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Histological identification of purified and cryopreserved allogeneic hepatocytes following transplantation in a murine model without host immunosuppression

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Abstract Hepatocyte transplantation is a conceptually attractive alternative to whole organ grafting for some inborn metabolic errors and for fulminant liver failure. However, studies of the immunogenicity of transplanted allogeneic hepatocytes have yielded contradictory results. In these experiments, the effect of purification and cryopreservation of the hepatocytes on the ability of these cells to engraft in the mouse allogeneic recipients without immunosuppression was studied. BALB/cByJ mouse crude (unpurified), modified (purified or cryopreserved), or dead (irradiated) hepatocyte preparations labeled with fluorescein dye CFSE were infused either into the portal vein or into the spleen parenchyma of the recipient CBA mice. A histological examination revealed normal appearance of engrafted modified hepatocytes with no signs of acute rejection up to 21 days posttransplant. Many of the intrasplenically implanted hepatocytes migrated into the hepatic sinusoids. The modified hepatocytes showed intact ultrastructural appearance 7 days after transplantation. The numbers of inoculated crude hepatocytes rapidly declined with signs of dense infiltration of mononuclear cells in the graft indicating destructive response. The fluorescence of dead hepatocytes was undetectable. These results suggest that reduced immunogenicity may be responsible for the longer survival time of inoculated, purified or cryopreserved hepatocytes with no adverse morphological effects.

Key words Allogeneic hepatocytes · Transplantation · CFSE · Murine model

Introduction

Hepatocyte transplantation is being developed as a bridge to solid organ grafting. Potential advantages of the use of this method include long-term hepatocyte cryopreservation and in vitro cell manipulation to decrease immunogenicity [1, 4]. However, the use of hepatocyte transplantation clinically has been hindered by limited survival of the cells in vivo. The ideal site for hepatocyte transplantation is the liver itself where the inoculated cells may function in their natural environment, receiving the hepatotrophic factors important for cell differentiation. The disadvantage of intrahepatic placement of the grafts is the difficulty of differentiating donor from host hepatocytes, unless the donor viable cells are labeled with specific markers [9, 16]. Another site widely used for hepatocyte transplantation is the spleen. There are many reports describing long-term survival of the hepatocyte grafts in the splenic pulp of syngeneic recipients, although the majority of inoculated cells translocate into the liver [8, 11]. Rejection of the transplanted allogeneic hepatocytes remains the major problem in several animal models. Addition of immunosuppressive drugs has shown only limited success [15, 17]. The elimination of hepatocyte immunogenicity would permit long-term graft survival without the hazards of using non-specific immunosuppressants.

Hepatic parenchymal cells express class I, not class II, major histocompatibility complex antigens. MHC class I antigens, are of themselves, weak immunogens, but freshly isolated hepatocyte preparations are contaminated with nonparenchymal cells including Kupffer cells, billiary epithelial cells or endothelial cells that express MHC class II antigens and may function as antigen-presenting cells (APC). Available evidence indicates that the lymphoblastic cells and vascular endothelial cells serve as the most efficient stimulators of the immune response [3]. Depletion or inactivation of antigen presenting cells from the graft may prevent initiation of the alloresponse. We have previously shown that single cell suspensions of allogeneic purified or cryopreserved mouse hepatocytes are less immunogenic in vitro when compared to the crude, unmodified preparations [13, 14]. In the present study we investigated the effect of various modifications of the allogeneic hepatocyte preparations on the ability of these cells to engraft after either intrahepatic or intrasplenic transplantation in the mouse. Specifically, we tested whether allogeneic modified hepatocyte grafts can be morphologically detected at the transplant site without host immunosuppression.

Materials and methods

Animals

Adult BALB/cByJ (H-2^d) and CBA (H-2^k) male mice (Jackson Laboratories-Bar Harbor, ME) were used as the hepatocyte donors and recipients, respectively. All animals, 8 to 6 weeks old, were maintained in 12 h dark/light cycles and had free access to food and water. The animal care and experiments were conducted in accordance with the guidelines of the "Principles of laboratory animal care" (NIH publication No. 86–23, revised 1985) and approved by the Animal Care and Use Committee of the University of Colorado School of Medicine.

Chemicals

Media used for the hepatocyte isolation including Hanks' balanced salt solution (HBSS), Leibovitz (L-15) and media supplements (EGTA, glucose, dimethyl sulfoxide) were purchased from Sigma Chemical Corp. St. Louis, Mo. Fetal bovine serum (FBS) was from Hyclone Laboratories, Inc. Logan, Utah. Collagenase (type A) was obtained from Boehringer Mannheim Corp. Indianapolis, Ind. Percoll was purchased from Pharmacia Biotech. Inc. Newark, NJ. Fluorescent dye CFSE was from Molecular Probes, Inc. Eugene, Ore. All antibodies were supplied by PharMingen San Diego, Calif.

Hepatocyte isolation and preparation

Hepatocytes were isolated by an in situ modification of the perfusion technique described by Seglen [19]. Briefly, a 24-gauge intravenous catheter was inserted into the portal vein and the liver was perfused with calcium free HBSS supplemented with 0.5 mM EGTA for 10 min and then with 0.05 % collagenase dissolved in L-15/glucose media at 37 °C. The vena cava was cut to allow for drainage. After dissociation, the cell suspension was filtered through 250 μ and 100 μ nylon mesh and then centrifuged twice at 40 × g for 3 min at 4 °C. After the last wash, the cells were suspended in L-15/glucose media. Viability and yield were determined by trypan blue staining. Four different hepatocyte preparations were investigated:

(1) Crude, unpurified hepatocytes, washed and filtered through the nylon mesh only.

(2) Hepatocytes purified by passing the cell suspension twice through 70 % Percoll gradient. Cells were centrifuged at 60 × g for 10 min at 4 °C. The supernatant with damaged or dead hepatocytes, nonparenchymal cells and debris was removed and pelleted hepatocytes were washed twice (at 50 × g for 3 min) in L-15/glucose medium. Percoll-purified hepatocytes were also enriched by elimination of MHC class II antigen expressing cells by panning with CD45 antibody. The purity of the preparation was determined by cytospin of 10^5 cells followed by the CD45 staining. Expression of the hepatocyte MHC antigens was determined by fluorescence-activated cell sorting (FACS) analysis. The cells were labeled with fluoresceine-conjugated goat-antirat IgG (control preparations) or with fluorescein conjugated anti-class I (H-$2D^d$) or anti-class II (I-A^d) monoclonal antibodies. Flow cytometry was performed using a Coulter EPICS-C using argon laser with standard optics.

(3) Hepatocytes processed as in # (2) then cryopreserved. Briefly, the cells were suspended at a density 4×10^6/ml of L-15/glucose medium, and 1 ml of hepatocyte suspension aliquots were transferred into the 2 ml cryogenic vials. Equal volume of cryoprotectant mixture containing 40 % L-15 medium (vol/vol), 20 % dimethyl sulfoxide (vol/vol), and 40 % FBS (vol/vol) was slowly added to the cells. Computerized controlled-rate freezing apparatus (Cryomed, Forma Scientific, Marietta, OH) was used to cryopreserve the cells. The initial freezing rate was 3 °C/min. Cryopreserved hepatocytes were quickly placed into the liquid phase of a LN_2 tanks where they were stored for one month. For cell thawing, the vials were removed from LN_2 and immediately thawed in 37 °C water bath with gentle mixing. The cells were gently pipetted into the 50 ml sterile centrifuge tubes, slowly resuspended in ice-cold L-15/glucose medium, and then washed twice by centrifugation at 50 × g for 3 min. Pelleted cells were resuspended in ice-cold saline.

(4) Dead hepatocytes irradiated with the γ source.

Hepatocyte labeling with fluorochromes

Three million cells were suspended in L-15/glucose medium enriched with 5 % FBS and fluorescein dye 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE) was added from a stock solution to a final concentration of 5 μM/L. The cells were incubated for 15 min at 37 °C and then washed twice in L-15/glucose medium. Fluorescence of labeled cells was verified by a fluorescent microscope and FACS analysis.

Hepatocyte transplantation

Allograft recipient mice underwent a midline laparotomy under the pentobarbital anesthesia (25–30 mg/kg intraperitoneally). The stomach and intestines were moved away to expose the portal vein. CFSE-labeled hepatocytes (1.2×10^6) were infused by sterile techniques either into the liver via portal vein, cannulated with a 24-gauge butterfly needle, or directly into the spleen parenchyma.

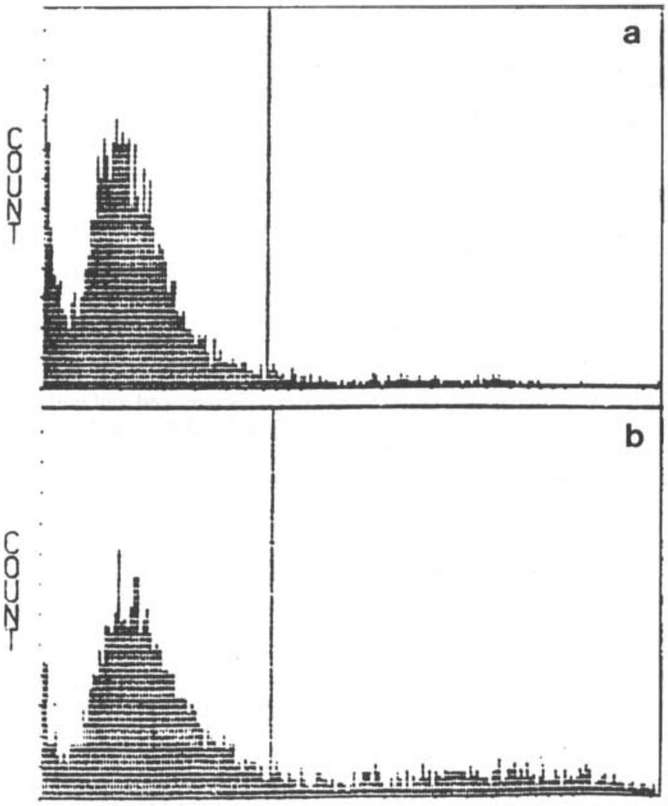

Fig. 1 Flow cytometric evaluation of MHC class II antigens on purified **a** and crude **b** hepatocytes. Staining of the cell preparations with fluorescein-conjugated anti-class II monoclonal antibodies

using a 27-gauge needle. CBA experimental mice received labeled or unlabeled purified hepatocytes (group I, $n = 5$), cryopreserved hepatocytes (group II, $n = 5$), crude hepatocytes (group III, $n = 5$), and dead cells (group IV, $n = 3$). Three mice from control group received sham operation (intraportal or intrasplenic injection of 0.2 ml saline).

Histology

Transplanted animals were sacrificed 7, 14 and 21 days after surgery. Spleen and liver were dissected and processed for the light microscopy using standard techniques; paraffin embedded sections were stained with hematoxylin and eosin (H & E) or by periodic acid-Schiff (PAS) procedure. Alternatively, several parts of dissected organs were snap frozen with OCT (Optimum Cooling Temperature) compound, cryostat sectioned, and examined by fluorescent microscopy to identify CFSE-labeled hepatocytes. Electron microscopy sections were dissected and fixed by immersion with 2.5 % glutaraldehyde. Thin sections were stained with uranyl acetate and lead nitrate and examined by a CM12 Philips transmission electron microscopy.

Results

The final cell suspension contained 75–80 % viable hepatocytes in crude preparations, 95–99 % viable cells after Percoll purification, and 80–89 % viable cells in cryopreserved samples. Contamination by CD45+ cells was 9.3 %, 0.7 %, and 0.5 %, respectively. Flow cytometric evaluation of MHC class I and class II antigens on crude, purified, and cryopreserved hepatocytes revealed differences between these preparations: 11.1 %, 2.8 %, and 1.8 % cells in analyzed preparations were positive for MHC class II antigens, respectively (Fig. 1 a, b). CFSE labeling efficiency was up to 98 % as demonstrated by fluorescent microscopy before transplantation. All animals survived following intrasplenic injection until sacrificed. One mouse injected intraportally (group III) was found dead 24 h after surgery with severe signs of hemorrhages.

Recipients from groups I and II

No signs of rejection were visible on gross examination of resected organs. Histological examination revealed the presence of CFSE-labeled hepatocytes up to 21 days posttransplant (Fig. 2 a). Although morphometric analysis was not performed, there were no significant differences in the histological demonstration of purified and cryopreserved transplanted hepatocytes. Single colonies of inoculated cells were scattered throughout the splenic parenchyma one week after intrasplenic injection (Fig. 2 c). Some of these cells were binucleate. The 14 day–old grafts formed aggregates in the splenic red pulp. There was no evidence of graft destruction with only sparse, noninvasive mononuclear cell infiltration (Fig. 2 e). Periodic acid-Schiff reaction stained intracellular red granules of glycogen in the transplanted hepatocytes (Fig. 2 d); the diastase extraction on duplicate paraffin sections did not show PAS staining. Three weeks after transplantation, hepatocyte grafts were still distinguishable. However, the numbers of fluorescent cells were smaller. Many of the intrasplenically implanted hepatocytes migrated to the recipient's liver and concentrated in hepatic sinusoids. Fluorescence of these cells was still visible 21 days posttransplant. After intraportal injection, CFSE-labeled hepatocytes were grouped in small diffused clusters of 5–10 cells, mostly at the periphery of the lobes. The largest numbers of labeled cells were identified 7 days after transplantation. These cells were very bright and formed morphologically distinct aggregates surrounded by single cells and concentrated in the sinusoids, mostly under the liver capsule. The hepatocyte grafts demonstrated a high incidence of stabilization and were free from infiltrating lymphocytic cells. The 14- and 21 day–old grafts were still observed, although

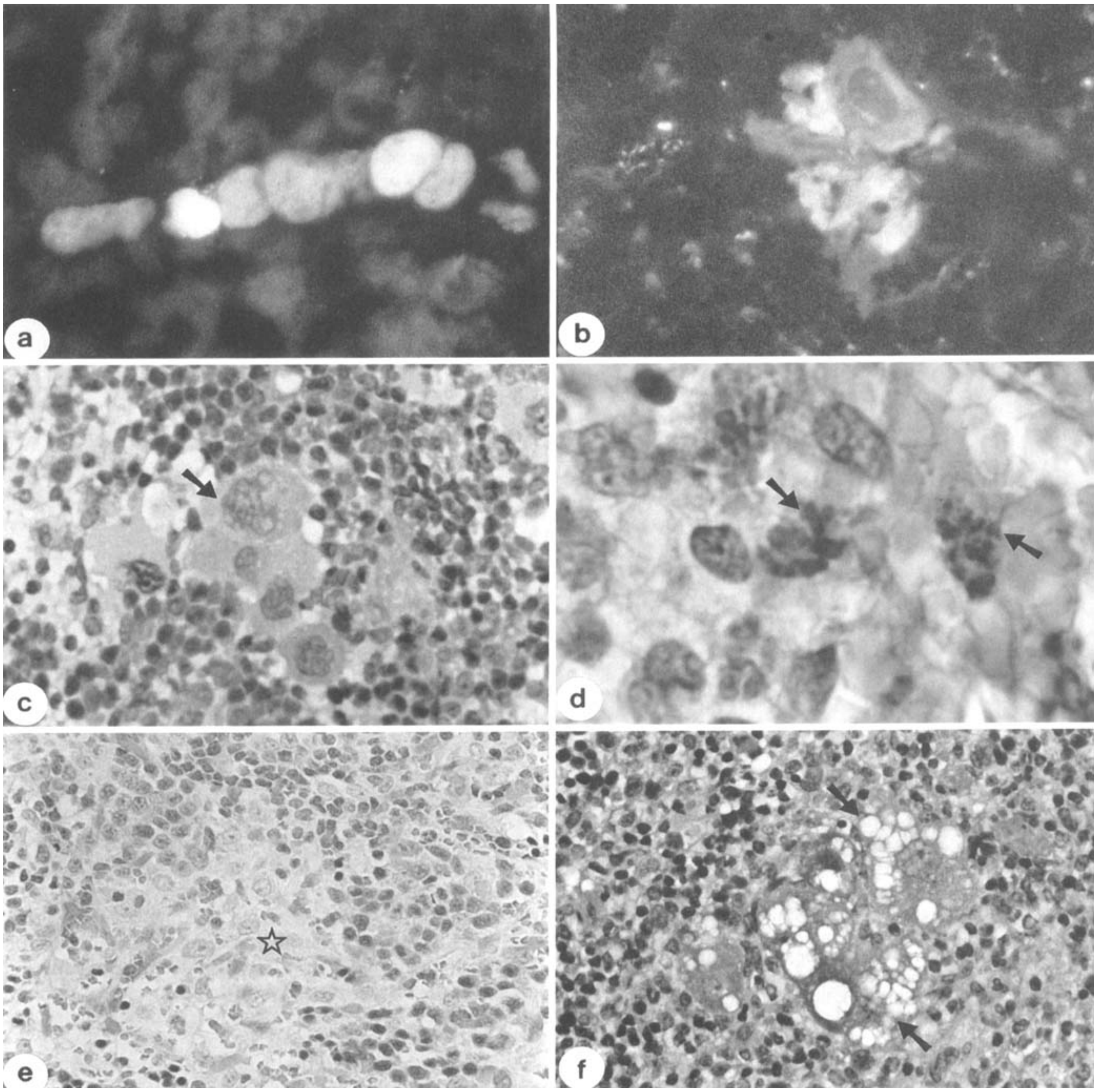

Fig. 2 Photomicrographs of transplanted hepatocytes: **a** CFSE-labeled modified hepatocytes 21 days post-intrasplenic inoculation, × 400; **b** CFSE-labeled modified hepatocytes in the liver 21 days post-intraportal infusion, × 400; **c** modified hepatocyte graft *(arrow)* in the splenic red pulp (H & E staining) 7 days posttransplant, × 400; **d** modified hepatocytes showing positive PAS reaction (intracellular glycogen, *arrows*) 14 days post-intrasplenic inoculation, × 500; **e** normal appearance of 14 day old modified hepatocytes (star) grafted in the splenic red pulp (H & E), × 200; **f** degenerative changes in crude hepatocytes *(arrows)* 7 days post-intrasplenic inoculation; note focal infiltration of mononuclear cells in the graft (H & E), × 200

their fluorescent appearance progressively decreased (Fig. 2b).

Recipients from group III

Microscopic analysis of the spleen 7 days after transplantation showed that inoculated hepatocytes were more dispersed, and their numbers rapidly declined with no subsequent appearance in the liver parenchyma.

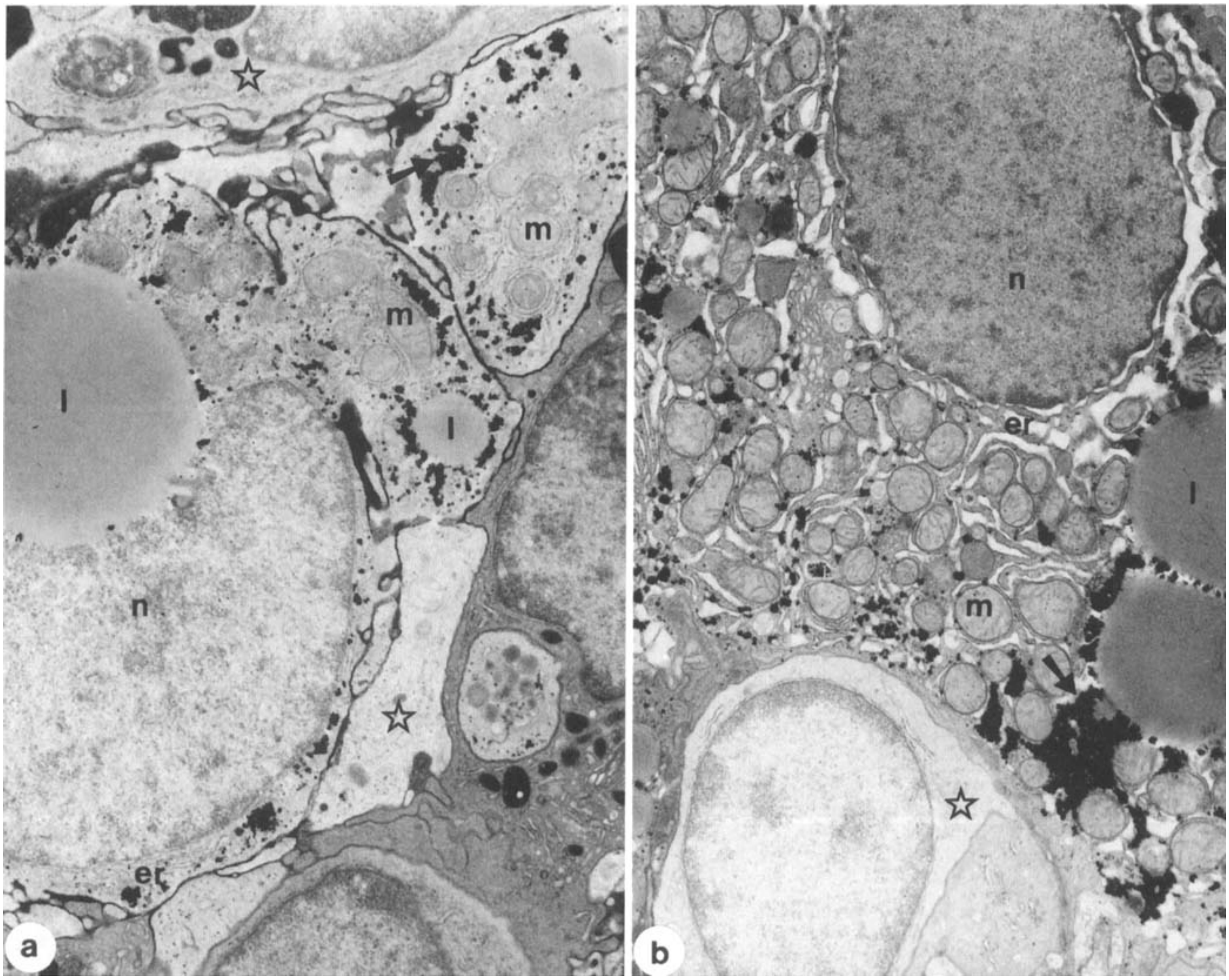

Fig. 3 Transmission electron micrographs of hepatocytes 7 days post-intrasplenic inoculation: **a** purified hepatocyte showing well preserved ultrastructure; **b** crude hepatocyte with signs of irreversible damage. Note the presence of mitochondria *(m)*, nucleus *(n)*, endoplasmic reticulum *(er)*, lipid droplets *(l)*, glycogen granules *(arrow)*, and adjacent splenic cells *(star)*, × 3000

In this case only a few cells were observed in the host spleen 7 days after inoculation. These cells were not positive for glycogen as demonstrated by PAS reaction, and their morphology showed signs of damage such as cytoplasmic vacuolisaton, macrovesicular steatosis, and necrosis. Many of the inoculated hepatocytes were swollen and pale-staining, with signs of balooning degeneration. Histological examination of the host spleen showed dense infiltration of mononuclear cells in the graft indicating destructive response (Fig. 2f). Biopsy of the liver 7 days after intraportal infusion demonstrated degenerative changes at the periphery of the lobes such as infractions and patchy necrosis. There was no morphological evidence of transplanted hepatocytes at 14 and 21 days after surgery.

Recipients from group IV

The fluorescence of the CFSE-labeled, dead hepatocytes was undetectable. All PAS reactions on the spleen sections were negative for glycogen.

Electron microscopy

The purified and cryopreserved cells showed intact ultrastructural appearance of normal hepatocytes 7 days after intrasplenic inoculation (Fig. 3a). However, 21 days after surgery (last day of histological examination) moderate signs of cellular derangement were found (slightly swollen mitochondria and increased number of large lipid droplets). The crude hepatocytes transplanted into the spleen and analyzed 7 days after surgery showed signs of irreversible damage to the ultrastructure including vesiculation of endoplasmic reticulum, depletion of glycogen granules, and condensation of mitochondrial matrix (Fig. 3b).

Discussion

The experiments reported here have shown that transplanted modified hepatocytes can be recovered from the spleen or liver of allogeneic mice recipients up to 21 days later without host immunosuppression. Labeling of hepatocytes with CFSE is a relatively new method that permits clear detection of transplanted cells by fluorescent microscopy [5, 12]. We utilized this method, which made the histologic identification after recovery of the liver (or spleen) much easier and more reliable. Histological examination revealed normal appearance of engrafted modified hepatocytes with no signs of inflammation or acute rejection. PAS reaction performed on the spleen sections demonstrated the presence of intracellular red granules of glycogen, indicating the metabolic activity of transplanted cells. Transmission electron microscopy analysis demonstrated that typical characteristics of the hepatocyte ultrastructure and graft integrity was maintained up to 3 weeks postoperative.

Experimental assessment of the hepatocyte immunogenicity is still controversial. It has been recently reported that the intrasplenic injection of hepatocytes into allogeneic host mice resulted in rapid hepatocellular graft destruction [2]. One possible reason for this discordance with our results might be the differences in the relative impurity of hepatocyte preparations used. These results are based on our previous in vitro evidence that Percoll-purified hepatocytes were incapable of stimulating an immune response although a standard (crude) hepatocyte preparation elicited a substantial cytotoxic response [13]. The second important observation was that cryopreservation substantially affected the measured cytotoxic activity, which suggests that cryopreserved hepatocytes are poor stimulators of in vitro cytotoxicity [14]. It is well established that lymphocyte activation in the immune response requires two signals: engagement of the T cell antigen receptor and a costimulator [7]. The liver parenchymal cells should have reduced immunogenicity because they are incapable of providing appropriate costimulatory signal. Since hepatocytes do not express MHC class II antigens, one of the strategies of allogeneic cell transplantation would be to purify the isolated hepatocyte preparations in order to remove contaminating cells that might initiate the immune response. We found out that other modifications of hepatocyte preparation, such as depletion of antigen presenting nonparenchymal cells (by panning with CD45 antibody) further reduces the immunogenicity. In this work, CD45 staining and flow cytometric analysis before transplantation showed that there were less MHC class II antigen-positive cells in the modified hepatocyte preparations compared to the crude hepatocytes. Reduced immunogenicity may be responsible for the longer survival time of inoculated modified hepatocytes with no adverse morphological effects in the host. However, it is important to emphasize that good survival and stabilization of the allografts in groups I and II can also be related to the better deposition and vascularization of modified hepatocytes, since damaged cells and debris were removed from the preparations. Recent studies indicate that distribution of transplanted hepatocytes is mechanically related to the structure of the graft [9, 10]. It has been well documented in mice and rats that intrasplenically transplanted hepatocytes are initially deposited in vascular spaces of the spleen and either translocate into the liver or are entrapped in the splenic red pulp. The presence in the graft of any dead or damaged cells may significantly disturb the process of polarization, cell-cell interactions, and integration of transplanted hepatocytes in the spleen or liver parenchyma. It is possible that the crude preparations of transplanted hepatocytes, contaminated with debris and cell fragments do not appropriately respond to regulatory and proliferative signals and exhibit a diminished functional and morphological capacity. Allogeneic hepatocyte acceptance may also be related to so-called hepatic tolerogenicity as shown by the fact that MHC mismatched liver grafts are spontaneously accepted without immunosuppression in many mouse and rat strains [6, 20]. The precise mechanism of this phenomenon is still unclear. According to recent reports, apoptosis of the infiltrating T cells may be responsible for the liver graft tolerance [18].

We have demonstrated a positive effect of pretransplantation hepatocyte purification and cryopreservation on the engraftment in allogeneic recipient after intrasplenic or intraportal injection. The model used in our experiments seems to be of value for further investigating the in vivo alloreactivity to isolated murine hepatocytes. Development of techniques to reduce or eliminate the immunogenicity of hepatocytes will be a huge step toward clinical use of hepatocyte transplantation. However, the important clinical end point of hepatocyte transplantation is the demonstration of the allograft activity without addition of immunosuppressive drugs. Further investigation is needed to evaluate a functional model of allogeneic hepatocyte transplantation and to determine what factors regulate the survival of the APC-depleted hepatocyte allografts in non-immunosuppressed recipients.

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