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Intraperitoneal hepatocyte transplantation: morphological results

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Summary. The value of isolated intraperitoneal hepatocyte transplantation as a temporary support in acute hepatic failure is controversial since the functional capacities and survival rate of auxiliary transplanted liver cells are uncertain. It was the aim of this study to investigate the survival rate of intraperitoneally (i.p.) transplanted hepatocytes and microcarrier attached hepatocytes. In 25 rats (group A) an i.p. hepatocyte transplantation and in 30 rats (group B) an i.p. microcarrier attached hepatocyte transplantation was performed and the animals were killed at 3 h, 12 h, 24 h, 3 and 7 days (group A) and 1, 2, 3, 7, 14 and 28 days (group B) after transplantation. Histological investigation showed that transplanted hepatocytes undergo complete cell necrosis within 3 days (groups A and B). Liver cell necrosis was followed by a peritoneal reaction resulting in granuloma formation in both groups. Since liver cell necrosis occurred soon after transplantation and could not be avoided by attachment of liver cells to microcarriers, it can be concluded that metabolic support from transplantation cannot be expected in acute hepatic failure.

Key words: Intraperitoneal hepatocyte transplantation – Microcarrier – Peritoneum – Metabolic liver support

Introduction

For more than 20 years auxiliary hepatocyte transplantation has been proposed as therapeutic approach in chronic and acute liver failure, or for the correction of inborn enzyme deficiencies (Berg et al. 1985; Groth et al. 1977). The spleen (Darby et al. 1986; Henne-Bruns et al. 1989; Mito et al. 1979; Sommer et al. 1979) and the peritoneum (Demetriou et al. 1986; Makowka et al. 1980; Sutherland et al. 1977; Thul et al. 1983; Vacanti et al. 1988) have been investigated as sites for implantation of isolated liver cells. The advantage of intrasplenic transplantation is the good vascularization of the spleen, an intraperitoneal injection allows the possibility of injecting a larger volume of hepatocytes.

For clinical application and functional support in acute hepatic failure it can be calculated that 20% of the liver parenchyma (5×10^{10} cells, approximately 500 ml hepatocyte suspension) has to be replaced. It has not been shown that such a large quantity of hepatocytes can survive for an extended period in the peritoneal cavity, and thus the aim of our study was to investigate the morphological changes and the survival rate of intraperitoneally (i.p.) transplanted liver cells. We also evaluated whether the survival rate of i.p. transplanted hepatocytes could be prolonged by attachment of the liver cells to microcarrier, as suggested by Demetriou et al. (1986).

Materials and methods

Male inbred Lewis rats weighing 200–300 g (obtained from the Zentralinstitut für Versuchstiere, Hannover, FRG) were used as liver cell donors and recipients. Animals were housed under standard conditions with free access to food and water and fasted 24 h before operation. There were two experimental groups. In group A, i.p. transplantation of $2–5 \times 10^8$ isolated hepatocytes was carried out and autopsy and morphological examination of the peritoneum was performed after 3 h ($n=5$), 12 h ($n=5$), 24 h ($n=5$), 3 days ($n=5$) and 7 days ($n=5$). In group B, i.p. transplantation of 7 ml microcarrier-attached hepatocytes was performed. Autopsy and morphological examination of the peritoneum was performed after 1 ($n=5$), 2 ($n=5$), 3 ($n=5$), 7 ($n=5$), 14 ($n=5$) and 28 ($n=5$) days. Group C was a control group with transplantation of 7 ml microcarrier suspension. Autopsy and morphological examination of the peritoneum was performed after 1 ($n=5$), 2 ($n=5$), 3 ($n=5$), 7 ($n=5$), 14 ($n=5$) and 28 ($n=5$) days).

Hepatocytes were isolated according to the collagenase digestive method of Berry and Friend (1969). Under ether anaesthesia the abdomen was opened by a midline incision. Cannulation of the portal vein was followed by a perfusion of the liver for 5 min with oxygenated Hank's solution and for 10 min with oxygenated Hank's solution containing 0.075% collagenase [type I, Sigma, St.

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Louis, Mo., USA (pH = 7.4, 37° C, flow rate 50–60 ml/min)]. The liver was removed and gently racked with a steel comb to isolate the hepatocytes. The hepatocyte suspension was washed twice with Hank's solution, centrifuged (50 *g*) and resuspended. Then $2–5 \times 10^8$ hepatocytes with a viability of 75%–85% after trypan blue exclusion test (0.5% Boehringer, Mannheim, FRG) were obtained from a rat liver. For experimental group A $2–5 \times 10^8$ hepatocytes were injected under ether anaesthesia into the peritoneum of the recipients within 1 h after isolation.

The preparation of microcarriers (Cytodex 3, Pharmacia, Freiburg, FRG) and incubation with the isolated hepatocytes were performed according to the technique described by Demetriou et al. (1986). One gram of microcarrier type Cytodex 3 was hydrated in 125 ml phosphate buffered saline (PBS) Dulbecco's medium (Gibco, Karlsruhe, FRG) for 90 min at 37° C using siliconized glassware to prevent adhesion of the microcarriers. The buffer was decanted and the microcarriers washed with 80 ml Kotake's medium (Kotake 1981) and 20 ml fetal calf serum (FCS). After 60 min incubation at 37° C using a microstir (Wheaton), the medium was decanted and the total volume was brought to 35 ml adding Kotake's medium. In animals of group C 7 ml microcarrier suspension was injected under ether anaesthesia into the peritoneum. For incubation of isolated hepatocytes with microcarriers the carriers were hydrated with Dulbecco's and Kotake's medium and FCS as described before. The medium was than decanted. Twenty millilitres of FCS, 170 ml Kotake's medium and the hepatocyte suspension which was filled up to 10 ml (containing $2–5 \times 10^8$ cells) were added. The suspension was incubated in the microstir (250 rounds/min) at 37° C for 150 min in a 5% carbon dioxide atmosphere.

At the end of the incubation period a probe was stained with trypan blue to evaluate the hepatocellular attachment. After sedimentation of the microcarriers and hepatocytes the medium was decanted and the suspension brought to a volume of 35 ml adding Kotake's medium. Microcarrier-hepatocyte suspension (7 ml) was injected under ether anaesthesia into the peritoneum of group B animals.

Animals were killed by deep ether anaesthesia according to the experimental protocol. The peritoneal cavity was examined and the hepatocyte/microcarrier aggregates were excised. For light microscopic examination the tissue was fixed in 3.5% buffered formalin, embedded in paraffin and stained with haematoxylin. Immunohistological examinations on paraffin-embedded sections were performed with the antibodies KL1 (pan cytokeratin; Dianova, Hamburg, FRG) and V9 (vimentin; Boehringer, Mannheim, FRG) using the ABC method (ABC kit; Vectastain, Camon, Wiesbaden, FRG). For electron microscopic examinations the tissue was primarily fixed in 2.5% buffered glutaraldehyde followed by fixation in 1% osmium tetroxide. After critical point drying in Frigen (11–13) probes were sputtered and examined with a Novascan 30.

Results

In Group A autopsy 3 h after i.p. hepatocyte transplantation revealed liver cells aggregated mainly in the omentum. Small aggregates were also present in the right subphrenic area and between the small bowel loops. Ascites (1–2 ml) were present in all animals, but absorbed after 12 h. After 12–48 h aggregates showed an increasing consistency. By day 3 after transplantation the aggregates appeared as white i.p. granulomas with adhesions to the bowel. The peritoneum showed a slight inflammatory reaction which had subsided by day 7. From the 7th to 28th day central necrosis encapsulated by fibrotic tissue was observed in larger granulomas. In groups B and C 24 h after transplantation of the hepatocyte-microcarrier suspension a large aggregate was found at the omentum and some small aggregates between the bowel loops. A slight peritoneal inflammation was observed until day 3.

Aggregates were of increasing consistency from day 1 to 28. In contrast to the findings in group A, no central necrosis was observed even in large granulomas, although fibrotic encapsulation could be seen after the first week.

In sections from animals from group A killed within 3 h after i.p. transplantation hepatocytes are observed aggregated at the omentum. About 50% of liver cells appear shrunken, the nucleus pyknotic (Fig. 1a). By 12 h a marked reduction of vital appearing hepatocytes as well as a slight granulocyte infiltration can be noticed (Fig. 1b). Within 24 h a peripheral zone with about 50% vital hepatocytes surrounded by granulocytes can be differentiated from a central area consisting mainly of necrotic liver cells, already showing cellular lysis (Fig. 2a). By day 3 four zones can be differentiated in each i.p. granuloma (Fig. 2b); a peripheral thin layer of connective tissue (zone A) surrounds necrotic liver cells (zone B). Deep to this a dense granulocyte wall (zone C) invades a central area (zone D) of necrotic hepatocytes.

By day 7–28 the peripheral fibrotic capsule thickens. Calcification and macrophages can be identified surrounding necrotic hepatocytes and there is a granulocyte infiltrate (Fig. 2c). Probes taken from the hepatocyte-microcarrier suspension prior to i.p. transplantation confirm the attachment of liver cells to the carrier surface in group B (Fig. 3a). Electron microscopy of microcarrier-attached hepatocytes shows that the attached liver cells make an impression in the carrier surface (Fig. 3b). In sections 24 h after i.p. transplantation only a few vital hepatocytes are observed. Most liver cells show pyknotic degeneration of the nucleus or lysis of the cellular membrane. Liver cells are found lying in clumps between the carriers. Few granulocytes are seen around the hepatocytes (Fig. 4a). Within 48–72 h the granulocyte infiltration increases remarkably (Fig. 4b). Liver cells show either pyknosis of the nucleus or lysis of the cellular membrane. By 1–4 weeks the microcarrier-hepatocyte aggregate is encapsulated by a thick layer of fibrotic tissue. Beneath this three zones can be differentiated (Fig. 5); a peripheral area consisting of encapsulated microcarriers (zone A), a dense granulocytic infiltrate (zone B) surrounding a central area (zone C) of necrotic liver cells and microcarriers. Magnification of encapsulated microcarriers in zone A reveals an epithelioid cell layer surrounding the carrier (Fig. 6a). Immunohistological staining is negative for cytokeratin (Fig. 6b) and positive for vimentin (Fig. 6c) demonstrating the mesenchymal origin of the epithelioid cells.

In animals undergoing unloaded i.p. microcarrier transplantation a similar reaction with granulocyte infiltration and encapsulation of the microcarriers was observed, as described above.

Discussion

In this study, i.p. transplantation of isolated hepatocytes or microcarrier-attached hepatocytes resulted in short-

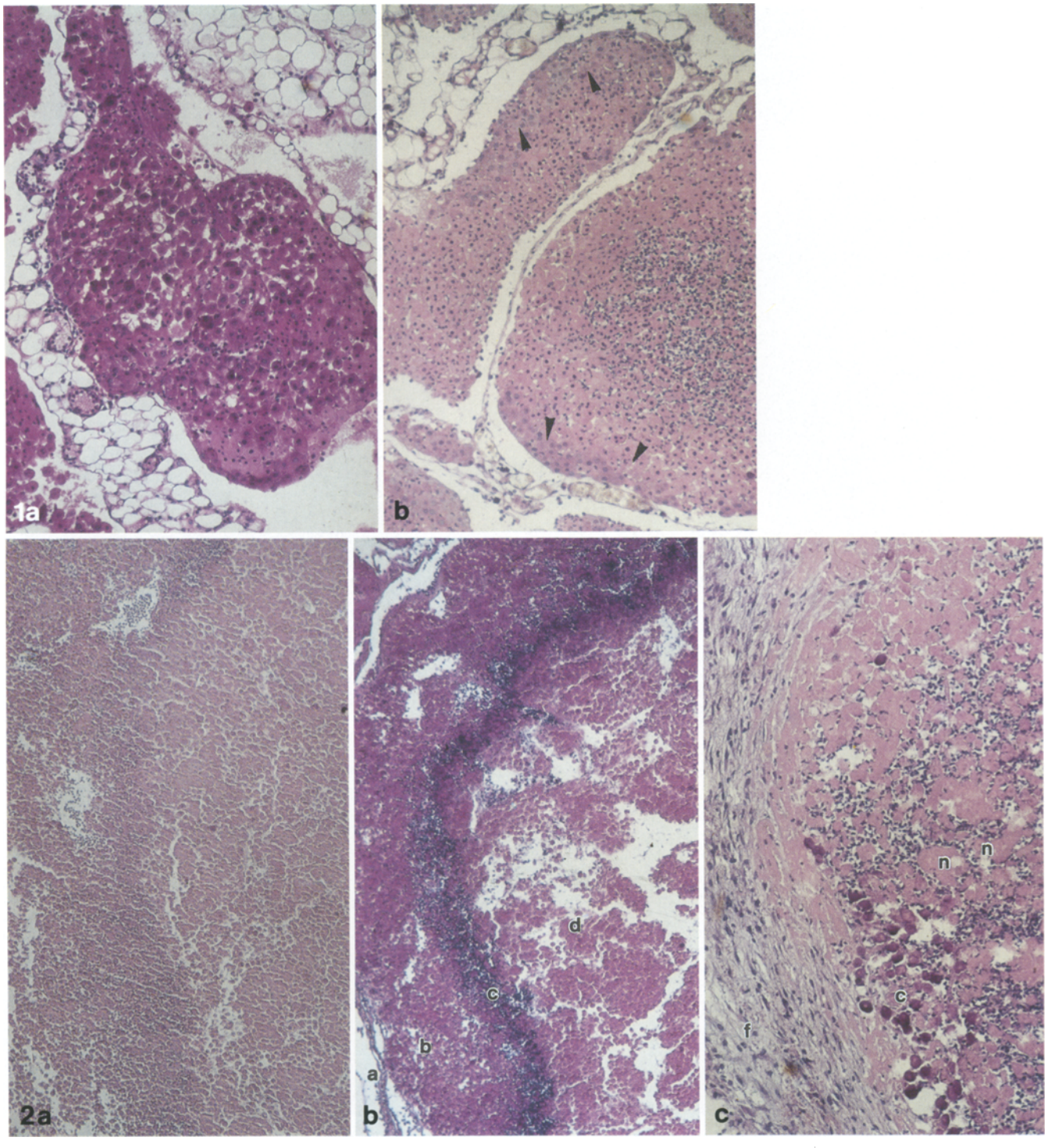
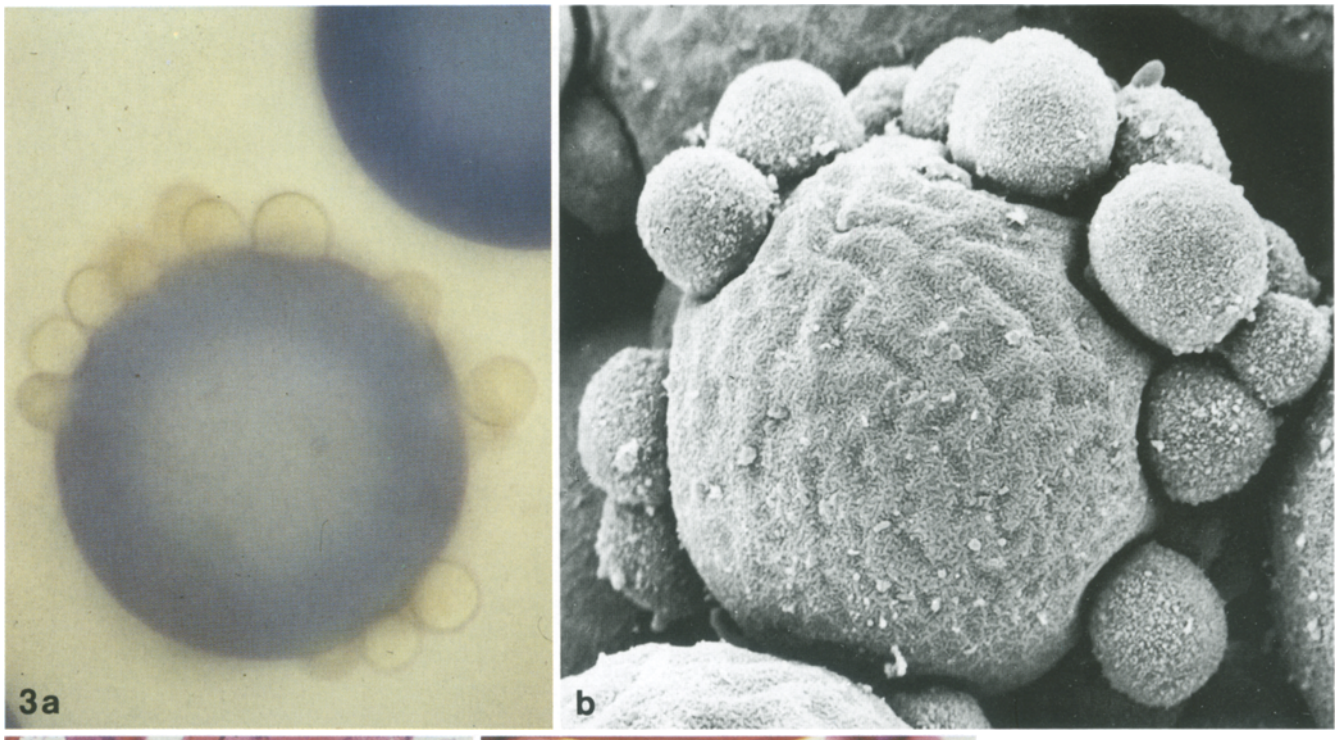


Fig. 1a, b. Histopathology after intraperitoneal hepatocyte transplantation. **a** Aggregation of hepatocytes at the greater omentum 3 h after injection. About 50% vital liver cells. HE ×40. **b** Aggregation of hepatocytes at the greater omentum 12 h after injection. Few vital hepatocytes (*arrowhead*) and slight granulocyte infiltrate. HE ×40

Fig. 2a–c. Histopathology after intraperitoneal hepatocyte transplantation. **a** Aggregate of liver cells 24 h after injection; peripheral granulocyte infiltration; only very few vital hepatocytes were observed in the centre of the aggregate. HE ×16. **b** Intraperitoneal aggregate 3 days after transplantation: *a*, peripheral zone of connective tissue, *b*, necrotic liver cells, *c*, granulocytes, *d*, necrotic liver cells. HE ×16. **c** Intraperitoneal granuloma 7 days after injection: *f*, fibrous capsule, *c*, calcifications, *n*, necrotic liver cells. HE ×40. (Reduced to 90%)



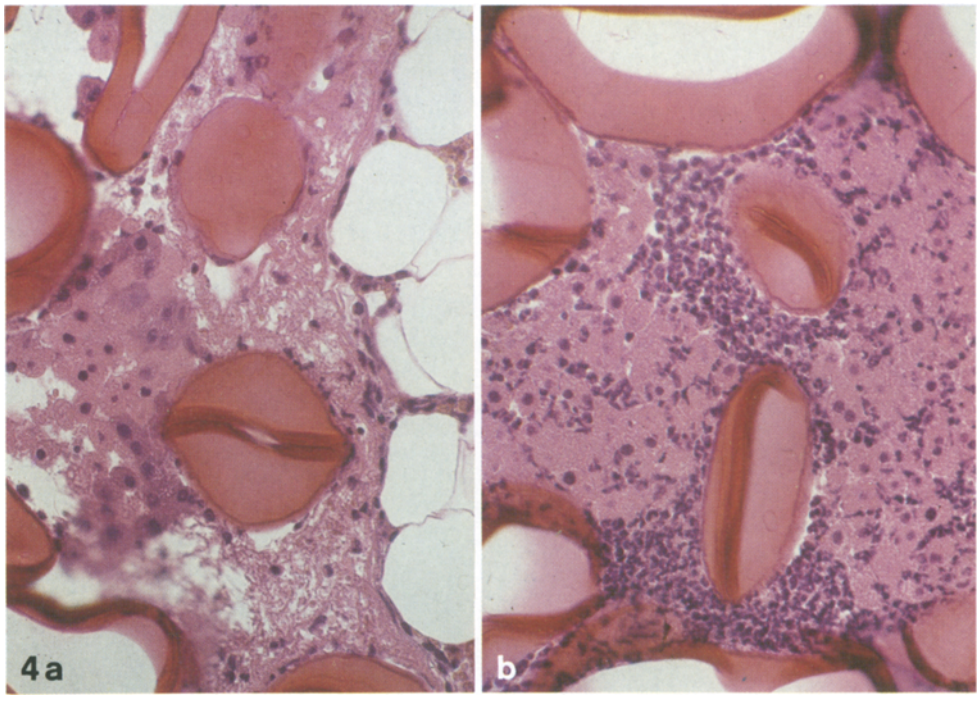


Fig. 3a, b. Histology 2 h after incubation of isolated hepatocytes with microcarriers.
a Methylenblue staining prior to injection: good aggregation of liver cells at the carrier surface ×100.
b SEM: Attached hepatocytes impress the carrier surface ×2575. (Reduced to 90%)

Fig. 4a, b. Histopathology after intraperitoneal transplantation of microcarrier attached hepatocytes.
a 24 h after injection: mainly necrotic liver cells between the microcarriers. HE ×100.
b 48 h after injection: necrotic liver cells surrounded by granulocytes between the carriers. HE ×100

term survival of transplanted liver cells, followed by a peritoneal foreign body reaction with granuloma formation and encapsulation of the necrotic liver cells and microcarriers.

In various publications (Makowka et al. 1980; Sommer et al. 1979; Thul et al. 1983) a reduction of the lethality rate in experimentally induced hepatic failure was described after i.p. hepatocyte transplantation, although in none of these studies were macroscopic findings at autopsy or histological investigations reported. Only Sutherland et al. (1977) described small i.p. nodules, consisting of fibrotic tissue at autopsy 5 days after i.p. hepatocyte transplantation. Although a reversal of acute hepatic failure could not be related to a living liver cell transplant, the above-mentioned studies suggested a benefical effect of i.p. transplanted hepatocytes. Early cell necrosis after i.p. hepatocyte grafting was also described by Vacanti et al. (1988) after transplantation of

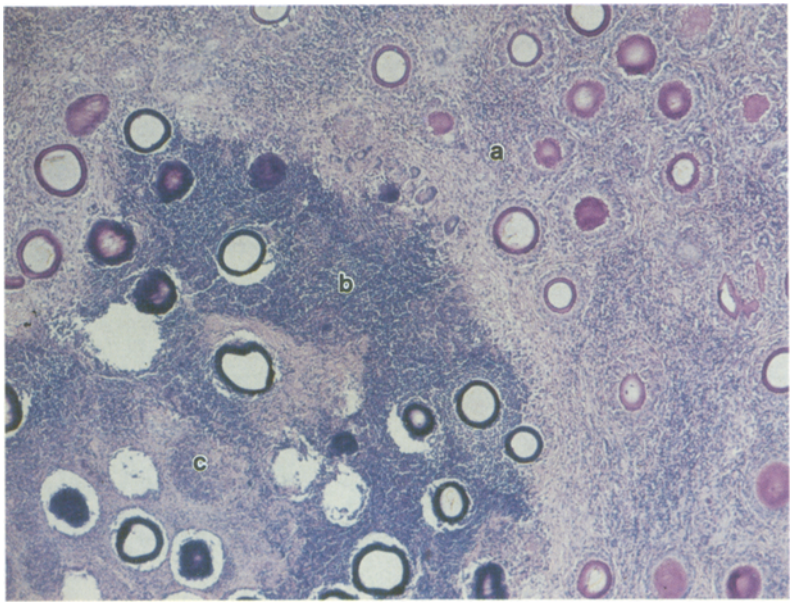


Fig. 5. An intraperitoneal granuloma 4 weeks after transplantation of microcarrier attached hepatocytes. *a*, microcarriers encapsulated by epithelioid cells; *b*, dense granulocyte infiltrate; *c*, necrotic liver cells are lying between the carriers. HE ×16

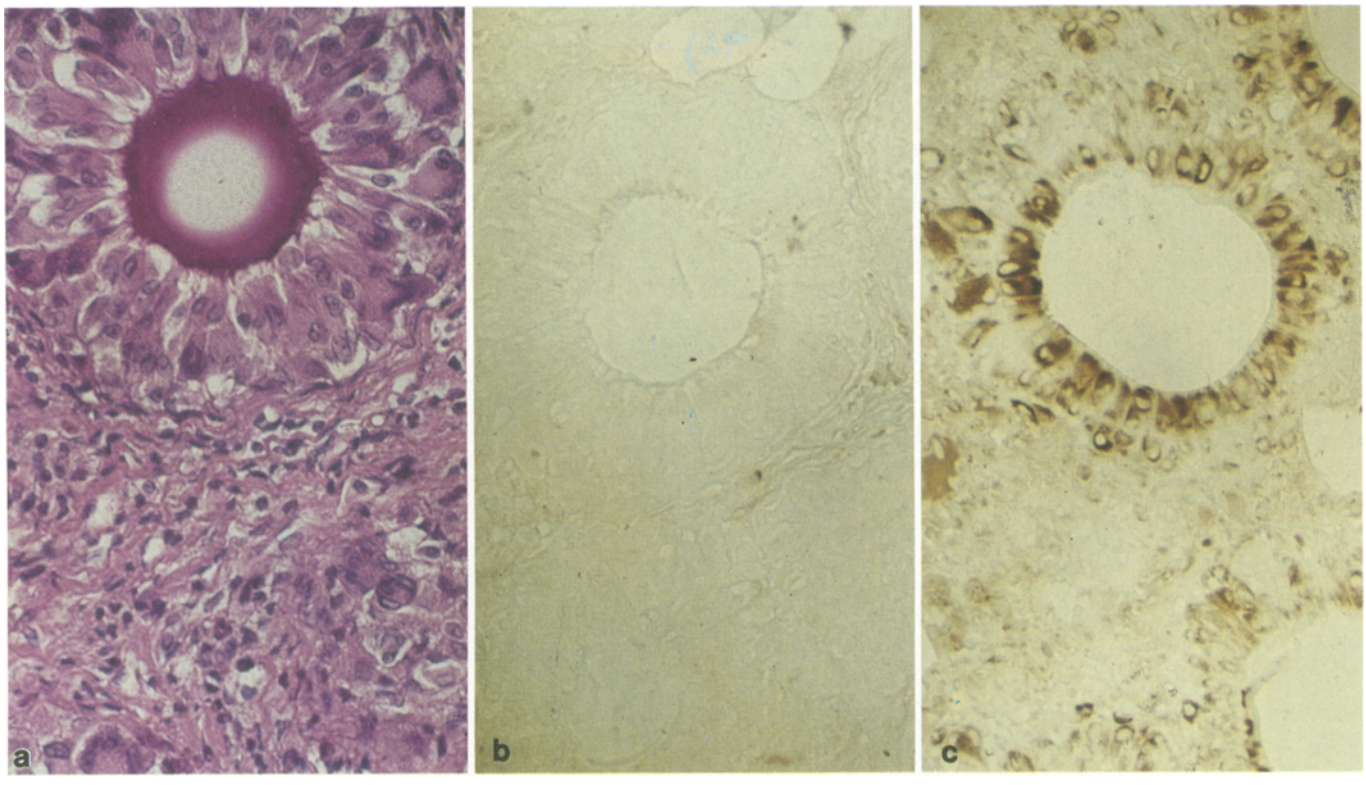


Fig. 6. Histopathology from a peripheral area of an intraperitoneal granuloma 2 weeks after i.p. transplantation of microcarrier attached hepatocytes. **a** Microcarriers encapsulated by an epithelioid layer of macrophages. HE ×100. **b**, **c** Immunohistological staining ×100, **b** = Cytokeratin negative, **c** = Vimentin positive (Reduced to 90%)

polymer-attached hepatocytes. In his study viable hepatocytes could only be identified in the peritoneum in 3 of 66 rats, 7 days after transplantation.

Our own results show that hepatocytes aggregate in the peritoneal cavity within a few hours after injection. Only dead hepatocytes could be found in the centre of the liver cell aggregates after 24 h. We therefore conclude that the diffusion distance from the peritoneal surface to the centre of the aggregate is too long for substrate supply of the transplanted liver cells; vascularization of

the peritoneum could not be observed at that time. Early cell necrosis of transplanted liver cells was not avoided by attachment of the hepatocytes to microcarriers. Demetriou (1986) described the encapsulation of microcarriers by epithelioid cells which which were thought to be liver cells after transplantation of microcarrier-attached hepatocytes. In our study we were able to confirm the finding of encapsulation of microcarriers, but as the immunohistological investigations demonstrated, the epithelioid cells are clearly of mesenchymal origin and are therefore probably macrophages.

In acute liver failure hepatic support can only be obtained by a large mass of viable liver cells. Neither i.p. hepatocyte transplantation nor transplantation of microcarrier-attached liver cells produced prolonged survival of the grafts. Thus a therapeutic approach of this type does not appear to be feasible in acute hepatic failure.

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