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Authors
Namikawa, R
Weilbaecher, K
Kaneshima, H
et al.

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Long-Term Human Hematopoiesis in the SCID-hu Mouse

By Reiko Namikawa, Katherine N. Weilbaecher, Hideto Kaneshima, Edwin J. Yee, and Joseph M. McCune

From SyStemix, Inc., Palo Alto, California 94303

Summary

Coimplantation of small fragments of human fetal thymus and fetal liver into immunodeficient SCID mice resulted in the formation of a unique structure (Thy/Liv). Thereafter, the SCID-hu mice showed reproducible and long-term reconstitution of human hematopoietic activity. For periods lasting 5–11 mo after transplantation, active T lymphopoiesis was observed inside the grafts and cells that were negative for T cell markers were found to have colony-forming units for granulocyte/macrophage (CFU-GM) and erythroid burst-forming unit (BFU-E) activity in the methylcellulose colony assay. In addition, structures similar to normal human bone marrow were observed inside the Thy/Liv grafts, consisting of blast cells, mature and immature forms of myelomonocytic cells, and megakaryocytes. These data indicate long-term maintenance, in vivo, of human progenitor cells for the T lymphoid, myelomonocytic, erythroid, and megakaryocytic lineages. The role of the implanted fetal liver fragments was analyzed using HLA-mismatched Thy/Liv implants. The HLA type of the liver donor was found on T cells and macrophages in the graft. In addition, cells grown in the methylcellulose colony assay and cells in a bone marrow-like structure, the "thymic isle," expressed the HLA type of the liver donor. Thus, the Thy/Liv implants provided a microenvironment in which to follow human hematopoietic progenitor cells for multiple lineages. The formation of the Thy/Liv structures also results in a continuous source of human T cells in the peripheral circulation of the SCID-hu mouse. Though present for 5-11 mo, these cells did not engage in a xenograft (graft-versus-host) reaction. This animal model, the first in which multilineage human hematopoietic activity is maintained for long periods of time, should be useful for the analysis of human hematopoiesis in vivo.

In vivo experiments with the laboratory mouse have contributed much to our understanding of the mammalian hematopoietic system. Adoptive transfer of congeneic hematopoietic cells into lethally irradiated hosts has shown that some populations of cells have the capacity to differentiate along multiple lineages. Mature progeny are thereafter assayed by sampling cells in the peripheral blood and/or colonies formed in the spleen (1). In this fashion, considerable information has been gathered about murine hematopoietic progenitor cells (2–5). The in vivo effects of hematopoietic growth factors have also been demonstrated in experiments with laboratory animals (6–9).

Methods acceptable for use in the mouse cannot be easily transferred to man. As a result, analogous aspects of human hematopoiesis are much less well defined. Several attempts have been made instead to transfer critical elements of the human hematopoietic system into a mouse (10–12). After intravenous inoculation of human bone marrow cells into immunodeficient *bg/nu/xid* mice, human colony-forming units for macrophage and granulocyte/macrophage (CFU-M and CFU-GM),[1] and erythroid-burst forming units (BFU-E) were later recovered from the mouse bone marrow (12). In another experiment, progenitor cells from human fetal liver were administered intravenously to immunodeficient C.B-17 *scid/scid* (SCID) mice (13) that had been previously engrafted with human fetal thymus. Later, the maturation of human T cells and myelomonocytic cells could be observed (14). Although each of these experimental systems did show evidence of human hematopoiesis, neither provided conditions necessary for long-term reconstitution with human cells of multiple lineages. Likely, human hematopoietic stem cells were either not provided, not maintained, and/or unable to differentiate as they normally would.

Given the complex regulatory mechanisms that surround hematopoiesis, it seems likely that human stem cells would best survive within a physiologic microenvironment that is

[1] *Abbreviations used in this paper:* BFU-E, erythroid burst-forming unit; CFU-c, colony-forming units in culture; CFU-GM, CFU for granulocyte/macrophage; gw, gestational weeks; TN, triple negative.

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human. In each of the examples cited above, such a microenvironment was not provided. We report now that when intact fragments of human fetal liver and thymus are coimplanted into the SCID mouse, structural elements similar to human bone marrow are observed to form and to remain functional for periods of time as long as 15 mo. These elements are associated with multilineage human hematopoietic differentiation. This animal model should provide a powerful tool for the analysis of the physiology and pathophysiology of human hematopoiesis.

Materials and Methods

Construction of SCID-hu Mice. Homozygous C.B-17 *scid/scid* mice (SCID) were bred, treated with antibiotics as described (14), and used when 6–8 wk old. Methoxyflurane anesthesia was applied during all operative procedures. Before implantation, cells from human fetal thymus (14–23 gestational weeks [gw]) or liver (18–23 gw) were typed for MHC class I alloantigens on a FACS. Thymus and liver were cut with a sharp blade into small pieces (~1 × 1 × 1 mm). One to two of these fragments were implanted under the kidney capsule of SCID mice with a 18-gauge trocar. Attention was paid to confirm that these fragments stayed close together under the capsule. Mice receiving thymus fragment alone were designated Thy/−; those implanted with a combination of thymus and liver fragments were designated Thy/Liv. Based on the HLA allotype analysis, Thy/Liv implants that were allogeneic with respect to one another were prepared so that cell movement could later be traced.

Analysis of Thymic Grafts. The mice were killed by cervical dislocation and the graft under the kidney capsule was removed under sterile conditions and placed in HBSS (without phenol red; Gibco Laboratories, Grand Island, NY) supplemented with 2% FCS and 10 mM Hepes (HBSS-FCS). Approximately one-third of the tissue was frozen for immunohistology; the remainder was teased into a single cell suspension for analysis by immunofluorescence (for phenotype) and by methylcellulose culture (for the presence of colony-forming units).

Antibodies. Mouse mAbs against human T cell markers and human MHC class I antigens were directly conjugated with FITC or PE. These included OKT3 (CD3), OKT4 (CD4), OKT8 (CD8), W6/32 (monomorphic class I determinant), MA2.1 (HLA-A2, B17), BB7.2 (HLA-A2), BB7.1 (HLA-B7, Bw42), MB40.2(HLA-B7, B40), and GAPA3 (HLA-A3). FITC- or PE-HLe1 (CD45) and LeuM1 (CD15) were purchased from Becton Dickinson Immunocytometry Systems, San Jose, CA. The mouse mAb MEM-43, which stains all hematopoietic cells of human origin, was kindly provided by Dr. V. Hořejši, Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Praha, Czechoslovakia (15). The mouse mAb Ly5.1 was obtained from Dr. G. Spangrude, Rocky Mountain Laboratories, Hamilton, MT; it reacts against most of the hematopoietic cells in the SCID mouse. Both MEM-43 and Ly5.1 were biotinylated. All antibodies and reagents were titrated and used at saturating concentrations.

Immunohistochemistry. Phenotypic characterization of cells in biopsy sections from the grafts was carried out using standard techniques. In brief, 6-μm frozen sections were prepared, fixed with acetone, and then sequentially incubated with a first-stage mAb followed by biotinylated horse anti-mouse IgG (Vector Laboratories, Inc., Burlingame, CA). Alkaline phosphatase (ALP)-conjugated avidin (Caltag Laboratories, South San Francisco, CA) was added and visualized using the substrate naphthol-AS phosphate (Sigma Chemical Co., St. Louis, MO) with Fast Blue BB salt (Sigma Chemical Co.). Endogenous phosphatase activity was blocked by adding levamisole (0.25 mM) in the reaction.

Colony-forming Units in Culture (CFU-c) Assay. Selection for cells that were triple negative (TN) for CD3, CD4, and CD8 was performed by immunomagnetic bead separation (5, 16). The cell suspension was incubated first with a cocktail of antibodies, FITC-OKT3, -OKT4, and -OKT8, and then with magnetic beads coupled to sheep anti-FITC antibodies (Advanced Magnetics, Cambridge, MA). $CD3^+$, 4^+, and 8^+ cells were removed by magnetic depletion. The degree of enrichment of the TN cells was determined afterwards by FACS. The TN fraction as well as unseparated populations were assayed for the presence of clonal hematopoietic progenitor cells in semisolid medium, using previously described methods (17). Briefly, TN cells or whole thymocytes were plated in 35-mm petri dishes at a concentration of 5–10 × 10^4/ml or 1 × 10^6/ml, respectively, in 1-ml cultures consisting of 1% methylcellulose (1,500 centipoises, Sigma Chemical Co.) in IMDM (Gibco Laboratories) containing 20% FCS, 0.05 mM 2-ME, 200 mM L-glutamine, 0.8% lept-albumin (Armour Pharmaceutical, Tarrytown, NY), 0.08% NaHCO3, and human recombinant erythropoietin (Amgen Biologicals, Thousand Oaks, CA) at the concentration of 1 U/ml, and 10% Mo-conditioned media (18). The methylcellulose cultures were incubated at 37°C in a humidified atmosphere of 5% CO_2 in air and were counted after 10–14 d to determine the total number of colonies per dish. CFU-c were characterized as having greater than 30 cells and consisted mainly of granulocytes and macrophages (CFU-GM) or multiple clusters of erythroid cells (BFU-E), although other lineages could be observed in some of the colonies. The number of the colonies was normalized per 10^5 pure TN cells based on the results of FACS analysis of the cells after magnetic bead separation.

Immunofluorescence Analysis. Immunofluorescence analysis with a single laser FACScan (Becton Dickinson Immunocytometry Systems) was performed with the cell suspensions from the grafts and cells recovered from CFU-c. Thymocytes from the grafts were analyzed for their CD4/CD8 profiles with PE-OKT4 and FITC-OKT8. Cells from CFU-GM and BFU-E colonies in the methylcellulose cultures were picked with a narrowed glass pipette and pooled. They were then stained with biotinylated MEM-43 or Ly5.1 followed by FITC-avidin (Caltag Laboratories) as the second stage. For the analysis of allogeneic Thy/Liv constructs, the HLA types of the thymocytes and the cells recovered from methylcellulose culture were determined with mAbs against allotypes of MHC class I antigens. Cells were resuspended in HBSS-FCS containing 1 μg/ml propidium iodide and analyzed with gates set to exclude debris, clumps, and dead cells.

Results

Coimplantation of Human Fetal Liver and Thymus Results in Active Human Hematopoiesis. SCID-hu mice were constructed by implanting small fragments of human fetal thymus and/or fetal liver beneath the kidney capsule. When thymus implants were engrafted alone (designated Thy/−), most (>90%) were observed to initially grow and then recede. Within 3 mo, the grafts were small and firm by gross examination. Cortical thymocytes were depleted at that time (Fig. 1 *A*) and human T cells were no longer detectable in the peripheral blood. In a minority of cases (<10%), Thy/− grafts were maintained as long as 11 mo. In these animals, cortical and

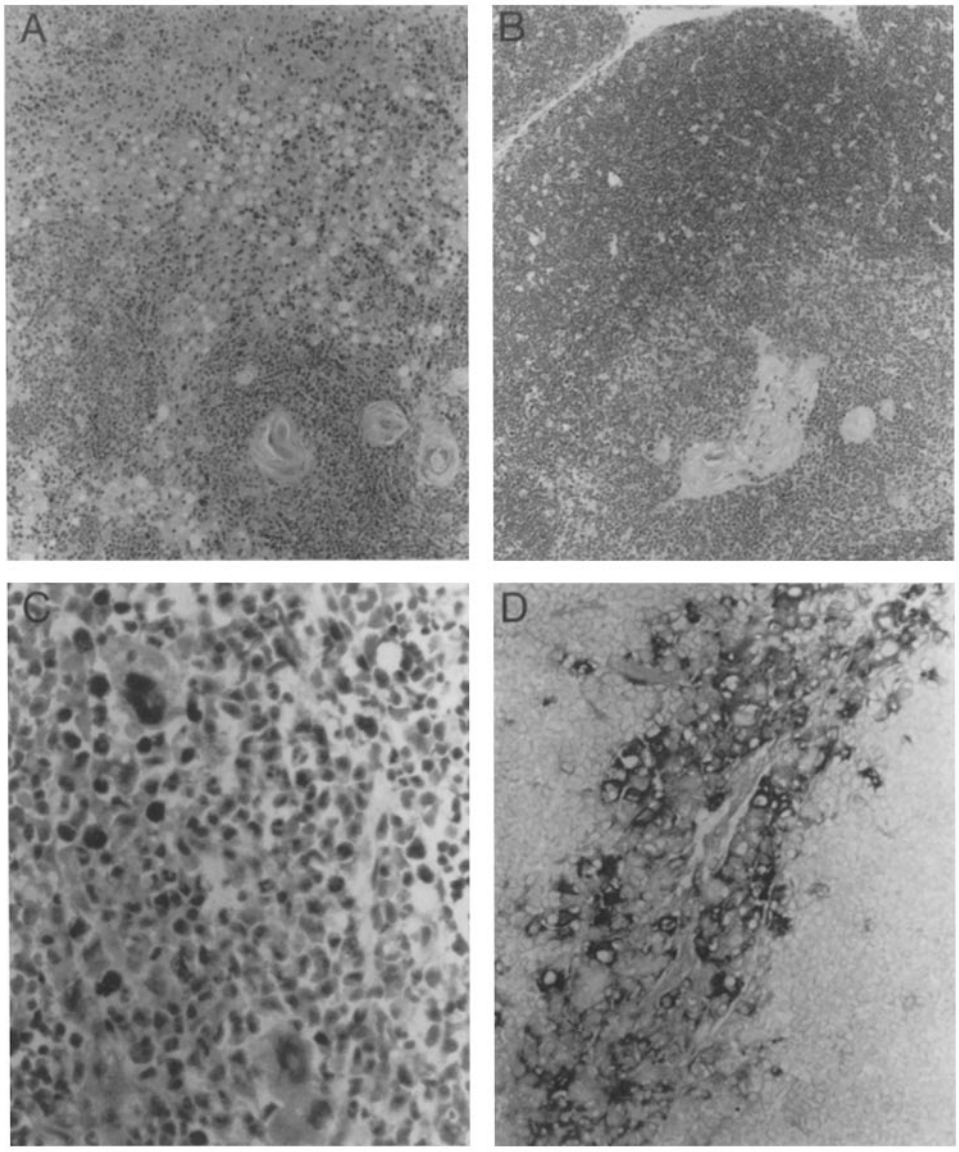

Figure 1. Histological features of the grafts. (*A*) Typical histology of Thy/− grafts without hematopoietic activity, 3 mo after implantation. The cortical area is mainly composed of epithelial cells (*upper half*); small number of lymphocytes are observed in the medulla (*lower half*) (Giemsa staining, ×80). (*B*) Typical histology of Thy/Liv grafts with hematopoietic activity, 6 mo after implantation. Both cortical (*upper half*) and medullary (*lower half*) areas are filled with thymocytes and the structure of the thymus is indistinguishable from that of a normal thymus (Giemsa staining, ×80). (*C*) The structure of the thymic isle. Blast cells, mature and immature forms of myelomonocytic cells, and megakaryocytes are observed (Giemsa staining, ×320). (*D*) Immunoalkaline phosphatase staining of thymic isle with Leu-M1. Most of the cells are positive for myelomonocytic lineage marker, Leu-M1. (×200).

medullary thymocytes remained intact and human T cells continued to mature into the peripheral circulation (see Table 1).

Continuous human T lymphopoiesis occurred much more frequently when intact fragments of fetal thymus and liver were coimplanted into the same SCID-hu mice (designated Thy/Liv). In 50% of animals, human T cells were detectable in the peripheral circulation at 6 mo (and in some cases, for periods of time as long as 15 mo). On gross examination, the grafts in these animals were large and soft. Microscopically, the histology showed a thymus structure which was indistinguishable from that of a normal human thymus (Fig. 1 *B*). Active T lymphopoiesis was reflected in cortical and medullary areas that were filled with thymocytes. In contrast to the depletion of cortical double-positive cells observed in most Thy/− grafts, FACS analysis of Thy/Liv grafts showed CD4/CD8 profiles containing double-positive thymocytes comparable to the levels found in normal fetal thymus (Table 1). Hepatocytes were not observed in the Thy/Liv grafts, although structures similar to biliary ducts were sometimes observed in the periphery of the grafts. Much more frequently, areas containing hematopoietic blast cells, immature and mature forms of myelomonocytic cells, and megakaryocytes were observed (Fig. 1 *C*). Most of the cells found in these areas (designated thymic isles) were positive for the human myelomonocytic marker, Leu-M1 (CD15) (Fig. 1 *D*). Some, including multi-nucleated large cells (as shown in Fig. 1 *C*), were also stained with a mouse mAb against the platelet related antigen, gpIIb (CD41) (AMAC, Inc., Westbrook, ME). Thymic isles localized in the septal area of the thymic implant, particularly in areas in which the septum invaginated the thymic medulla (Fig. 4 *D*). Microscopically, these areas had a structure similar to that of normal human bone marrow. Thymic isles were also observed in the rare Thy/− grafts that sustained hematopoiesis for long periods of time.

These findings suggested that Thy/Liv grafts were associated with multilineage human progenitor cell activity. To demonstrate this activity directly, cells from the grafts were preenriched for TN cells by negative selection for T cell markers (CD3, CD4, CD8), and then plated in the methylcellulose colony assay. The results, summarized in Table 1, showed that 8 of 10 grafts (80%) contained progenitor cell activity for CFU-c. Colonies of both the CFU-GM lineage and the BFU-E lineage were observed in most cases of the grafts, 6–11 mo after transplantation. The cells recovered from CFU-GM and BFU-E colonies were pooled and stained with either the human-specific antibody, MEM-43, or the mouse-specific antibody, Ly5.1, to confirm that they originated from human progenitors (Fig. 2). The vast majority of these cells (>70%) were positive for MEM-43 in all the samples analyzed (n = 20); none of them had a significant number of mouse cells stained with Ly5.1. Even when most of the pooled colonies were BFU-E, most of the cells were still positive for MEM-43. These results strongly suggested that the cells derived from CFU-GM and those from BFU-E were of human origin.

Human Hematopoietic Progenitor Cells in the Thy/Liv Grafts Are Derived from the Fetal Liver. Coimplantation of syngeneic fetal liver and thymus resulted in reproducible and long-term human hematopoiesis, whereas implantation of thymus alone usually did not. To test the possibility that hematopoietic progenitor cells and/or stromal cells from the fetal liver were responsible for this activity, SCID-hu mice were prepared with combinations of human fetal liver and thymus that were allogeneic with respect to human MHC class I antigens. mAbs against marker class I antigens were then used to trace cell movement.

In 23 of the 41 allogeneic Thy/Liv grafts analyzed (56%), long-term T lymphopoiesis (5–11 mo) was documented both by the presence of mature human T cells in the peripheral circulation and by the maintenance of a cortical component of immature $CD4^+/CD8^+$ thymocytes. In addition, hematopoietic progenitor cells could be detected with a frequency (16 of 19, or 84%) similar to that of syngeneic Thy/Liv grafts (80%). Some of those results are summarized in Table 2. The HLA types of medullary thymocytes and the cells recovered from CFU-c assay were analyzed. Invariably, it was found that progenitor activity in the Thy/Liv implants was derived from the fetal liver. A representative experiment is shown in Fig. 3. In this case, the thymus donor was HLA-$A3^+$, $A2^-$, $B7^-$ (stained with GAPA3); the fetal liver donor was HLA-$A3^+$, $A2^+$, $B7^-$ (stained with GAPA3 and MA2.1).

Table 1. *CD4/CD8 Profile and Progenitor Cell Activity in Fetal Thymus, Thy/−, and Thy/Liv Syngeneic Grafts*

Construct	Incubation	Thymocytes*				CFU-c(/10^5 TN‡)	
		$4^+/8^+$	$4^+/8^-$	$4^-/8^+$	$4^-/8^-$	CFU-GM	BFU-E
Ungrafted thymus	(19 gw)	85.9	6.9	3.0	4.1	20.5	15.0
	(22 gw)	72.9	17.4	4.0	5.6	16.9	10.0
Thy/−	9–11 wk	2.5	32.2	49.7	15.9	ND	ND
	(10 cases)	±6.1§	±13.8	±15.1	±10.9	ND	ND
Thy/−	11 mo	70.1	24.6	3.9	1.4	1.3	8.7
	11 mo	61.1	31.8	5.5	1.7	0.0	0.7
Thy/Liv	11 mo	71.9	20.4	6.7	1.0	6.5	6.9
	11 mo	70.4	21.9	7.0	0.8	12.0	15.0
	11 mo	60.2	29.2	9.2	1.5	0.0	0.0
	7 mo	78.1	17.0	3.7	1.2	3.0	36.8
	7 mo	77.5	17.4	4.3	0.8	17.0	50.8
	7 mo	74.0	19.7	4.5	1.9	5.6‖	3.2‖
	6 mo	73.1	20.5	5.2	1.2	4.0	22.7
	6 mo	72.2	22.2	4.5	1.1	44.6	60.8
	6 mo	49.5	33.5	15.1	1.9	2.6	6.9
	6 mo	7.0	20.0	8.8	64.2	0.0	0.0

* Phenotype of thymocytes, $4^+/8^+$; CD4 and CD8 double-positive, $4^+/8^-$; CD4 single-positive, $4^-/8^+$; CD8 single-positive, $4^-/8^-$; CD4 and CD8 double-negative.
‡ $CD3^-$,$CD4^-$,$CD8^-$ triple negative population.
§ Standard deviation.
‖ Calculated per 10^6 whole thymocytes.

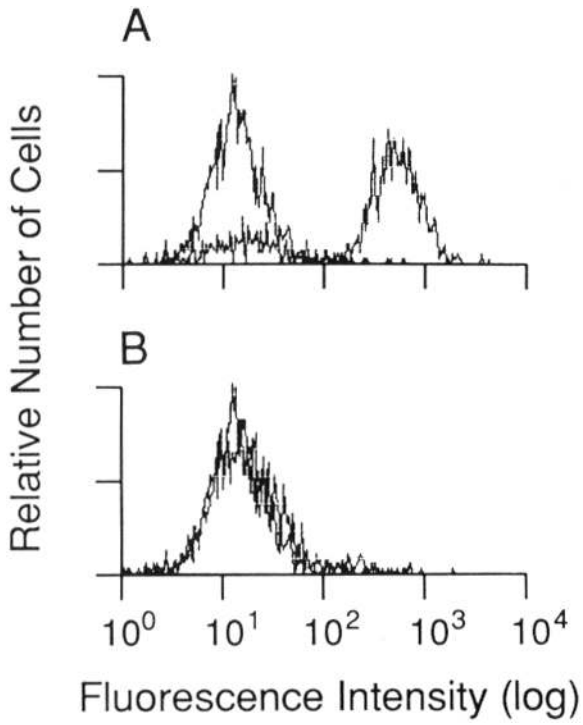

Figure 2. Immunofluorescence analysis of the cells recovered from CFU-c assay. The cells recovered from the methylcellulose culture were stained with either human-specific antibody, MEM-43 (*A*), or mouse-specific antibody, Ly5.1 (*B*). Cells stained with FITC-avidin as a control showed minimum reactivity (0.16%). Most of the cells were positive for MEM-43 (76%), whereas a small population of the cells were positive for Ly5.1 (3.8%).

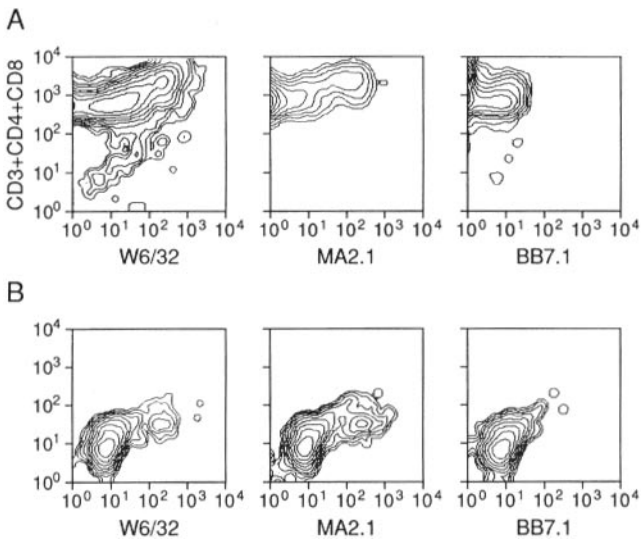

Figure 3. Analysis of HLA type of thymocytes (*A*) and CFU-c (*B*) originating from allogeneic Thy/Liv graft, 6 mo after implantation. The thymus donor was positive for GAPA3 and the liver donor for both GAPA3 and MA2.1. Antibody BB7.1, which was irrelevant to both donors, was included as a control. (*A*) The thymocyte suspension was stained with the cocktail of PE-OKT3, -OKT4, and -OKT8 and FITC-W6/32, -MA2.1, or -BB7.1. Those that express MHC class I antigens (defined by W6/32) were positive for the HLA type of the fetal liver donor (MA2.1). (*B*) Cells recovered from methylcellulose culture were stained with FITC-W6/32, -MA2.1 or -BB7.1. Cells positive for W6/32 and the fetal liver donor type (MA2.1) were observed.

6 mo after coimplantation of these organs, FACS analysis of medullary thymocytes demonstrated high level staining with the anti-HLA class I framework antibody, W6/32, and the antibody specific for the fetal liver donor, MA2.1 (Fig. 3 *A*). When $CD3^-$, $CD4^-$, $CD8^-$ thymocytes were plated in methylcellulose, CFU-GM, and BFU-E formed. These cells, too, were stained with MA2.1 and positive for HLA-A2 (Fig. 3 *B*).

The allogeneic coimplants of fetal liver and thymus were examined by immunohistology. In a representative example shown in Fig. 4, the thymus donor was HLA-$B7^+$ (stained with BB7.1 and MB40.2) and the fetal liver donor was HLA-$A2^+$ (stained with MA2.1 and BB7.2). 7 mo after coimplantation, biopsy sections were stained with either antibody. Epithelial cells in the cortex and medulla were still HLA-$B7^+$ (Fig. 4 *A*). Most of the other cells in the graft were derived, however, from the HLA-$A2^+$ fetal liver donor. These included medullary thymocytes, macrophages in the cortex (Fig. 4 *B*), as well as the diverse populations of cells found within the thymic isles (Fig. 4, *C* and *D*).

The results of the HLA typing of thymocytes, macrophages in thymus, and cells recovered from CFU-c assay are summarized in Table 2. Cortical macrophages and medullary thymocytes showed the HLA type of the fetal liver donor in all cases analyzed by immunohistology. Although medullary thymocytes were observed by FACS analysis to have a chimeric status in some cases, the major population was shown by HLA typing to originate from the fetal liver donor. Mixed cell populations comprised of CFU-GM and BFU-E were also demonstrated to originate from fetal liver donor. However, these were likely to represent the HLA types expressed on granulocyte/macrophage lineage cells because not all of the cells from BFU-E may express HLA molecules. Thus, in association with the microenvironment of the fetal thymus, progenitor cell(s) carried within the fetal liver fragments were able to give rise to long-term T cell maturation as well as to differentiation along the myelomonocytic and possibly erythroid pathways.

Absence of Graft-versus-Host Disease in SCID-hu Mice after Long-Term Hematopoietic Reconstitution. The SCID-hu mice described in this report had mature human T cells in the peripheral circulation for long periods of time. In other experiments, these cells have been shown to be functional, responding in vitro to mitogens as well as to antibodies against CD3 (Krowka, J.F., et al., manuscript in preparation). To evaluate whether these cells were reacting against the host mouse tissues, organs such as liver, skin, gut, spleen, and lung from a total of 10 SCID-hu mice were examined 5–11 mo after Thy/Liv implantation. Macroscopically and microscopically, no pathological changes of graft-versus-host disease (GVHD) were observed.

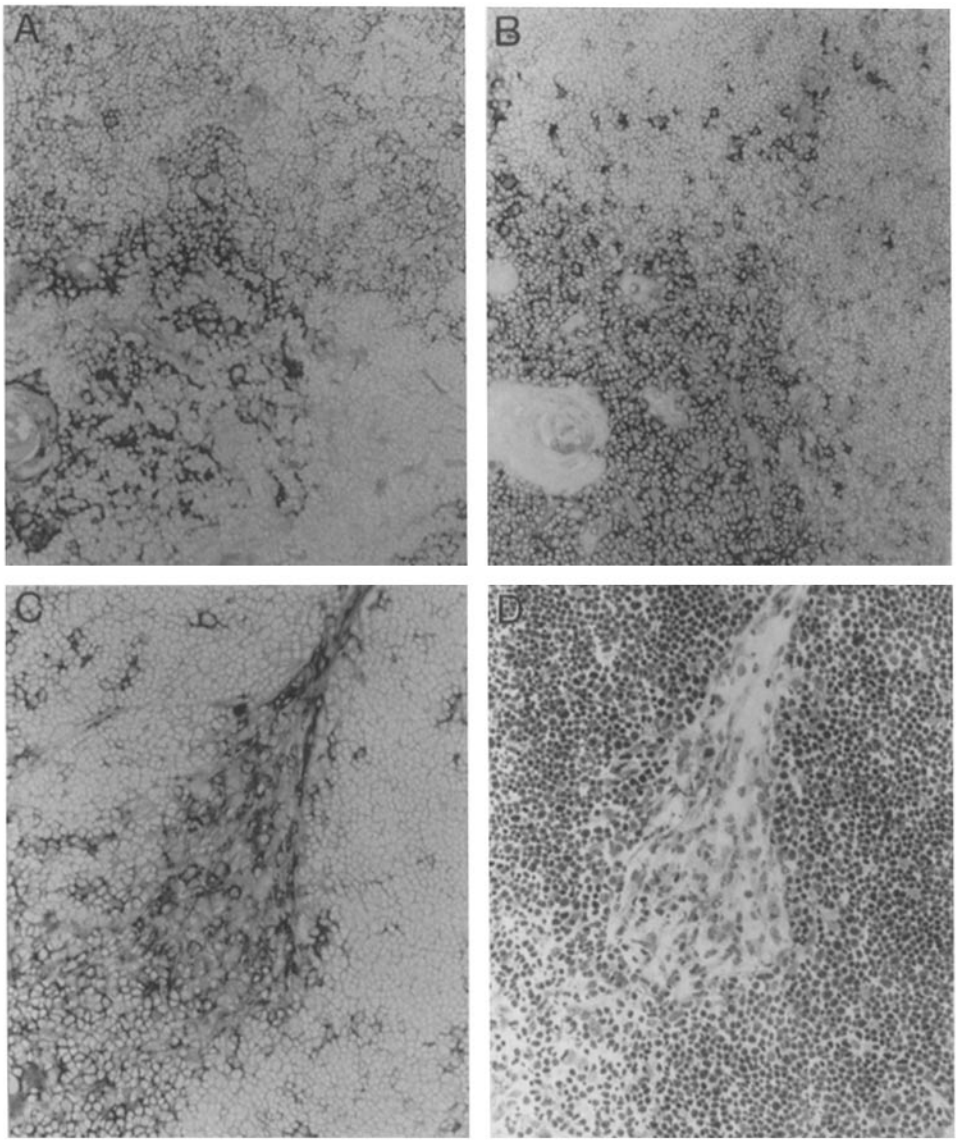

Figure 4. The expression of the MHC class I antigens in an allogeneic Thy/Liv graft, 7 mo after implantation, as analyzed by immunoalkaline phosphatase method. The thymus donor was positive for HLA-B7 (stained with BB7.1 and MB40.2) and the fetal liver donor was positive for HLA-A2 (stained with MA2.1 and BB 7.2). (*A*) Section was stained with BB7.1. Epithelial cells in both cortical (*upper half*) and medullary (*lower half*) areas were positive for thymus donor type, HLA-B7 (×120). (*B*) Section was stained with MA2.1. Medullary thymocytes (*lower half*) and macrophages with dendritic shape in the cortical area (*upper half*) were positive for the liver donor type, HLA-A2 (×120). (*C*) Section was stained with MA2.1. Cells in the thymic isle (*center*) as well as medullary thymocytes (*lower left*) were positive for the liver donor type, HLA-A2 (×160). (*D*) Histology of the thymic isle shown in *C*. The cluster of myelomonocytic cells is located in the septal area adjacent to the medullary area of the thymus (Giemsa staining, ×160).

Discussion

In this report, we describe an improved construction of the SCID-hu mouse. When human fetal liver was transplanted into the SCID mouse as an intact organ along with human fetal thymus, human hematopoiesis was established reproducibly, along multiple lineages, and for long periods of time. The two organs joined as unique Thy/Liv structures, which often developed focal areas of hematopoietic activity. Microscopically, these areas (thymic isles) were similar to normal human bone marrow, containing blast cells, immature and mature forms of granulocytes, and megakaryocytes. When plated in the methylcellulose colony assay to test for hematopoietic progenitor cell activity, the Thy/Liv grafts yielded CFU-c for the myelomonocytic and erythroid lineages. In vivo, progenitors to the T lymphoid lineage were evidenced by the continued production of immature cortical thymocytes and mature human T cells in the thymus and their progeny in the peripheral blood. All of these progenitor activities were maintained in the Thy/Liv grafts for periods of time ranging between 5 and 11 mo. Finally, all were derived from hematopoietic cells donated by the fetal liver, and not the fetal thymus, implant. These findings indicate that SCID-hu mice with human Thy/Liv coimplants contain stromal microenvironment(s) requisite for the long-term maintenance of human hematopoietic progenitor cells. As such, this animal model permits systematic evaluation of human hematopoiesis.

In previous studies with SCID-hu mice, long-term human hematopoiesis did not occur (14). Immature cortical thymocytes of the human thymic graft eventually became depleted; mature human T cells in the peripheral blood disappeared after 2–3 mo. These cells may have been destroyed, sequestered elsewhere, and/or simply not replenished. Given the design

Table 2. *CD4/CD8 Profile, Progenitor Cell Activity, and HLA Type of Various Lineages of Cells in Allogeneic Thy/Liv Graft*

	Thymocytes*				CFU-c(/10^5 TN‡)		HLA		
Incubation	$4^+/8^+$	$4^+/8^-$	$4^-/8^+$	$4^-/8^-$	CFU-GM	BFU-E	Mϕ§	T‖	CFU-c¶
8 mo	72.0	23.4	3.6	1.0	0.0	0.0	L‡‡	L/L	ND
7 mo	86.7	10.4	2.2	0.8	85.9	0.0	L	L/ND	L
7 mo	57.6	30.4	9.3	2.7	0.0	0.0	L	L/ND	ND
6 mo	79.9	14.9	4.5	0.7	0.0**	1.3**	L	L/L	ND
6 mo	79.8	14.9	3.7	1.5	19.3	38.5	L	L/L	L
6 mo	73.7	18.9	5.7	1.7	8.1	48.4	L	L/L	L
5 mo	85.0	8.8	5.6	0.6	4.8**	4.8**	L	L/L	ND
5 mo	82.8	11.4	5.3	0.5	6.5	3.5	L	L/L	L
5 mo	79.7	13.2	6.6	0.5	3.7	2.4	L	L/L	L
5 mo	76.2	14.1	8.9	0.8	0.8**	0.0**	L	L/L	ND
5 mo	67.2	24.4	7.4	1.1	0.0	1.0	ND	L/L	ND
5 mo	65.2	25.0	8.7	1.1	3.5	4.0	ND	L/L	L

* See same footnote of Table 1 legend.
‡ See same footnote of Table 1 legend.
§ HLA type of macrophages in thymus analyzed by immunohistology.
‖ HLA type of medullary thymocytes analyzed by immunohistology/FACS.
¶ HLA type of cells derived from CFU-GM and BFU-E analyzed by FACS.
** See footnote ‖ of Table 1 legend.
‡‡ HLA type of fetal liver donor.

of the experiment, the latter possibility seemed probable. The hematopoietic cell fraction of human fetal liver (containing progenitor cell activity) had been teased away from the stromal matrix of the organ. Once injected intravenously into SCID-hu mice with human thymic implants, long-term hematopoiesis did not ensue. Likely, hematopoietic progenitor cells had been provided in limiting numbers and/or without the necessary conditions for self-renewal.

In the current study, the fetal liver was instead implanted intact. Hematopoietic progenitor cells were introduced together with the hepatic stromal cells with which they normally interact. Technically and conceptually, this process is simple. With fewer manipulations, more of the elements necessary for active human hematopoiesis (most of which are unknown) may be implanted as a fully functional unit. The liver grafts do not grow on their own when placed beneath the kidney capsule of the SCID mouse. When, however, the fetal liver is coimplanted with grafts of human thymus, a novel structure (Thy/Liv) is formed. In many cases, this structure is associated with multilineage human progenitor cell activity when assayed in vitro. Functional hematopoiesis is then maintained in vivo for periods of time as long as 15 mo.

Multiple dynamics may underlie the genesis of the Thy/Liv structure. It is possible to envision a chimeric association between some cell populations of the fetal liver and those of the thymus. Fetal liver cells that move into the parenchyma of the thymus could thereafter be maintained. Coimplantation of fetal liver adjacent to thymus may also have an indirect effect, e.g., by way of diffusible factors. In all probability, these and other influences occur simultaneously. The hematopoietic cell fractions of the fetal liver donor, for instance, move into and/or through the thymus. Demonstrated in a transient assay before, this differentiation process is shown here to occur in a fashion which is both stable and reproducible. Over 50% of the Thy/Liv implants sustain human hematopoiesis. All of those analyzed in an allogeneic setting demonstrate repopulation by the hematopoietic cells from the fetal liver donor. It is not clear that the stromal cells of the thymic isles are also derived from the fetal liver donor. These cells may instead be thymic-derived and with diverse biologic potential. In the current studies, some SCID-hu mice with thymic implants alone showed the same, suggesting that the thymus itself has the microenvironment(s) that can support progenitor cells. Others have reported progenitor cell activity in fetal and neonatal thymus (19; Weilbaecher, K.N., unpublished observations) and the activity of thymic epithelial cells to induce myelomonocytic differentiation of progenitor cells (19).

Irrespective of the pathways by which the Thy/Liv implants are formed, the data suggest that they are suitable microenvironments for the long-term maintenance of human hematopoietic progenitor cells. Using a combination of in vitro and in vivo assays, maturation of these cells into the T lymphoid, erythroid, myelomonocytic, and megakaryocytic series was observed. It is not clear whether there is one long-lived population of pluripotent (stem) cells and/or multiple

populations of long-lived, lineage-restricted progenitors. It is now possible, however, to discriminate among these alternatives and to identify diverse subpopulations of cells by transferring them specifically into SCID-hu mice with Thy/Liv implants. Taking advantage of the unique microenvironment of these implants, the mouse could thus provide an in vivo read-out for human hematopoietic progenitor cells.

The improvements reported here in the SCID-hu mouse are still not complete. First, biologic variability remains between donor tissues, individual animals, and cohorts of animals. Even gross functions, such as the presence or absence of sustained hematopoiesis, are only observed in 50% of the animals. Even among these animals, there is a range in the level of reconstitution by donor fetal liver hematopoietic cells. Such biologic diversity is a reality in any complex system. Experimental designs must therefore be either independent of these variables or observant of their effect of the data. On a more general level, the hematopoietic function of the Thy/Liv transplants is in some obvious ways different than that which occurs physiologically in man. Even though human progenitor cells to multiple lineages can be maintained, not all of them differentiate with equal efficiency in vivo. When analyzed by FACS, human T cells are found in the periphery but no cells in other lineages, such as B cells, granulocytes, and macrophages are observed. Myelomonocytic cells and megakaryocytes are confined instead within the Thy/Liv parenchyma. Further, even those cell populations that do differentiate well in the SCID-hu (e.g., T cells) do not exist later in abundance. If these are points of limitation, they also serve as a foundation for further improvement. In a quantitative or qualitative sense, specific growth factors and/or cell populations may still be deficient. Once provided in appropriate amounts or anew, hematopoietic functions that are not now present may become apparent. If so, improvement of the SCID-hu mouse will be accompanied by additional insight into the complex regulatory mechanisms surrounding hematopoiesis per se.

A number of novel experimental opportunities are provided by the hematopoietic functions associated with the Thy/Liv grafts. In essence, this system provides the first small animal model for long-term human hematopoiesis. After engraftment, the human hematolymphoid organs may be analyzed or manipulated as a function of time. A descriptive purview of hematopoiesis can then be easily obtained. The differentiative fate of various cell populations can be followed. The interactive influences of stromal cells and/or their growth factors can be assayed. Upon definition of a baseline, it is also possible to study pathophysiologic insults to the process. As such, SCID-hu mice with long-term human hematopoiesis may provide an experimental system in which to study human disease states that are now hard to approach, e.g., those caused by infectious agents (HIV, CMV), drugs (AZT), neoplastic processes, and congenital hematologic defects (20–22, Kaneshima, H., C.-C. Shih, R. Namikawa, L. Rabin and J. M. McCune, manuscript submitted for publication).

Finally, the continual differentiation of human hematopoietic cells along the T lymphoid lineage provides a solid basis for studies on human immune function. As shown in this report, current data are most striking in the description of functions *not* present, to wit, the absence of GVHD. It is to be expected that mature human peripheral blood cells might initiate a xenograft reaction when injected into SCID mice, and indeed, histologic evidence of GVHD has been observed by some (23, 24) but not all (25) groups making "hu-PBL-SCID" mice. In contrast, similar histologic examination of SCID-hu mice shows no signs of GVHD, even in animals that have had circulating human T cells for 5–11 mo. Experiments are currently in progress to determine if this is due to a lack of function, or alternatively, to the acquisition of tolerance during the course of differentiation. These and other studies should contribute to our understanding of means by which to actively turn on or off specific human immune responses in the SCID-hu mouse.

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Address correspondence to Dr. Reiko Namikawa, SyStemix, Inc., 3400 West Bayshore Road, Palo Alto, CA 94303.

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