An Experimental Method for Rapid Growth of Liver in Spleen

The Survival and Proliferation of Chemically Induced Preneoplastic Hepatocytes in Spleen

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Cellular suspensions (2×10^6 cells) of isolated preneoplastic liver cells, obtained from carcinogen-treated rats, were injected in the spleens of syngeneic rats divided into groups on the basis of no treatment, partial hepatectomy (PH), and/or feeding regimens including 2-acetylaminofluorene (AAF). Recipient rats undergoing both PH and AAF showed significantly more rapid proliferation of the preneoplastic liver cell implant, compared with other treatment groups and control. The theoretic basis for this observation, supported by a large body of data derived from hepatocarcinogenesis, is as follows: The phenotype of the donor cells has been altered by chemical carcinogens such that the liver cells develop resistance to growth-inhibiting agents such as AAF. The recipient receives PH and AAF, the former creating a strong proliferative stimulus for hepatocytes, while the latter inhibits regeneration of normal liver cells but not those resistant to the mito-inhibitory effect of AAF, ie, the carcinogen-altered donor cells. These manipulations in donors and recipients thus create a selective environment in which the implant undergoes rapid proliferation. This model of resistance induction followed by selective proliferation, built upon the principles of carcinogenesis and applied to isolated liver cell transplantation, provides an experimental basis for achieving rapid liver growth of the splenic implant. (Am J Pathol 1983, 110:119–126)

A NEW EXPERIMENTAL TECHNIQUE that is receiving increasing interest of recent date is the implantation of a free graft of isolated liver cells into spleen.[1] Investigations using syngeneic rats have clearly demonstrated the spleen's ability to support the growth of isolated hepatocytes.[2–6] Mito and associates have shown that an inoculum of 5×10^6 normal rat liver cells injected into syngeneic rat spleen can proliferate over 17 months to occupy up to 40% of splenic parenchyma.[4] While incapable of bile formation and drainage, such a spleen containing liver (SCL) is nevertheless functional as assessed by ammonia tolerance testing and indocyanine clearance. Additional studies have confirmed that isolated liver cells transplanted into spleen as well as other sites maintain their physiologic and biochemical integrity.[7–22] Since an SCL, if successful, would find its greatest value in the treatment of severe, acute liver failure, it becomes mandatory that there be rapid proliferation of the hepatocyte implant to critical mass effective in assisting the failure liver. We will describe here an experimental method whereby prominent replacement of spleen by liver tissue is evident by 1 week following implantation. The basis for this

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approach is discussed as well as possible further applications.

Materials and Methods

Animals

Male Fischer-344 rats (Charles River) weighing 130 to 150 g were maintained on a high-protein (24%) semisynthetic diet (Bio-Serve, Inc.) and a daily cycle of alternating 12-hour periods of light and darkness. The animals were given food and water *ad libitum* and were acclimated to their environment for 1 week before beginning the experiment. A single carcinogen-treated rat served as a source for all isolated liver cell implants. We performed the experiment on three separate occasions, using a different single donor rat in each instance. All trials yielded comparable results. Measurements were obtained in each instance and statistically evaluated by the use of an image analyzer in one experiment.

Induction of Hyperplastic Nodules

Hyperplastic nodules were induced in the donor rat liver by the procedures outlined by Solt and Farber.[23] Two weeks after a single dose of diethylnitrosamine (Eastman Kodak Company, Rochester, NY, 200 mg/kg body weight, intraperitoneally) the animals were placed on a basal diet containing 0.02% 2-acetylaminofluorene (AAF) (Dyets Inc., Bethlehem, Pa) for 14 days. After a week on this dietary regimen, a partial hepatectomy (PH) was performed, and the rats were returned to the control basal diet after a further week on the AAF diet. Four months later, the liver containing hyperplastic nodules was used for the isolation of liver cells.

Isolation of Hepatocytes

A single cell suspension was prepared from the whole liver by the enzymatic perfusion technique of Berry and Friend[24] and Seglen[25] as modified by Laishes and Williams.[26] The hepatocytes, obtained as a pellet after centrifugation, were resuspended in ice-cold serum-free Williams' Medium E to give a cellular concentration of 10×10^6 viable cells per milliliter. Viable cells were measured by the trypan blue exclusion test as described by Laishes et al,[26] and the percentage of viability obtained for the present experiment was 80–85%. In the dissociation from the liver bearing hyperplastic nodules, no attempt was made to separate the nodule cells from the "normal" surrounding liver; and the cells implanted as such consist of a mixture of normal and nodule cells. In addition, the extent of contamination by nonparenchymal cells was not assessed, and hence bile duct cells were also co-implanted.

Implantation of Hepatocytes

The suspension of hepatocytes was kept on ice throughout the whole experiment with good maintenance of viability for at least 4 hours.

The host was anesthesized with ether, and a 4-cm incision was made in the midsection of the abdomen. We brought the spleen to the exterior by pulling the stomach out with blunt forceps. The hilar vesssels of the spleen were clamped with a 12-mm-long Heifetz intracranial aneurysm clip (Weck and Co., Inc., Research Triangle Park, NC). An aliquot of 0.2 ml of the cell suspension containing 2×10^6 cells was injected directly into the midinterior of the spleen through the capsule with a 30.5-gauge needle. A 5-mm-long Kleinert-Kutz microvessel clip (Weck and Co.) was then positioned over the injection site, and the clamp was applied as the needle was withdrawn. After 1–2 minutes, the larger clip was removed from the hilum, but the smaller clip was maintained at the injection site until a clot formed (usually another 1–2 minutes). In cases with a larger puncture, bleeding

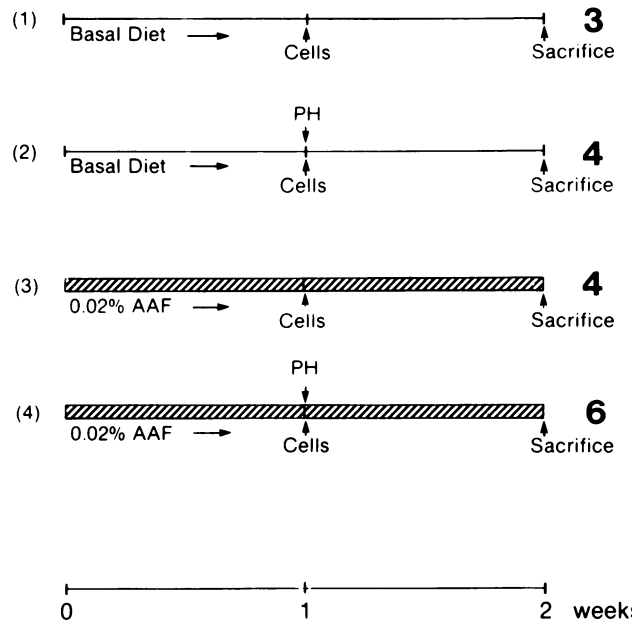

Figure 1—Diagramatic representation of various treatment protocols for control (1-3) and experimental recipients (4). Number of animals per treatment groups are shown on far right. *PH*, two-thirds partial hepatectomy. *AAF*, 2-acetylaminofluorene, represented by *hatched bars*. Donor hyperplastic liver cells are implanted 1 week after AAF treatment at the time of partial hepatectomy.

was stopped by the application of a small piece of Gelfoam (Upjohn) at the injection site.

Recipient Treatment Protocols

Four groups of recipient rats were used in this study. (Figure 1). One subset was given no pretreatment. A second underwent PH at the time of splenic implantation. A third group received AAF for 1 week before and after implantation. The final subset was given both AAF and PH as described for the second and third groups.

Processing of Tissues

Animals were sacrificed by cervical dislocation 1 week after implantation. The spleen was removed and laid on its dorsal surface. A longitudinal section (1–2 mm in thickness) was made along the midlongitudinal axis of the spleen and fixed in 10% buffered formalin for routine histologic examination. Five-micron-thick paraffin-embedded histologic sections were stained with hematoxylin and eosin (H&E) and periodic acid–Schiff (PAS) before and after diastase treatment, Prussian blue, Masson's trichrome, Gordon and Sweet's reticulin, and Hall's method for bile. Morphometric analysis was carried out on a Leitz ASM Image Analyzer on standard H&E sections.

Transmission Electron Microscopy

Small fragments of splenic tissue, no greater than 1 mm in greatest dimension, were immersed in fixative containing 2.5% glutaraldehyde in a 0.2 M cacodylate buffer at pH 7.4 for 2 hours. The tissue was then postfixed in 1% osmium for 2 hours at 4 C and embedded in Epon araldite. Semithin sections of plastic-embedded tissue were stained with toluidine blue. Ultrathin sections were cut on a Reichert OMU-3 ultramicrotome, stained with uranyl acetate

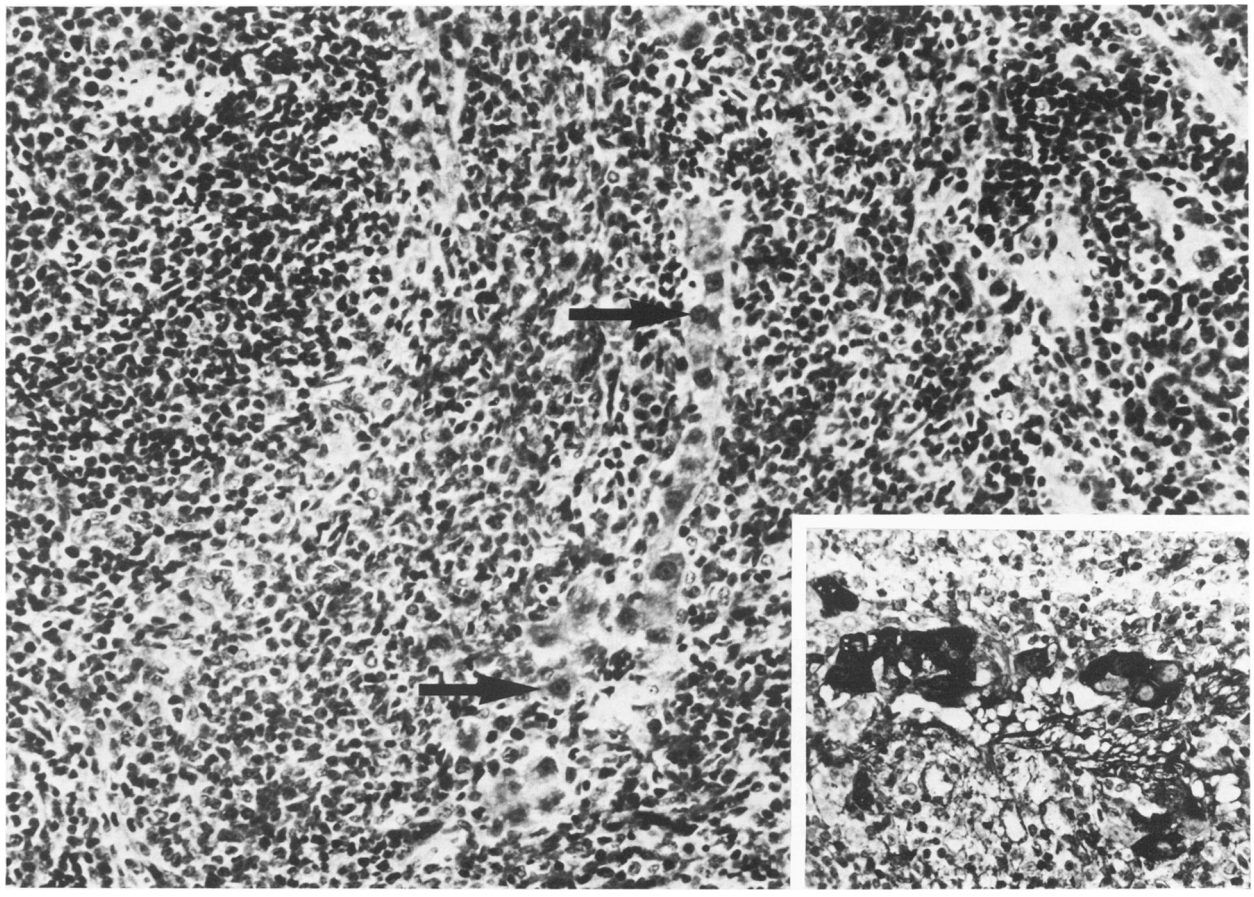

Figure 2—Spleen containing liver (SCL) 1 week after implantation, showing representative appearance of liver cells from Groups 1–3. Spleen contains small numbers of hepatocytes randomly distributed in red pulp (*arrows*). (H&E, ×100) **Inset**—Transplanted hepatocytes contain intracytoplasmic glycogen. (PAS, ×100) (With a photographic reduction of 7%)

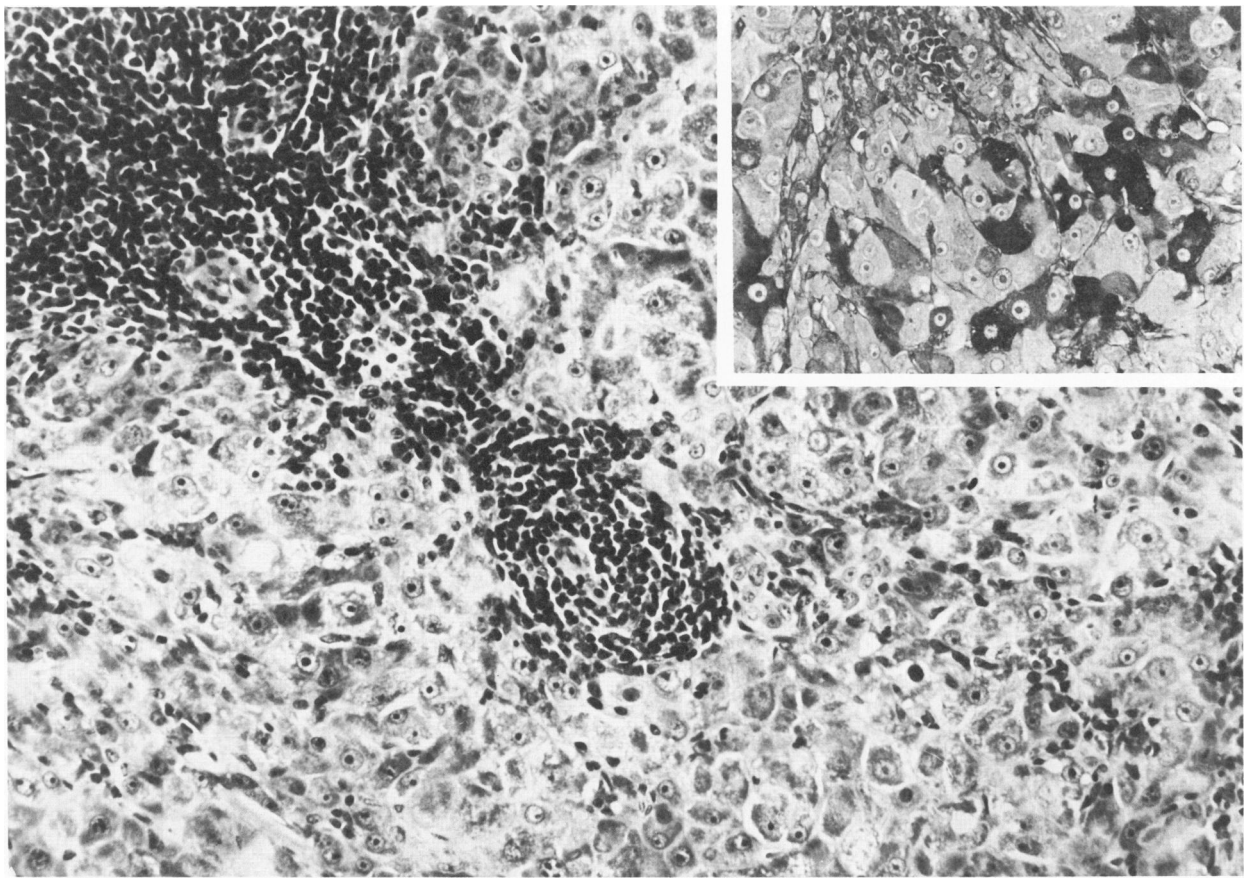

Figure 3—SCL from AAF and PH recipient of hyperplastic donor liver cells (Group 4). Sheets of hepatocytes are encroaching upon white pulp. (H&E, × 100) **Inset**—liver cells show marked variation in amounts of intracytoplasmic glycogen. (PAS, × 100) (With a photographic reduction of 7%)

and lead citrate, and examined under a Philips 301 electron microscope.

Results

The livers of donor rats, chosen for the experiment described in this report, contained many gray-white hyperplastic nodules. The gross and microscopic appearance of these 5-month-old, carcinogen-treated livers was typical of many such livers encountered in our laboratory[27-29] in which histologic investigation has failed to reveal any heptocellular carcinoma in this time interval.

In hepatized spleens removed at 1 week after-implantation it was not possible to grossly distinguish, with certainty, liver tissue from splenic parenchyma when one observed the cut surface of the spleen. However, on microscopic sections, liver cells were easily distinguished by virtue of their abundant cytoplasm, variable glycogen content, and epithelial

Table 1—Morphometric Analysis of Spleen Containing Liver 1 Week after Implantation

Recipient treatment	Total area of hepatocytes in relation to a standard section of spleen*
None	0.225 ± 0.110 (3)
PH only	0.175 ± 0.066 (4)
AAF only	0.273 ± 0.090 (4)
AAF and PH	2.195 ± 0.615 (6)†

* All values are in mm² and expressed as the mean ± SD; the number of samples is in parenthesis. Statistical comparisons were made by the Student *t* test. The total area of spleen examined was not statistically different in any of the 4 groups.

† $P < 0.01$.

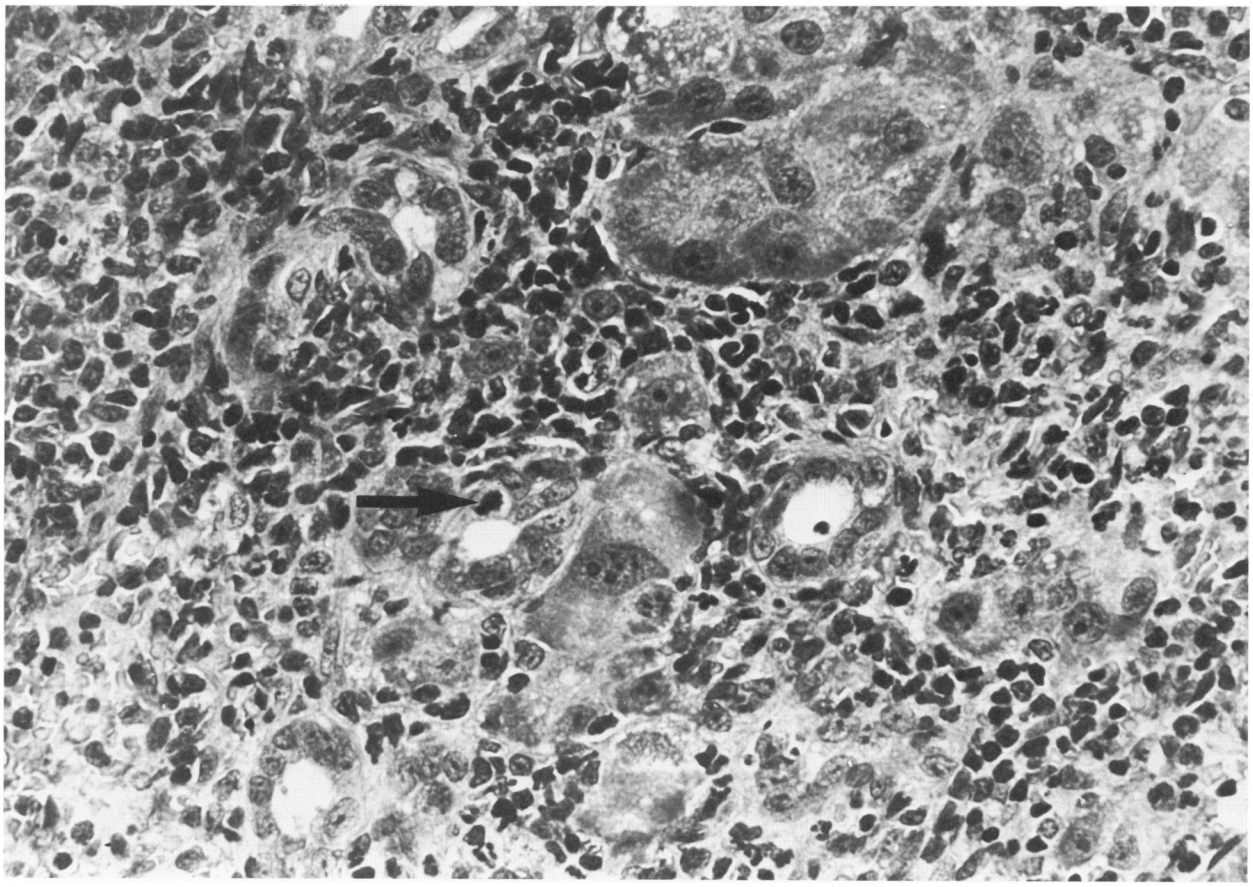

Figure 4—SCL from AAF and PH recipient of hyperplastic donor liver cells (Group 4). Bile ducts have formed with lining cells showing mitotic activity (*arrow*). (H&E, ×200) (With a photographic reduction of 7%)

growth pattern. In the no treatment, PH only, and AAF only groups, liver was present in the form of isolated small clusters of hepatocytes sparsely scattered in the red pulp (Figure 2). In the AAF and PH subset, however, the quantity of liver was much increased, taking the form of sheets of hepatocytes situated in red pulp and showing encroachment of white pulp (Figure 3). Mitotic figures were present but difficult to demonstrate. The total area occupied by hepatocytes was quantified morphometrically and is shown in Table 1. The AAF and PH recipients demonstrated approximately a tenfold increase in liver content. Scattered about the areas of liver tissue in rats that received AAF and PH were single and focal aggregates of bile ducts (Figure 4). However, no bile production was evident.

In fine structure the liver cells from control groups 1, 2, and 3 showed features of normal hepatocytes, including rough endoplasmic reticulin, abundant cytoplasmic glycogen, and large numbers of mitochondria. Bile canaliculi could be easily seen, but a paucity and blunting of microvilli was clearly evident. Liver cells in Subset 4 showed cells as described above; but in addition, hyperplastic liver cells, similar to those previously reported,[27] were seen. These cells possessed abundant smooth endoplasmic reticulin, intracytoplasmic filaments, and pericanalicular vesicles (Figure 5).

Discussion

Normal isolated liver cells, when implanted into spleen, will slowly continue to proliferate, so that over 17 months viable hepatic tissue comes to occupy

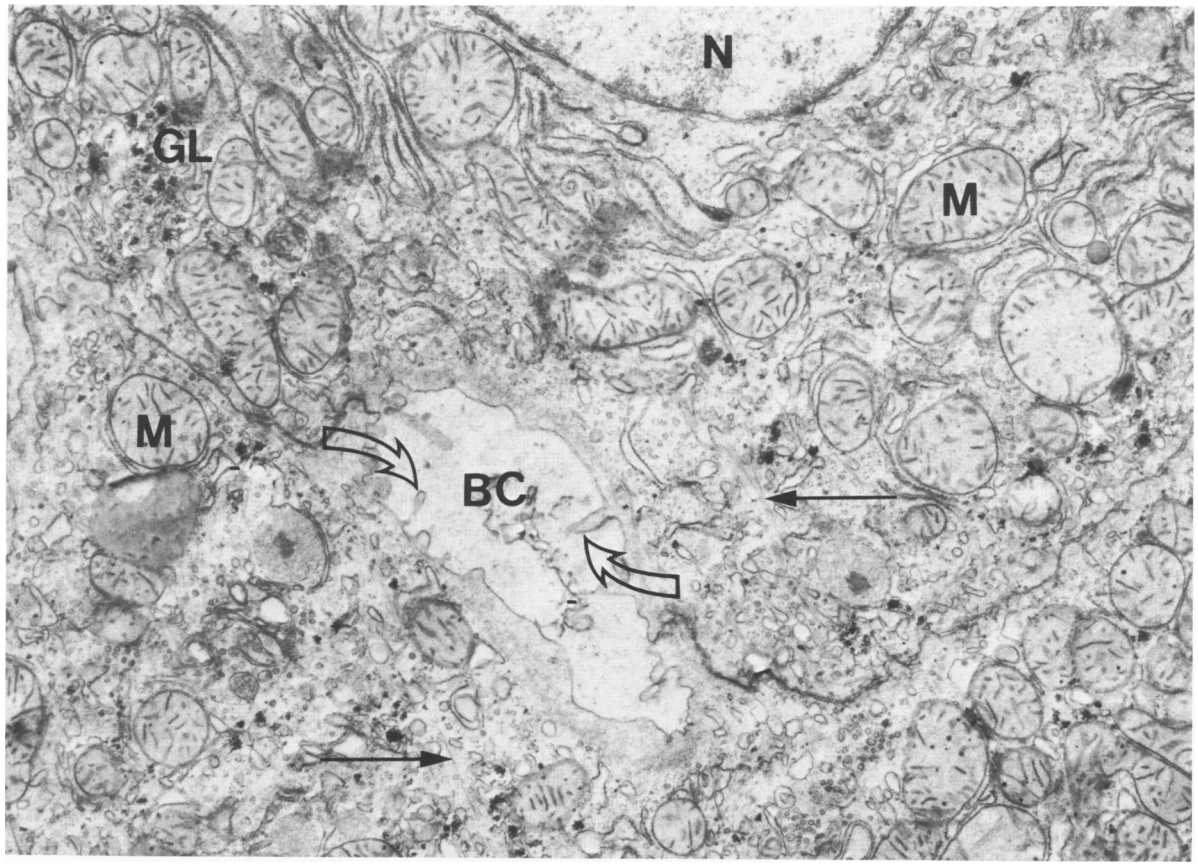

Figure 5 — Electron micrograph of SCL (Group 4). Hepatocytes possess abundant mitochondria (*M*), intracytoplasmic glycogen (*GL*), and microfilaments (*solid arrows*) and form bile canaliculi (*BC*). Note the paucity of microvilli projecting into the canalicular lumen (*open arrows*) *N*, nucleus. (× 14,250)

40% of the splenic parenchyma (without exceeding the original spleen weight.)[4] We attempted to see whether this process could be accelerated, we hoped to such a degree that significant amounts of liver tissue could be generated in the spleen over much shorter time periods.

While malignant liver cells might be useful from the point of view of rapid proliferation, uncontrolled growth and eventual metastasis countermand their use as donor cells. It is interesting that despite their malignant phenotype, hepatoma cells, when implanted subcutaneously, are effective in ameliorating the degree of jaundice when the congenitally enzyme-deficient Gunn rat is used as a recipient.[7] Drawing upon our experience in hepatocarcinogenesis, we undertook to see whether "preneoplastic" hepatocytes obtained from the livers of rats given certain chemical carcinogenic agents could survive when implanted into spleen and what the kinetics of their growth would be under suitable environmental conditions conducive to their proliferation.

Hyperplastic hepatocytes are "putative preneoplastic" liver cells that represent one of the many phenotypically different cell types encountered in the continuum from normal to overtly malignant cells.[28-30] While incapable of autonomous growth, under suitable conditions favorable to their growth, they manifest as focal proliferations within liver and from them evolve, through successive generations of hepatocytes, small numbers of cancer cells.[30] The sequence of events that underlies the change from normal to hyperplastic to malignant liver cells remains unclear. In this report we do not attempt to deal with this issue but rather make use of the enhanced pro-

liferative capacity and controlled growth of hyperplastic liver cells as donor hepatocytes in an animal model of SCL.

In this report we have shown that by using hyperplastic liver cells as donor hepatocytes and providing, in the recipient, the same selection pressure used to stimulate focal proliferation of hyperplastic hepatocytes, striking degrees of liver cell growth become possible. In 1 week an implant of 2×10^6 hyperplastic cells in an AAF-and-PH-treated recipient demonstrates a tenfold increase in liver content, as compared with similar donor inocula in partially or untreated recipients (Table 1). The cells that are injected represent a mixture of all cell elements from liver that contain hyperplastic nodules. It is conceivable that the increase in liver content in animals that received the total pretreatment might well represent preservation of the cells of the hyperplastic nodules. Careful histochemical and electron-microscopic evaluation would be necessary to clarify this point. Nevertheless, in 1 week approximately 10% of the area of a standard splenic histologic section taken through maximal SCL is occupied by viable liver tissue.

The possible operational basis for the observations described in this report is as follows. During the process of carcinogenesis the donor hepatocytes undergo a series of biomolecular alterations, resulting in a changed phenotype that is resistant to growth inhibition by carcinogens such as AAF.[31] It is this property of biochemical resistance that confers upon the implant the capacity to overcome growth inhibition in the recipient and thus result in rapid proliferation. In essence, our experimental model, founded upon basic principles in hepatocarcinogenesis, can be described as resistance induction/selective proliferation of the splenic implant. The proliferative stimulus of PH is advantageously directed upon the splenic implant of liver cells.

The ultimate goal of our research would be to develop a method of rapid liver cell proliferation in spleen suitable for human allotransplantation. Clearly many problems need to be overcome before our approach can become applicable. The most important of these obstacles is to circumvent the requirement for chemically induced preneoplastic donor hepatocytes. Preliminary work in our laboratory exploring alternate methods of resistance induction has, however, been most encouraging in this regard.[32] In summary, we feel that the concept of resistance induction/selective proliferation outlined in this report will serve as the basis for a model of rapid growth of liver cells in spleen.

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