electron micrographs demonstrated epithelial cells attached to a collagen-coated microcarrier surface; the fine granular surface on the microcarriers represents type I collagen, which is covalently-linked to dextran, whereas the coarse fibers present elsewhere represent newly synthesized collagen (Fig. 9).

Extracorporeal Liver Support Systems (Superfusion Column)

Bilirubin conjugation. Thin layer chromatography of azodipyrroles from the column eluent demonstrated two bands, one migrating with authentic biosynthesized standards of conjugated azodipyrroles, the other migrating with control unconjugated azodipyrrole. The band corresponding to conjugated azodipyrrole disappeared after incubation with beta-glucuronidase or NaOH, indicating that the conjugates are ester 1-0 acylglucuronides. High pressure liquid chromatography of underivatized column eluent demonstrated two peaks (Fig. 10), the first at 14 minutes corresponding to bilirubin diglucuronide and the second at 27 minutes corresponding to unconjugated bilirubin. There was a cluster of smaller peaks at 17–18 minutes, which most likely correspond to breakdown products of bilirubin monoglucuronides. The percentage of total bilirubin appearing as di-glucuronides as determined by the ratios of the areas under the peaks was 17%.

Eighty-three per cent of the [^{3}H]bilirubin added to the column was recovered in the eluent, and 21% (87 nmol) of this appeared as bilirubin mono- or di-glucuronide after a single pass through the column. Ninety-one per cent of the total conjugates was removed in the first 16 minutes (Fig. 11). Twenty-four per cent of the total conjugates appeared as di-conjugate and 76% as mono-conjugate. The proportion of total conjugates appearing as mono-

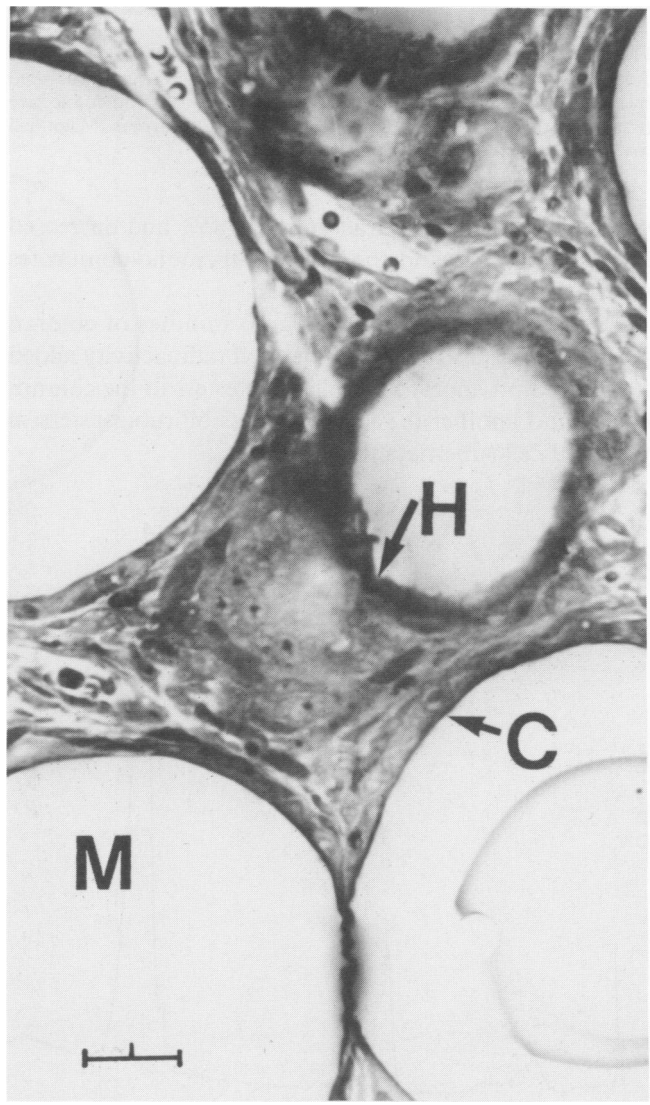


FIG. 8. Light microscopy photograph of section of microcarrier-attached liver cells 4 days after intraperitoneal transplantation. Both hepatocytes and connective tissue cells are seen: H = hepatocyte, M = microcarrier, C = collagen coating on microcarrier surface. A blood vessel is seen below the hepatocyte and close to the microcarrier surface. Magnified ×40.

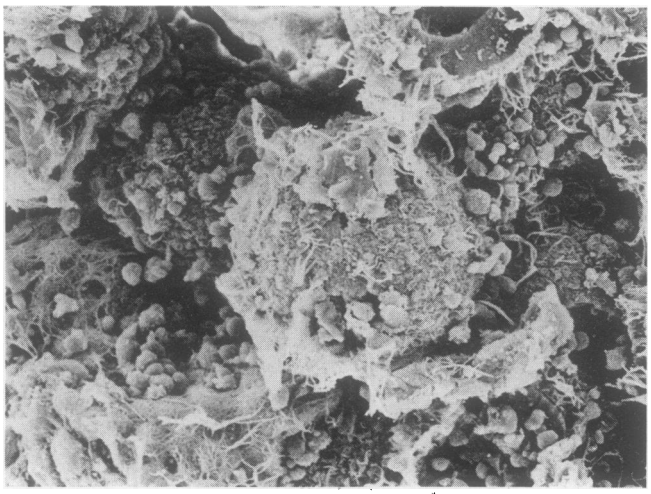


FIG. 9. Scanning electron micrograph of microcarrier-attached liver cells 5 days after intraperitoneal transplantation. The microcarriers are covered by a sheath of cells, fibrin, and collagen. There are cells and connective tissue filling the spaces among the aggregated microcarriers. Magnified ×450.

conjugates in the early fractions was 85% and decreased in later fractions, and the ratio of di-/mono-conjugates eventually was reversed (Fig. 11).

Bilirubin synthesis. Following 40 minutes of column perfusion, 1% (150 nmol) of the total radioactivity added to the column appeared as bilirubin esters in the column eluent and another 0.8% appeared as bilirubin esters in the lysed cell supernatant.

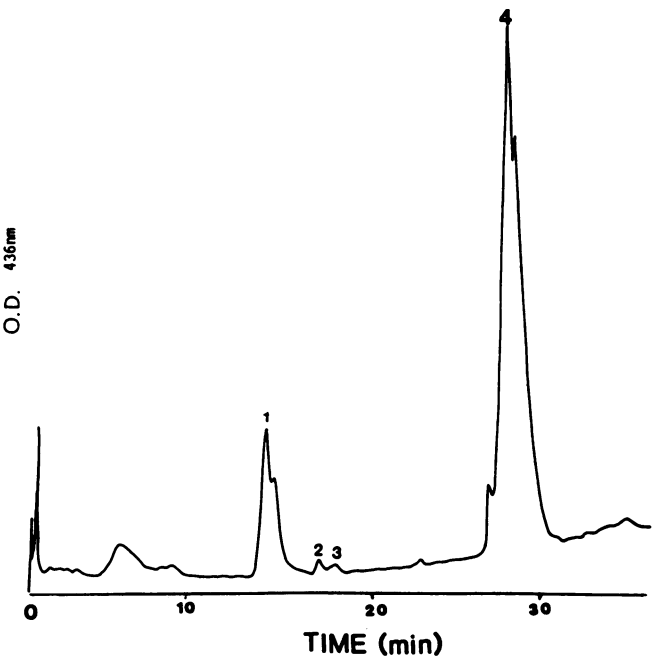


FIG. 10. Reverse-phase high pressure liquid chromatography of superfusion column eluent. 1 = di-conjugated bilirubin, 2 and 3 = bilirubin mono-conjugates, 4 = unconjugated bilirubin.

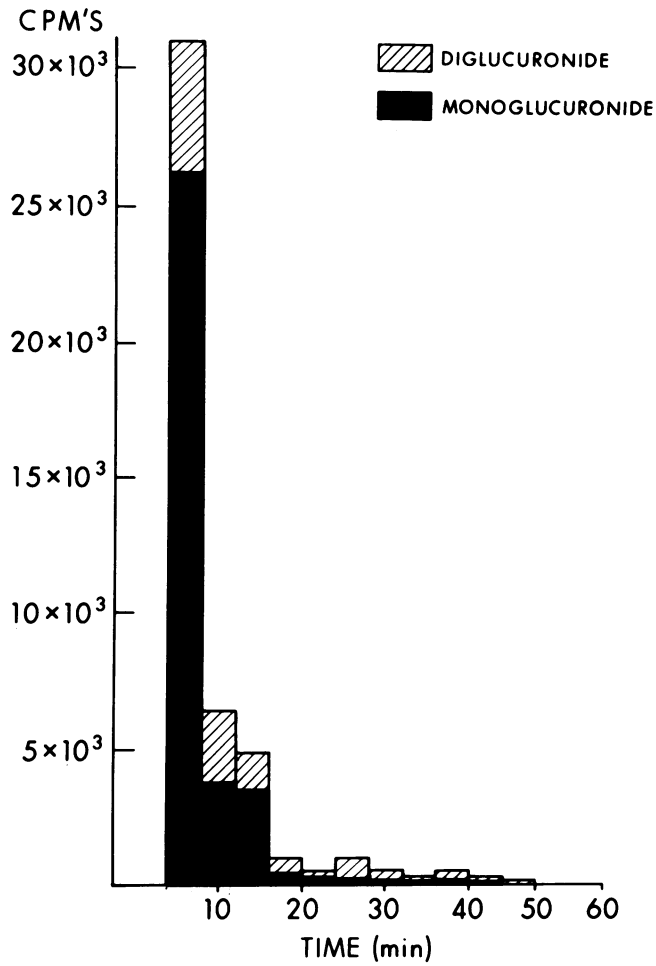


FIG. 11. Bilirubin conjugation by superfusion column. Total counts per minute of unconjugated bilirubin from 0.2 ml aliquots of 4 ml fractions. Relative proportions of bilirubin mono- and di-glucuronide are indicated for each fraction. Bilirubin was loaded onto the column at time 0 and allowed to diffuse into the microcarrier-attached hepatocytes, and fraction collections were started 4 minutes later.

Protein Synthesis

Following 1 hour of column perfusion, autoradiography and immune absorption of the electrophoretically separated cell lysate supernatant demonstrated incorporation of the radioisotope into albumin, UDPglucuronosyltransferase, and other proteins (Fig. 12).

Hepatocyte Storage

Cryopreserved microcarrier-attached normal Wistar (RHA) rat hepatocytes, stored for two weeks at −80 C, showed 55–60% viability and were transplanted intraperitoneally into four congeneic Gunn rats (1.5×10^7 cells/rat). All rats had both mono- and di-glucuronide bilirubin conjugates in their bile 10 days posttransplantation (demonstrated by thin layer chromatography with appropriate standards), the last day of study.

Discussion

The prognosis of acute liver insufficiency in humans is poor despite current attempts to provide metabolic support and/or detoxify the plasma to support the patient until liver regeneration can take place. Whole organ liver transplantation is the only method that has proved to be clinically useful in providing both acute and chronic hepatic metabolic support.[21] This procedure, however, is limited by the relative lack of donors, requirement for sophisticated technology and well-trained specialized support teams, technical difficulty, and high cost. Hepatocyte transplantation, if functionally successful, would be an attractive alternative. Transplantation of hepatocytes,[1,2] injection of various hepatocyte extracts,[22,23] and hepatocyte culture supernatants[22] have been reported to be effective in prolonging survival of rats with D(+)galactosamine-induced liver injury[3] and animals with acute liver ischemia.[24] In addition, hepatocyte transplantation has been used to transiently correct specific metabolic defects, *i.e.*, the unconjugated hyperbilirubinemia due to lack of UDPglucuronosyltransferase activity for bilirubin in the Gunn rat on a short-term basis.[25,26]

Experimental hepatocyte transplantation, in spite of early enthusiasm, has not proven to be an effective method of metabolic support in acute severe liver insufficiency or an adequate long-term replacement therapy in animals with specific metabolic defects.[27] Most investigators have used animal survival in the D(+)galactosamine model of acute liver injury as proof of viability and function of transplanted hepatocytes.[1,2,22] However, various cell extracts and tissue culture supernatants have been shown to be equally effective or in some instances more effective than intact hepatocytes in prolonging animal survival in this model.[22] The degree of liver injury varies according to the rat strain, age, dosage, and route of D(+)-galactosamine administration,[28] and the liver capacity to regenerate in response to exogenous factors derived from transplanted cells and extracts.[28,29]

Similar problems are encountered when using ischemic models of experimental liver injury[30,31]; that is, it is difficult to determine whether improved survival is due to intact functioning transplanted hepatocytes or to administration of hepatotrophic factors that accelerate liver regeneration and recovery and thus result in improved animal survival. Most studies reporting long-term success of hepatocyte transplantation have used histologic and morphologic criteria of viability alone as evidence for the persistence of hepatocyte function.[32,33] In a few studies, attempts to demonstrate persistent hepatocyte function were made; hepatocyte transplants were used to treat specific hepatocyte enzyme deficiencies. In these, the experimental model most commonly used was the Gunn rat. Several investigators showed transient decreases in serum

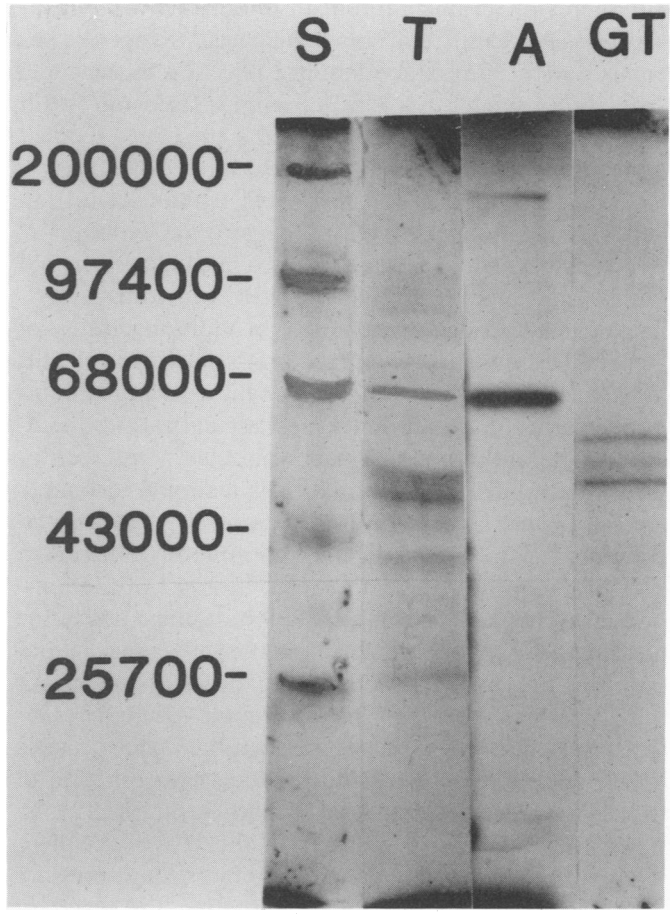


FIG. 12. Protein synthesis by superfusion column. Autoradiograph of microcarrier-attached hepatocytes removed from the column, released from the microcarriers by collagenase, and immunoabsorbed following electrophoretic separation. S = molecular weight standards, T = hepatocyte lysate; A = rat albumin; GT = rat bilirubin UDPglucuronosyltransferase. The figure shows that there was synthesis of albumin and bilirubin UDPglucuronosyltransferase by the hepatocytes on the column.

bilirubin levels in the Gunn rat following intraportal normal hepatocyte transplantation.[25,26] This was attributed to the presence of functional normal hepatocytes that have the ability to conjugate bilirubin. However, similar reductions in serum bilirubin were observed following splenectomy or intraportal injection of splenocytes, which suggests that the decreased serum bilirubin level was due to decreased red blood cell destruction.[34] Definitive proof of donor hepatocyte function in the Gunn rat requires identification of conjugated bilirubin in the host bile. This has been shown very recently[35] with short-term success; conjugated bilirubin was absent in the bile beyond the sixth day after allotransplantation.[35] Allogeneic hepatocyte transplantation has been used by most investigators and, as expected, is not successful in the long run.[35] Allograft rejection is delayed in immunosuppression, but experiments in which immunosuppression was carried out have utilized the acute injury models described above or mea-

surements of serum bilirubin in Gunn rats without attempting to identify bilirubin conjugates.[34] Syngeneic animals have been used to eliminate rejection in the acute liver injury models (D(+)galactosamine, ischemia) with the same limitations in assessing the presence of intact functioning hepatocytes as already outlined.[21,36]

Type I collagen gels from rat tail tendon have been reported to prolong survival of hepatocytes *in vitro*.[37,38] We hypothesized that collagen-coated microcarriers would improve hepatocyte survival *in vivo* by providing a physiologic matrix for cell attachment. In addition, clusters of hepatocytes attached to microcarriers may protect cells from host macrophages and phagocytic cells and also improve perfusion and promote neovascularization. In addition, use of the microcarriers would allow intraperitoneal injection of the cells, which is a simple technique and eliminates laparotomy and intrasplenic or intraportal administration of cells. In the experiments described in this paper, we were able to overcome most of the limitations of hepatocyte transplantation outlined above by: (1) Using Gunn rat recipients and congeneic normal donors that are genetically identical to the Gunn rats except for the presence of the bilirubin UDPglucuronosyltransferase locus. This eliminated the major obstacle of rejection and allowed study of long-term function of transplanted normal hepatocytes into Gunn rats. (2) Assaying for bilirubin conjugates in the bile of recipient Gunn rats by thin layer chromatography and high pressure liquid chromatography and quantitating the levels of mono- and di-glucuronide conjugates of bilirubin in the recipient rat bile following alkaline methanolysis in addition to measuring serum bilirubin levels. This is a direct measurement of transplanted hepatocyte function. (3) Using specific hepatocyte markers, *i.e.*, Tc99m-DISIDA uptake by the transplanted cells. (4) Using another animal model, the analbuminemic rat, which is unable to synthesize albumin, and following normal hepatocyte transplantation, to assess donor hepatocyte function by measuring and quantitating plasma albumin levels. In addition, Cyclosporin A was used in these experiments to prolong survival of allogeneic transplanted microcarrier-attached normal hepatocytes. We were able to demonstrate long-term survival and function of transplanted hepatocytes attached to collagen-coated microcarriers by showing presence of conjugated bilirubin in the bile of recipient Gunn rats, sustained decrease in serum bilirubin in Gunn rats, uptake of Tc99m-DISIDA by transplanted hepatocytes, and elevation of plasma albumin levels following transplantation in analbuminemic rats and by using morphologic criteria. In experiments reported elsewhere,[39] we have shown evidence of viable differentiated hepatocytes by utilizing immunocytochemical and electron microscopic techniques as long as 8 weeks following intraperitoneal normal hepatocyte transplantation into syngeneic rats. Using collagen-coated microcarrier-attached hepatocytes, we have thus developed a new simple method of intraperitoneal hepatocyte transplantation that allows long-term survival and function of transplanted cells.

There would be a major advantage for a clinically successful hepatocyte transplantation method if cells could be stored at low temperatures and be available for use at short notice. We carried out a limited experiment in which we were able successfully to transplant normal cryopreserved hepatocytes (−80 C, 2 weeks of storage) into Gunn rats as evidenced by the presence of conjugated bilirubin in the recipient rat bile. The large surface area/volume ratio of the microcarriers is advantageous for transporting and storing cells. Large numbers of cells (up to approximately 1×10^7 cells/ml) can be stored while still attached to the microcarrier matrix. The advantage of storing cells attached to a matrix (culture surface) rather than as a suspension is that cells are stored in a monolayer form, and, in addition, loss of cells associated with harvesting is avoided.[18] After thawing, the cells are already attached and ready to use. Availability of genetically defined hepatocytes for immediate use at short notice would be a major step forward in patient care.

Investigators have attempted to support metabolically humans or animals with acute liver insufficiency by utilizing various cross-circulation experiments,[40] extracorporeal assist devices,[41] hemoperfusion,[42] use of resins as adsorbents,[43] and chromatographic and other techniques.[44–46] None of these techniques is currently used widely clinically. An objective of this study was to utilize the microcarrier-attached hepatocytes in the development of an extracorporeal liver assist device. Other investigators[47] have used normal hepatocytes maintained for short periods of time on capillary tube membranes and perfused with blood from Gunn rats and were able to demonstrate the presence of bilirubin conjugates in the Gunn rat bile when such rats were attached to the perfusion system. A centrifuge was used by Eiseman and his associates[48] as a perfusion chamber for hepatocyte suspensions with maintenance of hepatic metabolic functions for up to 6 hours. We hypothesized that attachment of hepatocytes to a collagen matrix would allow optimal differentiation and expression of specific hepatocyte functional characteristics. We developed a "superfusion column" that allowed study of *in vitro* hepatocyte functions and could serve as a model for developing an extracorporeal liver assist device. Using this system, we were able to demonstrate that the microcarrier-attached hepatocytes on the column could synthesize bilirubin at rates similar to those found *in vivo*, conjugate bilirubin, and synthesize liver-specific proteins (albumin and bilirubin UDPglucuronosyltransferase). As a potential system for artificial liver support, our system offers several advantages over other

devices used in the past. The use of microcarrier-attached hepatocytes takes advantage of the entire metabolically intact cell to perform multiple specific liver functions. The system is not limited to specific excretory functions or enzymatic degradation. In addition, the system is simpler than whole liver perfusion. Further studies are in progress to determine its utility in various experimental animal models of severe liver insufficiency.

In conclusion, we have developed a technique that allows attachment of hepatocytes to collagen-coated microcarriers. Using this technique, we were able to develop a new method of successful intraperitoneal transplantation in the rat resulting in long-term hepatocyte viability and function, a new method of hepatocyte storage, and an extracorporeal liver superfusion column.

Acknowledgments

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Discussion

DR. WILLIAM V. MCDERMOTT, JR. (Boston, Massachusetts): I had the opportunity to read the manuscript that formed the basis for this excellent report you have just heard and that provided a summation of this long period of excellent and quite prodigious labors over a period of time. I will not comment on the hepatocyte microcarrier system; I will leave that to those who are expert in this field or who have a direct interest in this problem, perhaps through similar problems encountered by those investigators working with islet cell transplants. The objective of this report was to develop an extracorporeal support system that worked, and of course this has been an objective of many of us over many years.

(Slide) Some of the historical background, which I won't go into in detail, shows that in many instances it goes back to Claude Bernard's canine perfusion system and then carries through from the 19th century over the subsequent decades. You can see here that Ben Eiseman, as in so many instances connected with liver problems, was one of the pioneers in this system. As he developed this and began to use it in humans, he was of immense support and help to us in our work, and we collaborated and exchanged information over a period of time. Ben not only pursued this with the porcine and canine liver but also with hepatocytes.

(Slide) I would like to talk briefly about the extracorporeal support system that we utilized for a period of time. This is a description of the pig liver and its attachment in man and the various technological mechanisms in the clinical project.

(Slide) These are the first five patients whom we selected to be supported by this device. They all had a similar clinical situation, of deep, unresponsive coma and anuria or extreme oliguria, a combination which at that time was invariably lethal. This is the type of intermittent perfusion that we were using at that time.

(Slide) Without going into more than superficial details of this system and its clinical utilization, you can see here the effect on the bilirubin level of serial perfusion over several weeks in a patient.

(Slide) Some of the interesting things, some of which are still puzzling, show the protein characterization in the patient's sera, which I think demonstrates the presence of both porcine analbumin and alpha-1 and -2 globulins. The ascites is really the lymphatic flow from the extracorporeal liver. The bile made during the perfusion by the porcine liver shows the presence of human albumin, and again the globulins with human serum components.

One of the intriguing things was the antibody determinations that in all patients were negative by the relatively primitive techniques available at that time.

(Slide) In a brief summation of this, the pig liver produced bile containing human proteins and lymph containing both human and pig proteins and resulted in circulating pig albumin and alpha-2 globulin in the patients.

(Slide) Immunologically, although perfused and preterminal, these patients did have 7S gamma globulin prior to the perfusions and, we thought, had potential antibody formation capacity, but they showed no demonstrable antibody response despite serial testing over many weeks. I have no real explanation for this except perhaps a massive antigen overload.

I will conclude by saying that we gave this up for an obvious reason. Even though it was dramatically successful repeatedly and intermittently in restoring both renal function and responsiveness, none of the patients survived, and, at post mortem, microscopic studies all showed complete absence of hepatocytes and no evidence of regeneration. So, clearly, utilizing these terminal patients, we have felt that this type of support had no long-term clinical application.

DR. BEN EISEMAN (Denver, Colorado): Only those of us who have worked in the still unsolved area of artificial liver support can appreciate the significance of the advance made by this work.

One of the major problems that we found in our many trials illustrated by these slides (Slide) was to keep the liver cells suspension bound within the chamber. Dr. Levenson and co-workers have clearly shown this can be done by attaching the cells to a carrier.

I believe the autologous cells from a patient with liver failure will be of little help: they are too badly damaged. This will mean that the cells will either have a very short active life or immunosuppressives will have to be used in the clinical setting involving transplants.

I am delighted that this problem, which so long occupied our efforts, is yielding to the imaginative efforts of Stan Levenson and his colleagues.

DR. DAVID E. R. SUTHERLAND (Minneapolis, Minnesota): Dr. Levenson was kind enough to send me the paper, and I would like to discuss it and ask some questions that were raised in the presentation.

At the Transplantation Laboratories at the University of Minnesota, we have had a longstanding interest in hepatocyte transplantation. Ten years ago, Art Matas, who was with our group then, did some experiments in Gunn rats in which he transplanted hepatocytes, and he was able to effect a lowering of bilirubin that was not sustained. That was before Dr. Carl Hanson at NIH had developed the inbred strains of RAJ rats that Dr. Demetriou used to avoid the rejection problem and effect a 21-day reduction in bilirubin.

Dr. Marco Cavallini in our laboratory did auxiliary liver transplants in RAJ rats. He also did pancreas, heart, and kidney transplants, and he was able to show that even a kidney transplant would lower the bilirubin because there is glucouronyl-transferase in the kidney. However, ultimately even in liver transplant recipients the bilirubin increased, and it was thought that with the lack of portal perfusion these livers atrophied. I wonder if in these hepatocyte transplants this might be a problem if you carried out your experiments for longer than 21 days.

You did demonstrate that the hepatocytes actually function. One problem with hepatocyte transplant experiments that have been done in the past has been the use of a liver failure model. In liver failure models survival of the animals can be increased just by giving liver cell culture supernatants or other manipulations, and it appears that it may not be the hepatocytes that sustain the animal but some factors that accelerate liver regeneration.