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Genetics

Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation

(hepatocyte transplantation/gene therapy/α_1-antitrypsin/β-galactosidase)

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ABSTRACT **One approach to gene therapy for hepatic diseases is to remove hepatocytes from an affected individual, genetically alter them *in vitro*, and reimplant them into a receptive locus. Although returning hepatocytes to the liver itself would be advantageous, the feasibility of this approach has never been evaluated due to the inability to distinguish donor from host hepatocytes. To unambiguously identify transplanted hepatocytes after transplantation, and to better quantitate their number and degree of liver function, two transgenic mouse lines were generated in a C57BL/6 background. The first expresses the *Escherichia coli* β-galactosidase gene from the relatively liver-specific human α_1-antitrypsin (hAAT) promoter and allows transgenic hepatocytes to be readily identified after 5-bromo-4-chloro-3-indolyl β-D-galactoside staining; the second produces the hAAT protein under control of the same promoter, which enables hepatocyte survival and maintenance of liver function to be quantitated by measuring the serum levels of hAAT. Hepatocytes isolated from transgenic donors were transplanted into nontransgenic C57BL/6 recipients by intrasplenic injection. Surprisingly, a large fraction of these cells were identified within the liver parenchyma but not the spleen at 2 months after transplantation. The high levels of serum hAAT detected in transplant recipients were stable for >6 months, suggesting that established cells will survive indefinitely. These results have important implications for liver organogenesis and hepatic gene therapy.**

The liver is an attractive organ for gene therapy as there are numerous inherited diseases that affect genes expressed in hepatocytes. One approach is to remove liver cells from an affected individual, genetically alter them *in vitro*, and return them to the same patient. Although several previous studies (reviewed in refs. 1 and 2) have used histological techniques to demonstrate hepatocyte-like cells within the spleen (3–12), fat pads (13), pancreas (14, 15), or subrenal capsule (16), or on microcarrier beads in the peritoneum (17), after hepatocyte injections, the number of surviving cells and their degree of liver function have been impossible to assess due to the absence of quantitative biochemical markers and/or rejection of transplanted cells. Short-term (4, 17–20) or long-term (3, 14) reconstitution of some hepatic function has been demonstrated by transplanting normal hepatocytes into rats with a genetic deficiency of uridine diphosphate (UDP)-glucuronyltransferase, but it is unclear how many surviving hepatocytes were required to produce the slight decrease in serum bilirubin and increase in conjugated bilirubin in the bile. Finally, the possibility that transplanted hepatocytes might return to the liver has never been evaluated due to the inability to distinguish donor from host hepatocytes histologically.

Assessment of the potential efficacy of hepatocyte transplant for hepatic gene therapy requires knowledge of the number of genetically altered cells that can be transplanted, the degree of liver function that can be achieved per cell, and whether or not these cells can actually be returned to their normal locus, the liver. For example, deficiencies of serum proteins such as factor IX could be significantly ameliorated by transplanting 1% of a liver mass to almost any locus, if each cell could be programed to produce 5-fold or more functional protein than a normal hepatocyte. Conversely, other diseases such as glycogen storage diseases limited to the liver (e.g., von Gierke disease) would almost certainly require that a large fraction of hepatocytes be genetically altered and be located within the liver itself. To better evaluate these parameters, we generated two transgenic mouse lines in a congenic C57BL/6 background. The first line utilizes the previously described genomic clone of human α_1-antitrypsin (hAAT) (21), which is expressed almost exclusively in hepatocytes (21, 22). After transplanting hAAT-C57BL/6 hepatocytes into congenic C57BL/6 recipients, cell survival and maintenance of liver function were quantitated over time by measuring serum hAAT levels by radioimmunoassay (RIA). The second transgenic line contains the *Escherichia coli* β-galactosidase (β-gal) gene, again under the control of the liver-specific hAAT promoter. After transplanting hepatocytes from β-gal-C57BL/6 mice into congenic C57BL/6 mice, donor cells could be identified histologically with 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-Gal) staining. We demonstrate that 0.5% of the function of an entire liver can be maintained for at least 6 months after transplanting hepatocytes into the spleens of C57BL/6 mice. Furthermore, the majority of these cells are able to migrate into the liver parenchyma, raising the possibility that part of the liver itself could be replaced with genetically altered hepatocytes using this approach.

MATERIALS AND METHODS

RIA for hAAT. Serum of transgenic donors and of transplant recipients was assayed as described (23) using human-specific anti-AAT antibodies from Atlantic Antibodies (Scarborough, ME). All samples from a given transplant recipient were tested in the same assay in duplicate.

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Abbreviations: hAAT, human α_1-antitrypsin; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactoside; β-gal, β-galactosidase.

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Generation of Transgenic Mice. To create the line designated hAAT-C57BL/6, the 14-kilobase (kb) genomic clone for human AAT, which contains 2 kb of upstream sequence, the entire coding region and introns, and 2 kb of downstream sequence was injected into embryos of C57BL/6 mice (Sprague–Dawley) as described (21), and transgenic mice were identified by RIA of the serum for hAAT. To create the line designated β-gal-C57BL/6, a DNA fragment containing the 1.2-kb natural hAAT promoter (24) upstream of the *E. coli* β-gal gene (25) was injected into embryos and transgenic mice identified by Southern blot of tail DNA (26). [This construct was created by replacing the chloramphenicol acetyltransferase (CAT) gene of phAAT-CAT (24) with the 3.5-kb β-gal gene (25).] Only F_1 and later generations of mice were used as a source for donor hepatocytes.

Isolation and Transplantation of Hepatocytes. Hepatocytes were isolated from 4- to 12-week-old transgenic mice by collagenase perfusion (Boehringer, 0.3 mg/ml) for 20 min as described by Berry and Friend (27) except that perfusion was retrograde through the inferior vena cava with an outflow tract provided by severing the portal vein. Cells were rinsed once with cold minimal essential medium (MEM) with 10% fetal calf serum, rinsed once with cold MEM, resuspended at 1×10^7 or 2.5×10^6 cells per ml in MEM, and stored on ice until injection. Cells were injected in a volume of 200 μl with a 30-gauge needle into the portal vein of ether-anesthetized animals (12- to 20-week-old female C57BL/6 mice) with hemostasis obtained by applying Gelfoam (Upjohn) to the site of injection or injected into the inferior tip of the spleen with hemostasis obtained by ligating the injection site with 3-O chromic suture. Serum obtained by tail bleeding was assayed for hAAT by RIA. Splenectomies were performed under ether anesthesia.

X-Gal Staining. Eight-micron-thick frozen sections of liver or spleen were fixed with 1.25% glutaraldehyde in phosphate-buffered saline (PBS) on ice for 10 min and then stained overnight with X-Gal (Stratagene) as described (25). Plated hepatocytes (plated on Primaria plates for 4 hr in MEM with 10% fetal calf serum) were rinsed twice with PBS, fixed for 10 min with 0.5% glutaraldehyde at room temperature, and then stained with X-Gal.

RESULTS

Long-Term Expression of hAAT in Serum of Transplant Recipients. Long-term expression of hAAT after injecting 5×10^5 hAAT-C57BL/6 hepatocytes into the portal vein or 2×10^6 cells into the spleen is demonstrated in Fig. 1 *A* and *B*. The appearance of hAAT in transplant recipients was clearly due to expression of the transgene from donor hepatocytes, as C57BL/6 recipients that were transplanted with C57BL/6 hepatocytes had no detectable hAAT in their serum (not shown). For these experiments, we used hepatocytes from line 2452B, for which the average contribution to the steady-state *in vivo* hAAT level is 7.5 pg/ml per cell (see Table 1). Thus, we calculate that the level of 600 ng/ml observed 6 months after injecting into the portal vein corresponds to 8×10^4 surviving hepatocytes (16% of injected), assuming that the average hAAT production per cell is unchanged. Similarly, the level of 3.1 μg/ml observed after transplanting into the spleen corresponds to survival of 4.1×10^5 hepatocytes (20% of injected).

To directly compare the efficacy of portal vein and splenic injection for hepatocyte transplantation, experiments using both methods were done with cells from the same donor (Fig. 1*C*). The higher levels of hAAT in recipients for this experiment were consistently found when using line 2449 as the donor, whose hepatocytes express twice as much hAAT per cell as line 2452B. For splenic transplantation, the level of expression was directly proportional to the number of cells injected. Although on a per cell basis splenic and portal vein

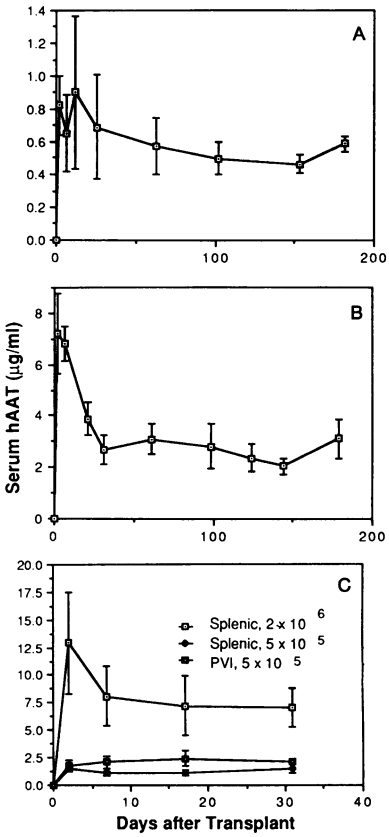

FIG. 1. Survival of transgenic hAAT-C57BL/6 hepatocytes after transplantation. (*A*) Hepatocytes (5×10^5) from hAAT-C57BL/6 donors (line 2452B) were injected into the portal vein of C57BL/6 mice. Serum was analyzed by RIA for hAAT, and the mean ± SEM is shown for six recipients. (*B*) hAAT-C57BL/6 hepatocytes (2×10^6; line 2452B) were injected into the inferior tip of the spleen of C57BL/6 mice. $n = 7$. (*C*) hAAT-C57BL/6 hepatocytes [2×10^6 ($n = 5$) or 5×10^5 ($n = 9$)] from line 2449 were injected into the spleen or cells (5×10^5; $n = 4$) were injected into the portal vein (PVI) of C57BL/6 mice. For all experiments, pretransplant serum was negative for hAAT, demonstrating the specificity of the antibody for the human protein. Average levels shown do not incorporate data from transplant recipients that had only transient expression of hAAT (≈25% overall); this decrease is probably due to technical difficulties, as it is not associated with production of antibodies directed against hAAT (data not shown).

injection again gave comparable results, the mortality rate of injecting into the portal vein was 40% using 5×10^5 cells and 100% using 2×10^6 cells, whereas it was only 10% after injecting either number of cells into the spleen. Thus, we conclude that splenic injection is the preferred route.

Localization of Transplanted Hepatocytes to the Liver Parenchyma. With the demonstration of long-term survival of transplanted hepatocytes, it is important to localize the cells

Table 1. DNA copy number and *in vivo* expression levels in transgenic hAAT-C57BL/6 mouse lines

Line	Serum hAAT, mg/ml	Gene copy number	hAAT production per cell, pg/ml per cell
2449	3	5	15
2452A	6	30	30
2452B	1.5	2	7.5

Serum hAAT was quantitated by RIA, and DNA copy number was determined by Southern blot of DNA obtained from tails of three different transgenic lines. The average contribution each cell makes to the steady-state *in vivo* hAAT serum level was determined by dividing the serum level for that particular line by 2×10^8, which is the estimated number of hepatocytes in an adult mouse.

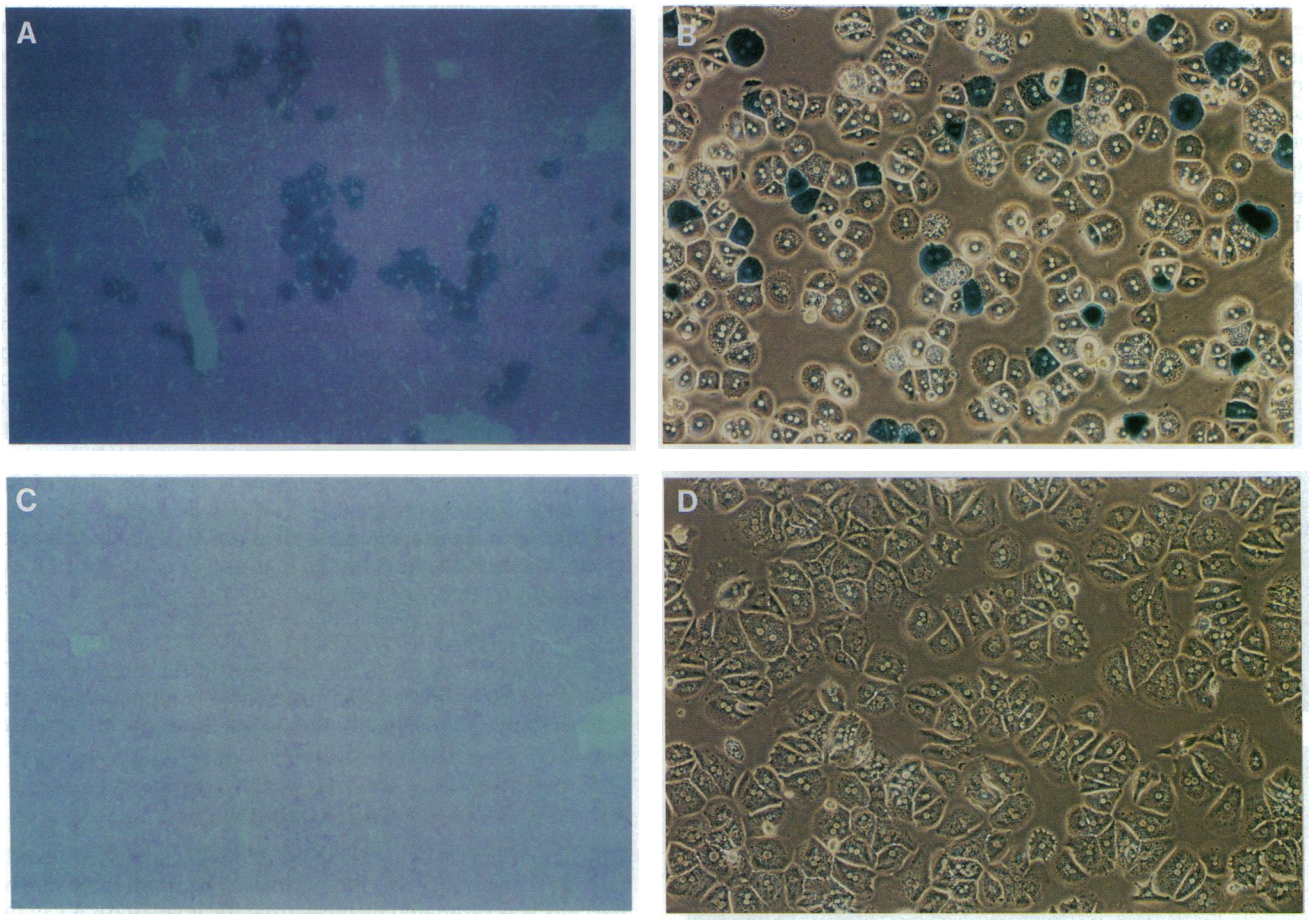

FIG. 2. X-Gal staining of transgenic β-gal-C57BL/6 and control livers. (*A*) Frozen sections of β-gal-C57BL/6 livers were fixed and stained overnight with X-Gal and then counterstained with nuclear fast red. (×130.) (*B*) Hepatocytes were isolated from β-gal-C57BL/6 livers by collagenase perfusion, plated on Primaria plates, and then stained with X-Gal. (×65.) (*C*) Liver from a nontransgenic mouse was treated as in *A*. (×130.) (*D*) Cultured hepatocytes from a nontransgenic mouse were treated as in *B*. (×65.)

in vivo. A second transgenic mouse line was generated to enable donor cells to be identified histologically after transplantation. The β-gal-C57BL/6 line contains 20 copies per haploid genome of the *E. coli* β-gal gene controlled by the 1.2-kb hAAT promoter; expression in these animals occurs almost exclusively in hepatocytes (data not shown). X-Gal staining of liver sections (Fig. 2*A*) or cultured hepatocytes (Fig. 2*B*) from β-gal-C57BL/6 mice shows that ≈10% stain blue, whereas extensive evaluation of liver sections (Fig. 2*C*) or cultured hepatocytes (Fig. 2*D*) from non-transgenic littermates has never identified a single blue cell. Expressing cells are scattered randomly throughout the liver, in a pattern that mimics that of the endogenous mouse AAT gene, as determined by *in situ* hybridization (22).

To localize transplanted hepatocytes, β-gal-C57BL/6 mice were crossed with hAAT-C57BL/6 mice to generate animals that express both transgenes. C57BL/6 recipients were transplanted with hAAT-C57BL/6–β-gal-C57BL/6 hepatocytes

Table 2. Quantitation of transplanted cells within the liver after splenic injection

Exp.	Cells injected, no. × 10^{-5}	Donor hAAT, mg/ml	Recipient hAAT, μg/ml	Overall frequency of donor cells	Blue cells per million hepatocytes plated ×10	Frequency of donor cells in liver	% surviving cells localized in liver
1	25	1.5	2	1/750	1290	1/775	96
2	9.6	6	3.8	1/1580	538	1/1859	85
	48	6	31.4	1/191	2520	1/396	48
	48	6	41.2	1/145	2900	1/345	42
3	25	1.5	13.5	1/111	3020	1/331	34
	20	1.5	4.5	1/326	520	1/1923	17

hAAT-C57BL/6–β-gal-C57BL/6 hepatocytes were injected into the spleens of C57BL/6 mice, and recipient animals were analyzed in two ways 8 weeks after transplantation. First, serum was tested for hAAT levels and compared with the appropriate donor to determine the estimated frequency of transplanted cells present anywhere in the recipient. Second, hepatocytes were isolated from recipients by collagenase perfusion of the liver, plated on Primaria plates, and stained with X-Gal. The frequency of donor cells in the liver was determined by counting the number of blue cells per 10^6 cells plated and then multiplying by 10 (only 10% of the transgenic hepatocytes stain with X-Gal). The proportion of all cells that are located in the liver was then determined by dividing the frequency of blue cells in the liver by the overall frequency of transplanted cells.

by either portal vein or splenic injection. Livers and spleens were sectioned 1–2 months after transplantation and stained with X-Gal. Blue cells were identified within the liver parenchyma after splenic (Fig. 3 *A* and *B*) or portal vein (data not shown) injection. Extensive histological examination of spleens after splenic injection, using X-Gal staining or a hepatocyte-specific glucose-6-phosphatase assay, failed to identify transplanted hepatocytes in two recipients and identified only a few small clusters in two others (data not shown).

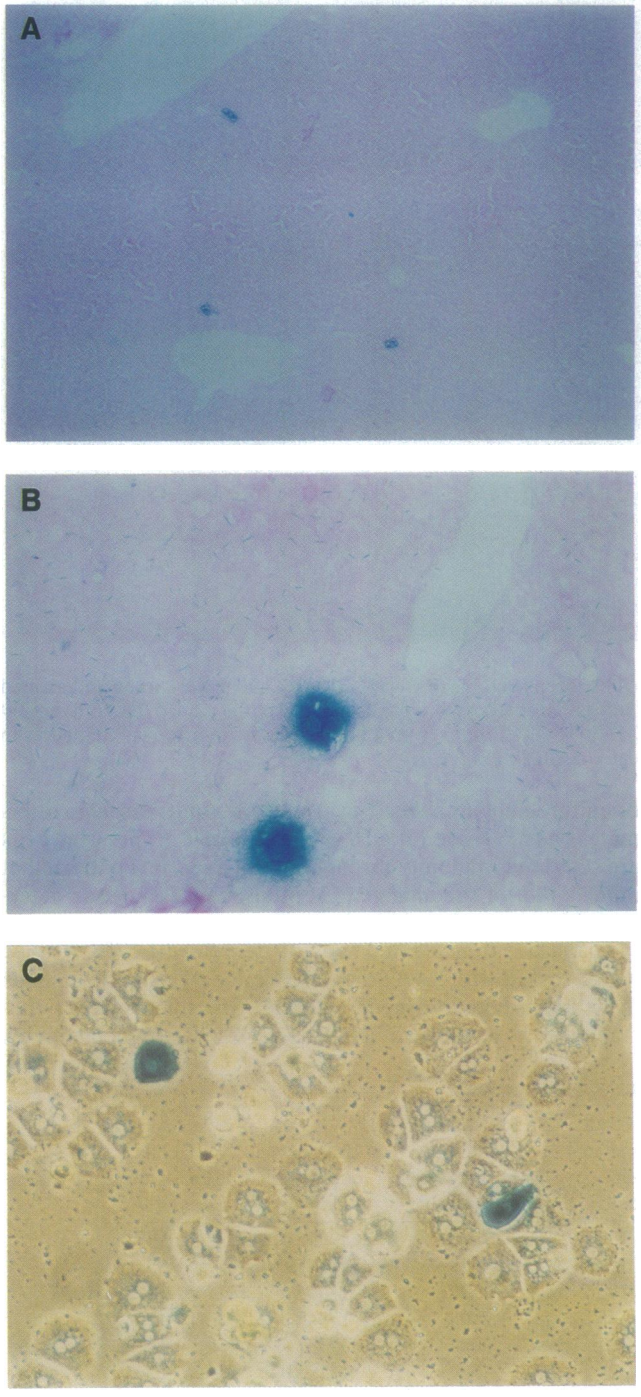

FIG. 3. X-Gal staining of livers and hepatocytes from transplant recipients. hAAT-C57BL/6–β-gal-C57BL/6 hepatocytes (2×10^6) were injected into the spleens of C57BL/6 mice and livers from the recipients were analyzed 8 weeks later. (*A* and *B*) X-Gal staining of frozen sections of a recipient liver. (*A*, ×65; *B*, ×330.) (*C*) X-Gal staining of cultured hepatocytes isolated by collagenase perfusion of the recipient liver. (×260.)

To better quantitate the frequency of donor cells in the liver, hepatocytes were isolated from livers of transplant recipients, and a known number of cells were plated and stained with X-Gal. Blue cells with a typical hepatocyte morphology were identified (Fig. 3*C*) and counted. Table 2 demonstrates the fraction of donor cells present in the liver (based on the number of blue cells) compared with the total number of cells calculated to be present (based on the hAAT levels). We estimate that, on average, about 50% of the surviving hepatocytes can be localized to the liver, where the frequency of donor cells is calculated to be as high as 1 in 350 of all hepatocytes. Whether or not the remainder of the transplanted hepatocytes reside elsewhere is unclear. Undercounting of the blue cells could contribute to this discrepancy. Alternatively, our assumption that the expression of hAAT per cell is unchanged before and after transplantation might not be the case. Despite these caveats, it is remarkable how well results of the two independent measurements agree with each other, and it is clear that a large fraction of the surviving hepatocytes localize to the liver.

Effect of Splenectomy on Survival of Transplanted Hepatocytes. Further documentation that spleens do not contain significant numbers of transplanted cells is shown in Fig. 4. Animals that were splenectomized 1 month after receiving a splenic injection of hAAT-C57BL/6–β-gal-C57BL/6 hepatocytes showed essentially no decrease in their serum hAAT levels; the slight increase in hAAT levels immediately after splenectomy is presumably due to an acute phase response (28).

DISCUSSION

Efficacy of hepatocyte transplantation was assessed quantitatively using transgenic mouse lines. The fraction of surviving cells was estimated by measuring the level of hAAT in the serum and comparing recipient values with those found in the donor line. With three different hAAT-C57BL/6 transgenic lines expressing hAAT at varying levels, ≈0.25–0.5% of a liver mass could be successfully transplanted by intrasplenic injection of 1–2% of a total mouse liver, representing an estimated survival rate of ≈25%. Although the relative number of surviving cells was constant with the three different transgenic hAAT-C57BL/6 lines, the absolute level of hAAT was directly proportional to the average level of expression per cell for that particular line. This demonstrates the feasibility of increasing the overall expression of a transplanted gene by increasing the expression per cell. Liver function was unambiguously maintained in the transplanted cells, as

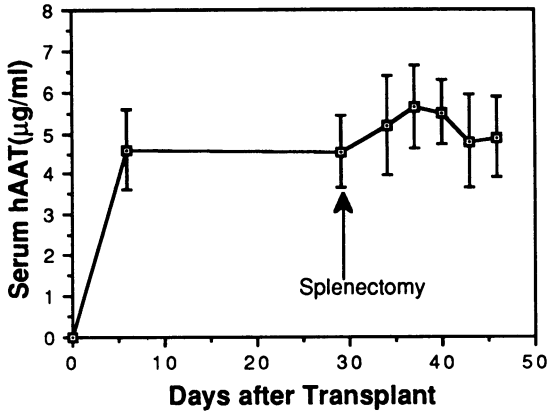

FIG. 4. Effect of splenectomy on hAAT levels. Four C57BL/6 mice were transplanted by splenic injection with 2×10^6 hepatocytes from hAAT-C57BL/6 (line 2449)–β-gal-C57BL/6 mice. One month after transplantation, they were splenectomized and serum hAAT levels were determined. The arrow denotes the time of splenectomy.

expression from the hAAT promoter occurs almost exclusively in hepatocytes (21, 22). In 75% of transplant recipients, the level of expression observed at 2 weeks remained constant for at least 6 months, the duration of the study. The etiology of the decline in hAAT levels in the remaining 25% of recipients was unclear but did not appear to be due to development of high titers of antibodies (data not shown).

The most startling result of these studies is that the majority of hepatocytes injected into the spleen can migrate into the liver parenchyma, where they integrate into hepatic parenchyma rather than remain as intravascular emboli. Previous studies have lacked methods for identifying donor hepatocytes within the histologically indistinct recipient liver; the use in this study of the sensitive and absolutely specific β-gal staining procedure made such tracking trivial. Surprisingly, we were unable to identify significant numbers of transplanted hepatocytes within the spleen, using either X-Gal or glucose-6-phosphatase staining. These results are contradictory to some previous studies that demonstrate histologically large numbers of hepatocytes in spleens of rats (3–9, 11, 12) or pigs (10) several months after splenic transplantation. However, other studies have demonstrated that establishment of transplanted hepatocytes in rat spleens requires that liver damage be present at the time of transplant (29–31), a treatment that was not done to the mice in our study. Furthermore, Vroemen *et al.* (3) have determined that more than half of the function conferred by transplanting normal hepatocytes into spleens of UDP-glucuronyltransferase-deficient rats remains after splenectomy, and Gupta *et al.* (36) have obtained similar results after transplanting mouse hepatocytes expressing the hepatitis B surface antigen.

The ability of transplanted hepatocytes to function long-term in recipients may be a direct result of their incorporation into normal liver parenchyma. Use of such marked hepatocytes may be a useful tool for studying mechanisms of liver organogenesis and hepatic regeneration, using mature hepatocytes and putative hepatic stem cells. Finally, although the number of hepatocytes that can be successfully transplanted is still limited, the combination of judicious use of strong hepatic promoters and established methods for efficient gene transduction into hepatocytes *in vitro* (32–35) with multiple intrasplenic injections may contribute to the ultimate realization of gene therapy for hepatic deficiencies in the future.

Note Added in Proof. The transplanted mice in Fig. 1 were followed for 6 more months, and the serum hAAT remained at similar levels. Thus the transplanted hepatocytes persisted for a year *in vivo*.

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