1620

Murine Liver Allograft Transplantation: Tolerance and Donor Cell Chimerism

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Nonarterialized orthotopic liver transplantation with no immunosuppression was performed in 13 mouse-strain combinations. Two strain combinations with major histocompatibility complex class I and class II and minor histocompatibility complex disparity had 20% and 33% survival of more than 100 days, but the other 11 combinations, including four that were fully allogeneic and all with only class I, class II or minor disparities, yielded 45% to 100% survival of more than 100 days. Long-living recipients permanently accepted donor-strain heterotopic hearts transplanted on the same day or donor-strain skin 3 mo after liver transplantation, in spite of detectable antidonor *in vitro* activity with mixed lymphocyte reaction and cell-mediated lymphocytotoxicity testing (split tolerance). In further donor-specific experiments, liver grafts were not rejected by presensitized major histocompatibility complex class I–disparate recipients and they protected donor-strain skin grafts from second set (or any) rejection. Less frequently, liver transplantation rescued rejecting skin grafts placed 1 wk earlier in major histocompatibility complex class I, class II and minor histocompatibility complex, class II or minor histocompatibility complex–disparate strain combinations. Donor-derived leukocyte migration to the central lymphoid organs occurred within 1 to 2 hr after liver transplantation in all animals examined, persisted in the surviving animals until they were killed (>375 days), and was demonstrated with double-immunolabeling to be multilineage. The relation of these findings to so-called hepatic tolerogenicity and to tolerance in general is discussed. (HEPATOLOGY 1994;19: 916-924.)

We have proposed that a reciprocal migration of leukocytes of bone marrow origin between graft and host, with subsequent chimerism in both, is the first step toward donor-specific nonreactivity (tolerance) that may or may not require immunosuppression (1-7). According to this concept, the variable tolerogenicity and ease of "acceptance" of different organs merely reflect the comparative content of these multilineage leukocytes, of which the antigen-presenting dendritic cell defined as a distinct lineage by Steinman and Cohn (8, 9) is the most critical (1-7). In our study of liver transplantation in mice, we have examined the effect of major histocompatibility complex (MHC) on hepatic tolerogenicity and chimerism, with further attention to the lineages and traffic of the donor leukocytes.

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MATERIALS AND METHODS

Animals and Procedures

Ten- to 12-wk-old male mice with varying H-2 histocompatibility genotypes (Table 1) were purchased from the Jackson Laboratory (Bar Harbor, ME) and housed in a pathogen-free facility. All operations and procedures were carried out with mice under methoxyflurane (2.2-dichloro-1,1-difluoroethyl methyl ether) anesthesia.

Liver Transplantation. The allograft was placed in the normal location after removal of the recipient's liver. It was revascularized with a combination of suture and cuff techniques. The hepatic artery was not reconstructed. This operation has been described in detail elsewhere (10) and is fundamentally the same as developed first in dogs (11) and human beings (12), and then used experimentally in pigs (13) and other large animals. In mice (10) and rats (14), hepatic arterialization is not necessary. Cholecystectomy was performed, and bile duct patency was assured with a fine polyethylene tube stent. Immunosuppression was not used. Animals that died within 1 week after transplantation were classified as technical failures (10% to 15% of total operations) and excluded from analysis. Tissues were usually harvested after the animal had been killed, but in some cases wedge resection biopsies of the liver were performed at reoperation without the animals being killed.

Heart Transplantation. The intraabdominal operation was adapted from the rat procedure of Ono and Lindsey (15), with daily monitoring of the heart grafts by palpation through the abdominal wall. Rejection was defined by the cessation of cardiac impulses and confirmed by exploration and histological examination.

Skin Transplantation. A full-thickness skin graft from the donor tail (8 mm × 8 mm) was placed on the recipient's dorsal side by the method of Billingham et al. (16). The graft was held in place by the dressing and a tape for 7 to 8 days and inspected daily thereafter. Rejection was defined as the day of complete graft destruction.

Tissue Collections

With the few exceptions in which wedge biopsies were performed, the liver recipients were killed 1 hr to more than

TABLE 1. H-2 Haplotypes

Strains	H-2 haplotype	Alleles at H-2 loci			
		K	A	E	D
C57B1/10 (B10)	b	b	b	b	b
C3H	k	k	k	k	k
B10.BR	k	k	k	k	k
B10.AKM	m	k	k	k	q
A.TH	t_2	s	s	s	d
A.TL	t_1	s	k	k	d
A.SW	s	s	s	s	s
C57B1/6	b	b	b	b	b
B10.D2	d	d	d	d	d
B10.HTG	g	d	d	d	b
DBA2	d	d	d	d	d

TABLE 2. Primary monoclonal antibodies used for immunohistochemical studies

Specificity	Clone	Isotype
H-2K^b	AF6-88.5	Mouse IgG$_{2a}$[a]
H-2K^k	36-7-5	Mouse IgG$_{2a}$[a]
I-E^k	14-4-43	Mouse IgG$_{2a}$[a]
I-A^b	AF6-120.1	Mouse IgG$_{2a}$[a]
IgM (μ chain)		Goat IgG[b]
Thy 1.2 (T cell)	3D-H12	Rat IgG$_{2b}$[c]
iC3bR (Mø)	M1/70 (TIB128)	Rat IgG$_{2b}$[c]
F4/80 (Mø/DC)	HB 198	Rat IgG$_{2b}$[d]
(DC-associated antigen)	NLDC 145	Rat IgG$_{2a}$[e]
(DC-associated antigen)	2A1	Rat IgG$_{2b}$[f]
CD45R (B cell)	B220 (TIB 145)	Rat IgG$_{2a}$[a]

[a]PharMingen, San Diego, CA.
[b]Vector Laboratories, Burlingame, CA.
[c]Boehringer Mannheim, Indianapolis, IN.
[d]American Type Culture Collection, Rockville, MD.
[e]Accurate Chemical and Scientific Corp., Westbury, NY.
[f]Gift from Professor Ralph Steinman, Rockefeller University, New York, NY.

375 days after surgery. Lymphoid and nonlymphoid organs were harvested and placed in the embedding medium (Tissue-Tek O.C.T. Compound; Miles Inc., Elkhart, IN), snap-frozen in liquid nitrogen and stored at −80° C until sectioning. Cryosections were cut at 4 μm, mounted on precleaned slides, dried overnight at room temperature, fixed in acetone for 5 min and subsequently rehydrated with PBS.

Immunohistochemical Staining

Donor cells were localized within the recipient tissues and the liver allografts with the use of a direct immunoperoxidase procedure. Endogenous biotin was blocked with the avidin-biotin blocking reagent (Vector Laboratories, Inc., Burlingame, CA) for 20 min each. The tissues were incubated with nonfat dried milk for 20 min and, after two washes with PBS, were reacted for 45 min with the biotinylated donor-specific class I or class II monoclonal antibodies or an isotype-matched biotinylated control (negative-staining control) and then washed in PBS (twice for 5 min). Endogenous peroxidase activity in the tissues was then quenched with 0.6% H_2O_2 for 4 min and washed in PBS (twice for 5 min). The primary antibody was localized with streptavidin-conjugated peroxidase for 30 min and washed in PBS (twice for 5 min), and the reaction was developed with 3-amino-5-ethycarbizol (9 min). The slides were counterstained with Harris' hematoxylin and mounted with Gelvatol (Monsanto, St. Louis, MO).

A double immunofluorescence (DIF) procedure was used to identify the lineages of donor cells in recipient tissues and grafted livers. The procedure adopted to localize donor cells was identical to immunoperoxidase staining, except that endogenous peroxidase activity was not quenched, and biotinylated primary monoclonal antibody was visualized with streptavidin-conjugated Cy3 (Jackson Immunoresearch Laboratories, Inc., West Grove, PA).

After localization of the donor cells with the use of antidonor MHC class I or II antibodies (with the above DIF technique), IgM+ cells were identified by use of FITC-conjugated goat antimouse IgM (μ chain specific). T cells, B cells, macrophages and dendritic cell lineages were identified by use of an indirect IF technique with the rat monoclonal antibodies to mouse shown in Table 2. These primary immunoreactants were then visualized by using mouse-adsorbed, FITC-conjugated goat antirat IgG. Donor class I or II positive cells that were negative for the specific lineage marker being tested were stained pure red. Cells of the recipient origin and bearing the appropriate lineage marker stained green, whereas donor cells expressing the appropriate lineage stained yellow. Technical controls for each lineage marker included staining of normal B10 lymphoid tissue (positive control), substitution of isotype-matched irrel-

TABLE 3. Survival of liver allografts in different strains across various histocompatibility barriers

Strain combinations	MHC disparity	Liver graft survival (days)
C3H→B10	I, II, mHC	8,15,35,41, > 100
B10→C3H	I, II, mHC	18,51, > 100, > 100, > 100, > 100, > 100, > 100, > 100, > 100, > 100, > 100
BALB/C→C3H	I, II, mHC	8,13,15,15,16,52, > 100, > 100, > 100
C3H→C57B1/6	I, II, mHC	13,20,45, > 100, > 100, > 100, > 100
B10.BR→B10.D2	I, II	9,10,11,16,18,18, > 100, > 100, > 100, > 100, > 100
B10.BR→B10	I, II	17, > 100, > 100, > 100, > 100
A.TH→A.TL	II	7,14,14,18,83, > 100, > 100, > 100, > 100, > 100
A.TL→A.TH	II	55,56, > 100, > 100, > 100, > 100, > 100
A.SW→A.TH	I	16, > 100, > 100, > 100, > 100
B10.AKM→B10.BR	I	81, > 100, > 100, > 100, > 100, > 100
B10.D2→B10JHTG	I	14, > 100, > 100, > 100, > 100
B10.BR→C3H	mHC	22, > 100, > 100, > 100, > 100
DBA2→B10.D2	mHC	> 100, > 100, > 100

TABLE 4. Skin graft survival 3 mo after successful liver transplantation

Liver donor	Recipient	Disparity	Skin donor	Skin survival (days)
None	C3H		B10	13,13,15,18,19
None	C3H		B10.D2	12,13,13,14
B10	C3H	I, II, mHC	B10	39, > 100, > 100, > 100
B10	C3H		B10.D2	13,13,13
None	B10.BR		B10.AKM	20,20,23,23,23
None	B10.BR		B10.D2	13,13,14,14
B10.AKM	B10.BR	I	B10.AKM	20, > 100, > 100, > 100, > 100, > 100
B10.AKM	B10.BR		B10.D2	13,13,13,13
None	A.TL		A.TH	14,16,19,19,19
None	A.TL		BALB/C	12,13,13,13
A.TH	A.TL	II	A.TH	56,[a] > 100, > 100
A.TH	A.TL		BALB/C	12,12,12
None	C3H		B10.BR	20,22,22,23
None	C3H		B10.D2	13,13,13,13
B10.BR	C3H	mHC	B10.BR	> 100, > 100
B10.BR	C3H		B10.D2	13,13,14

[a]Animal died of intestinal obstruction with living skin graft.

TABLE 5. Liver transplantation 1 wk after skin grafting rescues some rejecting skin grafts

Skin graft (days)	Combinations	Disparity	Skin grafts rescued
-7	B10→C3H	I, II, mHC	1 of 4
-7	A.TL→A.TH	II	1 of 3
-7	B10.BR→C3H	mHC	2 of 3

evant antibodies (negative control) and staining of the liver allograft ("cross-reaction" control). The presence of the three distinct colors (meaning three types of cells) in lymphoid tissues, the complete absence of red or green staining in the negative controls and red-only staining of the bile ducts when the donor liver was reacted with both donor-specific monoclonal antibodies to MHC and leukocyte lineage markers were used to verify the results. Slides were viewed on a Nikon epifluorescent microscope (Nikon Instrument Group, Melville, NJ) equipped with a DAPI/FITC/Texas Red triple band pass filter (Fryer Co., Huntley, IL).

In some of the immunolabeling studies, class I and II surface antigen expression was augmented by an intraperitoneal injection of 4×10^5 units of recombinant interferon-γ (r-interferon-γ) (Schering-Plough, Kenilworth, NJ) 2 days before the animal was killed. Interferon-γ pretreatment did not change the pattern of chimerism, but it facilitated the ease of recognition of donor cells in long-term allograft recipients. The technique has been reported elsewhere (17).

In Vitro Immunologic Tests

Unidirectional mixed lymphocyte reaction was measured in recipients from nonrejecting strain combinations that were killed 30 days after liver transplantation for immunohistochemical studies. Irradiated (20 Gy) donor and third-party lymphocytes were used as stimulators and cells isolated from the recipients' spleens as responders. In addition, the cytolytic activity of the recipient's splenic lymphocytes toward donor and third-party targets was assessed in cell-mediated lympholysis (CML) assays. Effector lymphocytes were incubated with ^{51}Cr-labeled target cells at 100:1 effector/target ratios. More complete studies over the whole range of survival are reported elsewhere (18).

TABLE 6. Liver graft protects the second donor skin graft in presensitized recipient in B10.AKM→B10.BR strain combination

Day (−14 to −28)	First B10.AKM skin survival (days)	Day 0	Day (31-58)	Second B10.AKM skin survival (days)
First skin	18,19,20,20,20,22	–	Second skin	13,13,13,13,13,13
First skin	18,18,19,22,22	Liver	Second skin	46,[a] > 100, > 100, > 100, > 100, > 100, > 100, > 100, > 100, > 100

[a]Skin was rejected but animal survived.

TABLE 7. Survival of simultaneous heterotopic cardiac transplants of donor origin in recipients of liver allografts

Strain combinations	MHC disparity	Heart survival (days)	
		No OLT[a]	With OLT
B10→C3H	I,II,mHC	7,7,7,7,7,10,10	> 100, > 100
A.TH→A.TL	II	9,11,19,22,23	> 100, > 100
B10.AKM→B10.BR	I	9,25, > 100, > 100, > 100	–
B10.BR→C3H	mHC	13,13,13,13,14	> 100, > 100, > 100

[a]Orthotopic liver transplantation.

RESULTS

Survival After Liver Transplantation. Fatal rejection was consistently seen only with C3H→B10 and BALB/C→C3H (Table 3), but even in these groups 20% and 33%, respectively, of recipients (disparate at class I, class II and minor histocompatibility complex) had survival of more than 100 days. Fifty-five of 78 liver recipients in the other 11 groups whose donors had histocompatibility disparities ranging from fully allogeneic to minor survived more than 100 days. This outcome was accomplished more regularly when there was MHC class I and II or class II compatibility (Table 3), the strain combinations of which essentially all animals had permanent survival. However, 70% or better survival was also recorded in several strain combinations with disparate MHC class II, class I or both.

The results were influenced by the strain direction of the organ transfer. C3H→B10 transplantations fared badly (1 in 5 liver grafts accepted more than 100 days), whereas with B10→C3H 10 of 12 had more than 100-day survival. A smaller directional influence was seen with the A.TH→A.TL class II combination (Table 3).

Hepatic Tolerogenicity

After we determined the most predictably nonrejecting strain combinations by the foregoing studies, four pairings covering the spectrum of histoincompatibility were selected for studies of hepatic tolerogenicity for other grafts.

Skin Transplantation. Three months after successful liver transplantation, donor-strain skin grafts were accepted no matter what the MHC disparity, with only two exceptions in a total of 15 mice (Table 4). The delayed skin transplantations did not adversely affect the preexisting liver allografts. All third-party skin grafts were rejected at the expected times.

In 10 additional experiments with total, class II and minor incompatibilities, liver transplantation from the same donor strain was performed 7 days after rather than before skin transplantation. Four of the 10 skin grafts were rescued from their expected fate of rejection (which normally occurred at about 2 to 3 wk), but this outcome was accomplished more than once only with the B10.BR→C3H strain combination, which is minor histocompatibility complex incompatible (Table 5).

In further sensitization experiments with the class I–disparate B10.AKM→B10.BR strains, accelerated rejection was produced by repeat skin grafts (Table 6). The second-set rejections were prevented in nine of nine experiments by an intervening B10.AKM liver transplantation, which erased the memory of the previous exposure; eight of the nine skin grafts survived permanently (Table 6).

Heart Transplantation. Strong hepatic tolerogenicity was observed when donor-strain heart was transplanted on the same day as liver replacement. Instead of being rejected after 7 to 23 days (variable with different MHC disparities), all of the hearts protected by concomitantly transplanted livers survived permanently (Table 7).

Similar experiments were planned with the class I disparity of B10.AKM→B10.BR. However, the control cardiac grafts were permanently accepted by three of the five normal B10.BR mice (Table 7). This self-induction of tolerance by the heart made hepatic tolerogenicity studies impossible.

Histopathologic Evidence of Chimerism

Systemic chimerism was looked for in the organs of liver recipients 7 mo after transplantation in fully disparate B10→C3H (class I, II, minor disparate) combinations.

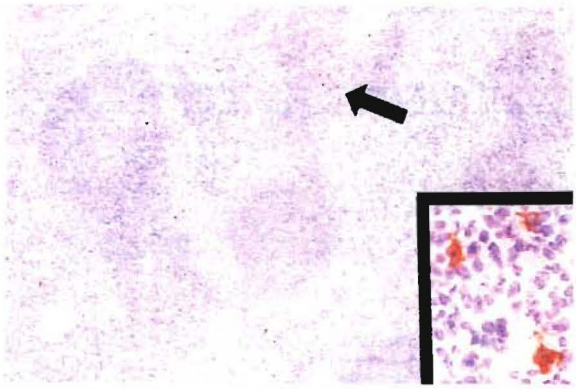

FIG. 1. Recipient spleen 7 mo after liver transplantation in the B10→C3H combination stained for donor-specific MHC class II [I-A^b] antigens. The majority of donor cells were found in the periarterial lymphatic sheath and the marginal zone (immunoperoxidase staining for I-A^b, counterstained with hematoxylin; original magnification ×40). *Inset:* (original magnification ×400) shows the individual cell staining *(arrow)* in greater detail.

Sparse donor-specific class I– or class II–positive cells could always be found. They were most easily detectable in the recipient's lymphoid organs (spleen, mesenteric lymph nodes and thymus) (Figs. 1, 2 and 3). Donor cells were also identified in small numbers in the recipients' peripheral lymph nodes, small bowel, skin, kidney, tongue, heart and lung.

The migrant donor cells found in recipient tissues long after transplantation were detected by double immunolabeling using antidonor MHC class I or II mAb and lineage-restricted leukocyte markers. The phenotype of donor cells identified included B cells (B220 and IgM positive), T cells (Thy 1.2 positive), macrophages (M1/70 and F4/80 positive) and dendritic cells (NLDC-145 and 2A1 positive). B cells were the most frequently detectable chimeric cells (about 60% of total), followed by T cells, dendritic cells and macrophages. These cells were the same lineages as demonstrated with flow cytometric analysis of nonparenchymal cells isolated from normal mouse liver (18). The multilineage nature of the chimerism was evident at all time points tested.

The chimeric B cells (Fig. 3A) were detected mainly in the B-cell follicles of the recipient spleen. Analogous homing of donor B cells to normal sites of trafficking was also noted in lymph nodes. Donor dendritic cells and T cells were most frequently located in the splenic periarterial lymphatic sheath, mingled with recipient T cells and dendritic cells (Fig. 3B and C).

In Vitro *Immunologic Reactivity*

Splenocytes of C3H recipients of B10 livers showed alloreactivity toward the irradiated spleen cells of both the donor (B10) and third-party (BALB/C) animals. The alloreactivity was similar to that of lymphocytes isolated from naive C3H animals (Table 8). The cell-mediated lymphotoxicity (CML) activity (CML) of the stimulated splenocytes from the liver-transplanted mice 30 days after transplantation was essentially the same against donor and third-party targets, whereas the killing of syngeneic targets was less than 10% (Table 9). The antidonor immunologic reactivity was maintained throughout the observation period from wk 1 through wk 12 in our study.

DISCUSSION

An unanticipated advantage of the mouse liver transplantation model was that permanent graft acceptance could be achieved in all strain combinations without immunosuppression and reliably so with most. Failure of success to correlate with class I MHC compatibility was nonsupportive of an earlier hypothesis that secretion by the liver of new soluble class I antigens is the basis of its tolerogenicity (19). The acceptance self-induced by the liver extended to other donor-strain tissues and organs, and could be used to rescue them from a second-set rejection caused by previous sensitization or less frequently from ongoing rejection. Thus, the full spectrum of "hepatic tolerogenicity" (5, 6, 20) was exhibited in the absence of immunosuppression. Liver transplantation without treatment is also possible in a few rat-strain combinations (14, 21, 22) but not in outbred large animals (including human beings) with the exception of pigs (13, 20, 23, 24).

The ability to induce tolerance is not unique to the liver, only much stronger than that possessed by other organs (1-6). Russell et al. (25) showed that mice that permanently accepted weakly histoincompatible kidneys without treatment were subsequently tolerant of skin from the donor strain. Corry et al. (26) described a mouse-strain combination that was nonrejecting for hearts, similar to one of our heart experiments in which there was an MHC class I disparity (BIO.AKM→ B10.BR). Though chimerism was not looked for in these earlier experiments, it is now the presumed explanation for the unexpected historical results.

The association of hematolymphopoietic chimerism with acquired tolerance (15, 27) and graft vs. host disease (28) was discovered by Billingham et al. (27). The cause-and-effect relation was formally verified by Russell (29), who reversed both tolerance and runt disease (graft-vs.-host disease) in the Billingham et al. (27) model by the simple expedient of eliminating the chimerism with antidonor leukocyte antibodies. However, because chimerism was not realized to be a feature of successful whole-organ transplantation, it has been widely assumed that the "acceptance" of organ grafts is by different mechanisms than successfully engrafted bone marrow. Revision of this entrenched misconception has been mandated by the recent demonstration with sensitive cytostaining and polymerase chain reaction techniques that microchimerism is invariably present after successful whole-organ transplantation in human beings (1-6) and rats (7).

In fact, the microchimerism observed in our mouse liver transplant experiments (reported here) was strik-

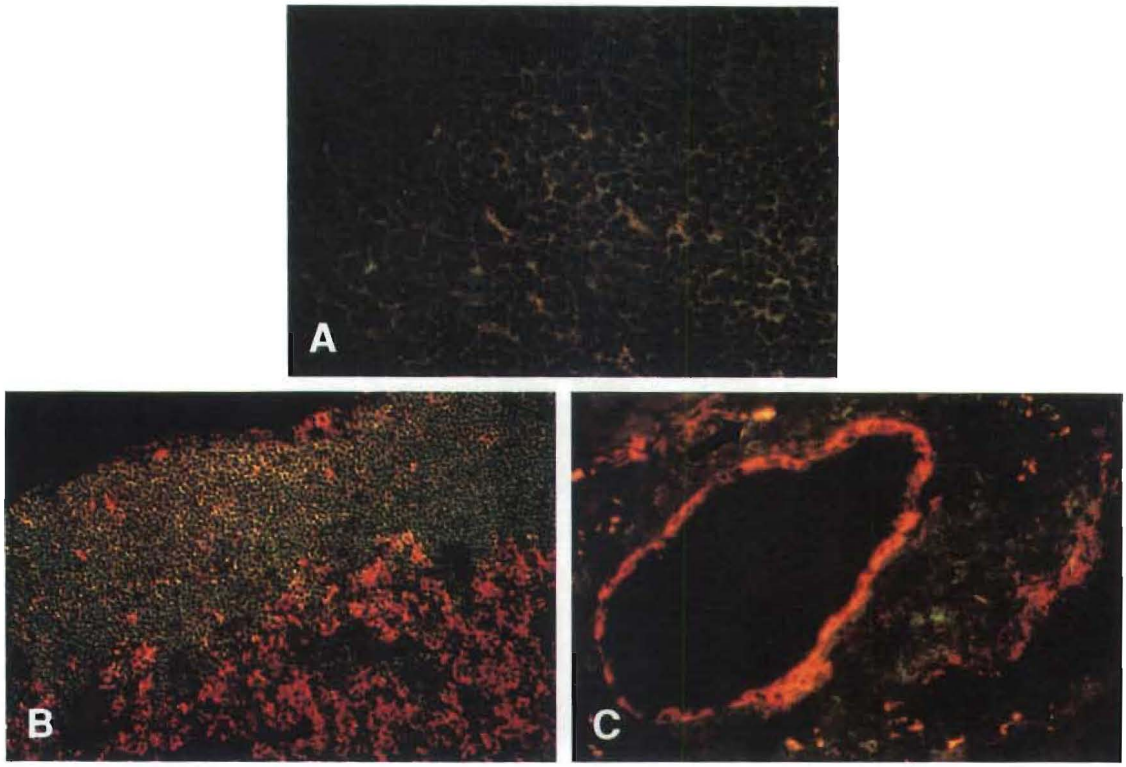

FIG. 2. Double immunofluorescent staining (DIF) controls for lineage identity of donor cells in recipient tissues. (a) No staining was seen in negative controls (see "Materials and Methods"; original magnification ×400). (b) DIF staining of normal B10 lymph node (positive control) with anti-I-A^b *(red,* non–B cells) and anti-B-220 *(green,* B cells). Note red cells in the paracortex, which are only positive for I-A^b; the green cells in the cortex, which are only positive for B-220; and the yellow cells (class II–positive B cells), which are double positive for both I-A^b and B-220 (original magnification ×200). (c) DIF staining of the allografted liver with anti-I-A^b *(red)* and anti-B-220 *(green)* served as an additional control (original magnification ×400). Note red-only staining of biliary epithelium (donor I-A^b) and green-only staining of infiltrating recipients B-220–positive B cells. Lack of double-staining in bile ducts excludes possibility of nonspecific cross-reactivity of immunohistochemical reagents used. *Arrow* highlights lipofuscin autofluorescence.

TABLE 8. Mixed lymphocyte response of spleen cells isolated from liver graft recipients (B10→C3H) 30 days after transplantation

Responders	Stimulators ^{3}H TdR incorporation[a]		
	C3H	B10	BALB/C
C3H (n = 3)	6,720 ± 653	28,921 ± 1,341	22,868 ± 1,562
C3H (>OLT) (n = 3)	9,169 ± 732	27,540 ± 2,731	22,595 ± 987

[a]Data expressed as mean cpm ± 1 S.D.

ingly similar to that reported many years ago by Liegeois et al. (30, 31) in mouse bone marrow experiments that were designed to explain why animals treated by Monaco and colleagues (32, 33) with antilymphocyte serum plus delayed (1 wk later) intravenous bone marrow had developed donor-specific nonreactivity to skin grafts. With karyotyping, Liegeois et al. (30, 31) demonstrated progressively declining numbers of replicating donor bone marrow cells in the recipients' spleens for as long as 134 days, a condition for which they proposed the term "microchimerism." The assumption by Liegeois et al. (30, 31) and others (34) that the decline in donor cells signaled the impending disappearance of these leukocytes obscured the importance of the early work.

In addition, it was not remotely suspected, until our recent clinical (1-6) and experimental (7) studies, that the finding of microchimerism after bone marrow infusion is mimicked by migratory donor leukocytes from whole organs. For the low-level chimerism (usually <0.1%) to have a potent and sustained tolerogenic effect, an amplification process is required. Our hypothesis (5, 7, 35) is that the donor leukocytes of bone marrow origin that are contained in all whole organs represent a functional fragment of the donor immune

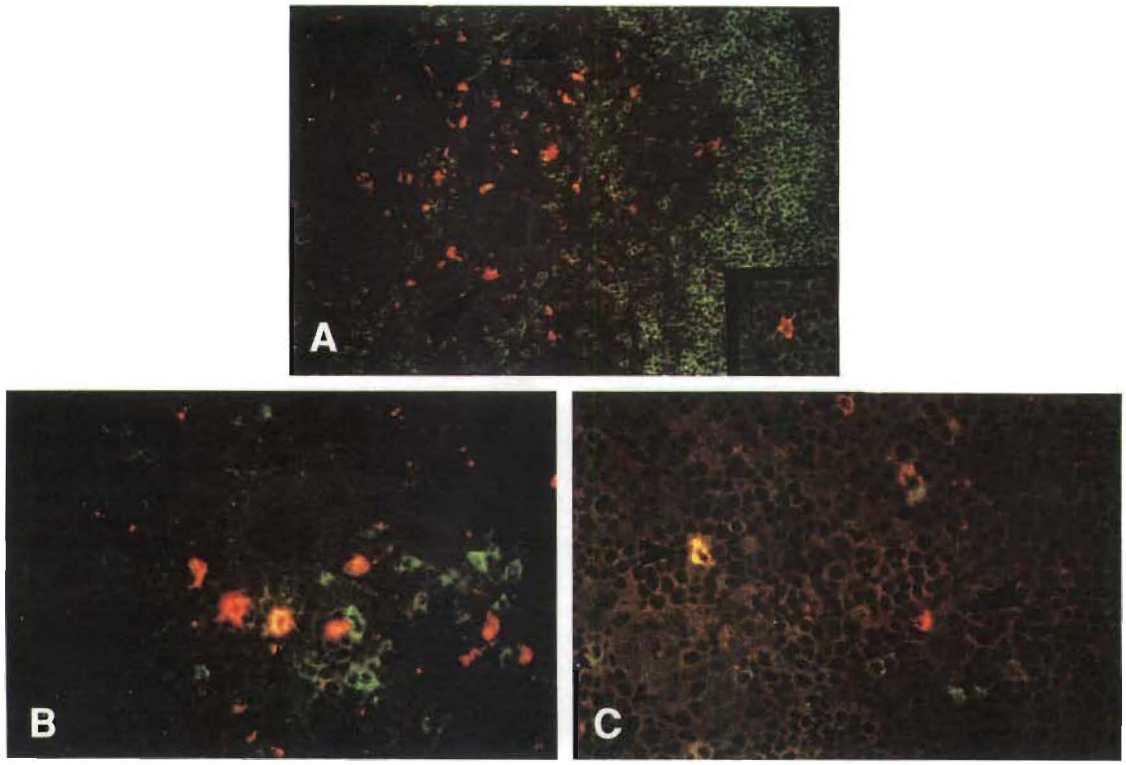

FIG. 3. Lineage identification of donor cells by double immunofluorescent staining (DIF) in recipients' spleen 7 mo after OLT in B10→C3H combination. (a) DIF staining for donor I-A^b *(red)* and B-220 (B cells, *green;* original magnification ×100). A splenic arterial branch is located in the center. Single-positive donor non–B cells *(large arrowhead, red-only)* and double-positive donor B cells *(small arrowhead, yellow)* are located in the PALS. *Inset:* Yellow double-positive donor–B cell in greater detail (original magnification ×400). (b) Donor leukocytes of dendritic cell lineage could also be detected by DIF labeling (original magnification ×400). Red-only donor cell in the PALS is positive for donor I-A^b *(large arrowhead),* whereas green-only cells stain positive with mAb 2A1 (dendritic cell marker). The double-positive *(bright yellow)* cell is a donor dendritic cell *(small arrowhead),* which is stained for both donor class II and dendritic cell–associated antigen 2A1. (c) Donor T cells were also detected with DIF staining (original magnification ×400). Note red cells *(large arrowhead),* which are positive for donor MHC class I antigens (H-$2K^b$) alone; green cells, which are positive with mAb Thy 1.2 (T cells) alone; and donor T cell *(bright yellow),* which is positive for both donor class I and Thy 1.2 *(small arrowhead).* The Thy 1.2–stained cell could be a fibroblast, but confirmatory evidence of a donor subpopulation of T cells was obtained by double labeling with donor class I and CD4 or CD8.

TABLE 9. Cytotoxic activity of spleen cells isolated from liver graft recipients (B10→C3H), 30 days after transplantation

Effectors[b]	n	% Specific ^{51}Cr release[a]		
		B10	C3H	BALB/C
Naive controls				
C3H-C3H	3	3 ± 1.2	2 ± 1.5	3 ± 1.9
C3H-B10	3	32 ± 4.5	3 ± 1.6	10 ± 2.0
C3H-BALB/C	3	7 ± 3.1	5 ± 3.5	33 ± 4.4
After OLT				
C3H-C3H	3	2 ± 1.2	2 ± 1.3	2 ± 1.3
C3H-B10	3	33 ± 3.1	3 ± 1.7	3 ± 1.8
C3H-BALB/C	3	3 ± 1.4	4 ± 2.1	30 ± 3.0

[a]Data expressed as mean ± 1 S.D.

[b]Effector cells isolated from either naive or liver transplant recipients were initially cultured for 4 days with γ-irradiated splenocytes from either naive syngeneic (C3H), donor (B10) or third party (BALB/C) animals and finally incubated with ^{51}Cr-labelled syngeneic, donor and third-party target cells at an E : T ratio of 100 : 1.

system, which for successful transplantation must be incorporated into the recipient's immune apparatus with receptor-ligand interactions of variable and changeable affinities.

In this "network" viewpoint (36-40), bidirectional redefinition of self by the coexisting immune systems is not unreasonable, nor is the possibility of their eventual cooperation and complete mutual assimilation. The

contention of Coutinho (37) that immunologic self-definition is maintained by self-assertion is compatible with our experimental evidence in rats (7) and in our more complete studies of the mouse liver model. These studies have not shown clonally deleted recipient populations but suggest instead a state of low-grade reciprocal stimulation (18).

The iterative and metadynamic properties implicit in such an immune network would explain not only the large influence of a small number of chimeric cells but also the donor specificity of the consequent tolerance. If the detached and assimilated fragment of the donor immune system is able to maintain the rest of its "self" in its new environment, a mirror image (recipient specific) effect would not be surprising, such as that implicit in the results of both our rat and mouse experiments. Arnold et al. (41) have speculated that donor- (or recipient-) specific nonreactivity is the end stage of a long process that cannot be accurately assessed in its evolution by current *in vitro* techniques (42-44). This situation was epitomized in our mice, which were shown to have normal antidonor activity by mixed lymphocyte reaction or CML testing at a time when their liver allografts were beyond the danger of fatal rejection and already were capable of shielding other normally rejected donor-strain tissues and organs from immunologic injury (18). This phenomenon has been called "split tolerance" (45, 46).

We believe that multilineage donor leukocyte chimerism is required for these changes in the immunologic environment to occur. Although the presence of dendritic cells was emphasized in our human cases (1-6) and rat liver transplant experiments (7), there was also morphological and functional evidence that T and B lymphocytes and macrophages were part of the chimeric population. A mixture of chimeric cell lineages in recipient tissues was demonstrated far more conclusively in the mouse experiments reported here, both early and late after liver transplantation. However, the exact quantitation of the lineage proportions at different time points remains to be done.

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