Lymphoid reconstitution of the human fetal thymus in SCID mice with CD34+ precursor cells

Brief Definitive Report

Lymphoid Reconstitution of the Human Fetal Thymus in SCID Mice with CD34$^+$ Precursor Cells

By Bruno Péault, Irving L. Weissman,* Charles Baum, Joseph M. McCune, and Ann Tsukamoto

*From SyStemix, Inc., Palo Alto, California 94303; and the *Howard Hughes Institute, Beckman Center for Molecular and Genetic Medicine, Stanford University School of Medicine, Stanford, California 94305*

Summary

The search for human hematopoietic stem cells has been hampered by the lack of appropriate assay systems. Demonstration of the ability of precursor cell candidates to give rise to T cells is of significant difficulty since dissociated in vitro cultured thymus stroma cells lose their ability to sustain thymocyte maturation. To define further the differentiative capacities of the rare human fetal liver and bone marrow cells that express the CD34 surface antigen and exhibit in vitro myeloid and pre-B cell activities, we have microinjected them into HLA-mismatched fetal thymus fragments, partially depleted of hematopoietic cells by low temperature culture. In vitro colonized thymuses have then been allowed to develop upon engraftment into immunodeficient SCID mice. Using this modification of the SCID-hu system, we show that low numbers of fetal CD34$^+$ progenitor cells can repopulate the lymphoid compartment in the human thymus.

The production of hematopoietic cells in blood-forming organs requires interactions between progenitor cells and stromal elements that can be, in some instances, experimentally reproduced in vitro. Bone marrow cells, cultured in a two-dimensional arrangement, retain their ability to support myelopoiesis and B lymphocyte production, yielding convenient in vitro stem cell assays in both murine (see reference 1 for a review) and human (2; Baum, C., and A. Tsukamoto, manuscript submitted for publication) models. In contrast, the maintenance of the three-dimensional structure of thymic reticulum appears to be a prerequisite for driving the full differentiation of stem cells into mature T lymphocytes. In the mouse, experimental T cell differentiation can be achieved in a quantitative manner by transfer of precursor cell candidates into irradiated hosts, either systemically (3) or by direct intrathymic injection (4, 5). Similar physiologic assays of putative T cell precursors in man are, of course, impossible. The SCID-hu mouse, a chimera obtained by transplantation of human hematolymphoid tissue into a congenitally immunodeficient host, may provide an in vivo culture system for dissecting the human T cell differentiation process (6). In this study, we have first colonized lymphocyte-depleted human thymus fragments in vitro by HLA-mismatched putative precursor cells, then transplanted them into C.B.17 *scid/scid* (SCID) mice to facilitate expansion and differentiation of the donor cell population. The candidate hematopoietic precursor cells we have first analyzed in this experimental system express the CD34 surface antigen and represent ~1–4% of bone marrow and fetal liver cells. CD34$^+$ cells can be shown to include colony-forming units endowed with myeloid (7–9) and pre-B cell activity (Baum, C., and A. Tsukamoto, manuscript submitted for publication); they also give rise to colonies of blast cells on bone marrow stroma capable of self renewal and multilineage commitment (10). These observations suggest that the CD34$^+$ cell pool may include multipotential stem cells.

Materials and Methods

Hematopoietic Depletion and Reconstitution of the Human Fetal Thymus. Individual lobules from 19–22-wk fetal thymuses obtained from elective abortions were placed on sterile nitrocellulose filter squares (1 cm × 1 cm) (Millipore Corp., Bedford, MA) supported by absorbent gelatin sponges (Gelfoam; UpJohn, Kalamazoo, MI), in 3.5-cm petri dishes containing 2 ml of RPMI, 10% heat-inactivated FCS, and penicillin/streptomycin, for 7 d at 25°C in a 5% CO_2 atmosphere. An oil-filled syringe (Hamilton Co., Reno, NV) with a screw-operated plunger was used to inject, through glass micropipettes, suspensions (0.5–1 μl) of HLA-mismatched CD34$^+$ or CD34$^-$ cells into low temperature–cultured thymus fragments. In vitro colonized thymus fragments were inserted under the kidney capsule of Nembutal-anesthetized CB17 *scid/scid* mice bred in our own facility.

Sorting of Hematopoietic Precursor Cells from Human Fetal Blood-forming Organs. Human fetal marrow cell suspensions were prepared from long bones of 15–20-wk-fetuses. The bones were split lengthwise, then placed in a 1-mg/ml solution of collagenase/dispase in RPMI 1640 for 30 min at 37°C, after which time the medullary cavity was flushed with medium (RPMI 1640 with Pen/Strep, 2-ME, and 10% FCS) to remove hematopoietic cells. Red blood

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/91/11/1283/04 $2.00
Volume 174 November 1991 1283–1286

cells were lysed by low osmolarity ammonium chloride treatment. Cell suspensions were mechanically prepared from livers of 14–20-wk fetuses, then placed on a Ficoll-Hypaque gradient (Sigma Chemical Co., St. Louis, MO) to remove hepatocytes, red blood cells, and debris.

$CD34^+$ and $CD34^-$ cells were isolated on a FACStar® (Becton Dickinson & Co., Mountain View, CA) from fresh fetal liver or bone marrow mononucleated cell suspensions, after labeling with a monoclonal anti-CD34 IgM (kindly provided by Dr. Irving Bernstein, Fred Hutchinson Cancer Center, Seattle, WA), then with a goat antibody anti-mouse IgM, non-crossreactive to human Igs, conjugated to PE or to FITC (Caltag Labs., South San Francisco, CA).

Analysis of T Cell Repopulation in Human Fetal Thymus Grafts. For analysis of chimerism, mice were killed by cervical dislocation and human thymus grafts dissected free of kidney tissue. Thymocytes were then mechanically dispersed and stained by two-color immunofluorescence, resuspended in the presence of propidium iodide, and analyzed on a FACScan® (Becton Dickinson & Co.). PE-labeled anti-CD3, anti-CD4, and anti-CD8 mAbs were purchased from Becton Dickinson & Co. Hybridomas secreting the following anti-MHC class I antibodies: BB7.2 (anti-A2), MA2.1 (anti-A2 and B17), GAPA3 (anti-A3), W632 (anti-A, B, C), BB7.1 (anti-B7), and MB40.2 (anti-B7 and B40) were obtained from the American Type Culture Collection (Rockville, MD). mAbs were purified from ascitic fluids and directly labeled with FITC.

Immunohistochemical Analysis of $CD34^+$ Cell-reconstituted Human Fetal Thymus Grafts. 6-μm cryostat sections of frozen grafts were air-dried and fixed with acetone. Rehydrated sections were incubated successively with mouse monoclonal anti-human class I antibody, biotinylated-horse anti-mouse Ig antibody (Vector Labs., Inc., Burlingame, CA), and avidin-alkaline phosphatase at room temperature. For development, a mixture of Fast Blue BB salt (2.5 g in 50 μl dimethylformamide) and naphtol AS phosphate (2.5 mg in 50 μl dimethylformamide) was used, in 2.5 ml of 0.05 M propanediol buffer, pH 9.75, containing 60 μl of 10 mM levamisole to block endogenous enzyme activity. Sections were then post-fixed with 20% formalin for 5 min, washed in distilled water, counterstained with hematoxylin, and mounted in gelatin-glycerol.

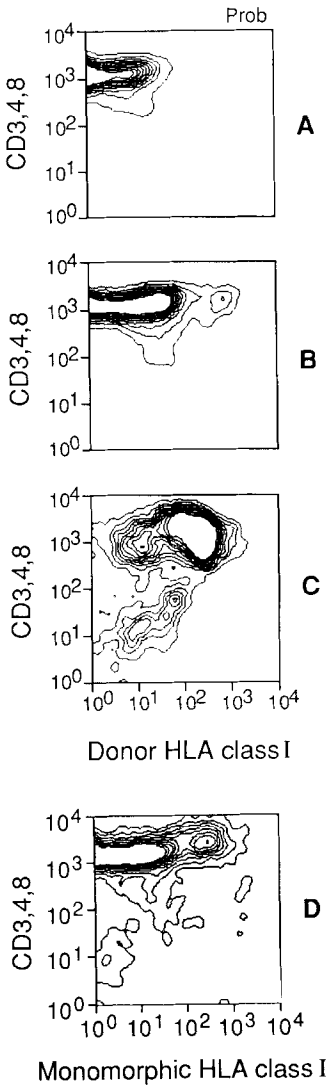

Figure 1. Lymphoid reconstitution of the 20-wk human fetal thymus after in vitro intrathymic transfer of 10^4 fetal bone marrow (22 wk) $CD34^+$ cells and engraftment into SCID mice for (*A*) 1 mo, (*B*) 6 wk, and (*C*) 4.5 mo. (*D*) Surface HLA class I antigen distribution in a cell suspension from a fresh 19-wk fetal thymus.

Results and Discussion

Reducing the size of the thymus hematopoietic cell compartment is a prerequisite to experimental reconstitution and can be achieved by low temperature in vitro organ culture (11). Human thymus tissue cultured at 25°C appeared as a loose epithelial network, showing large lymphocyte-free areas (not shown), as previously described in the mouse model (11). HLA-mismatched $CD34^+$ or $CD34^-$ cell suspensions sorted from fresh human fetal liver or bone marrow were delivered by microinjection to the depleted thymus pieces. After engraftment under the kidney capsule of SCID mice, low temperature–cultured, in vitro colonized thymus fragments were vascularized and grew. At intervals, grafts were harvested, reduced into cell suspensions, and analyzed for chimerism in the T cell compartment. Using a two-color immunofluorescence assay class I HLA antigens served as markers for donor vs. host-derived lymphocytes. The initial phase of thymus lymphoid repopulation from injected donor cells was difficult to trace given the fact that nonmature and immature cortical thymocytes express very low quantities of class I antigens. Chimerism could be evidenced when the progeny of transferred cells was differentiated far enough to express detectable amounts of class I molecules, which occurred 3–4 wk after the engraftment of thymus colonized with 10^4 $CD34^+$ cells (Fig. 1 *A*). From this time onwards, the donor-derived population was observed to expand and mature, with larger numbers of thymocytes in the graft expressing higher amounts of the donor class I antigens. After 6 wk, the donor-derived thymocyte population in the graft was similar to that of a fresh normal thymus, with respect to its range of CL I antigen expression (Fig. 1, *B* and *D*). Eventually, the progeny of the transferred $CD34^+$ cells appeared as populations of mature-type T lymphocytes, expressing high levels of class I antigens (Figs. 1 *C* and 2, *A–C*). These cells were analyzed by three-color immunofluorescence and found to include, in the expected proportions, donor-derived elements that were either $CD4^+$ or $CD8^+$ (not shown). In contrast, no T cell repopulation was ever observed after transfer of fetal liver or bone marrow $CD34^-$ counterpart cells, which were analyzed in the same conditions in all experiments. The successful

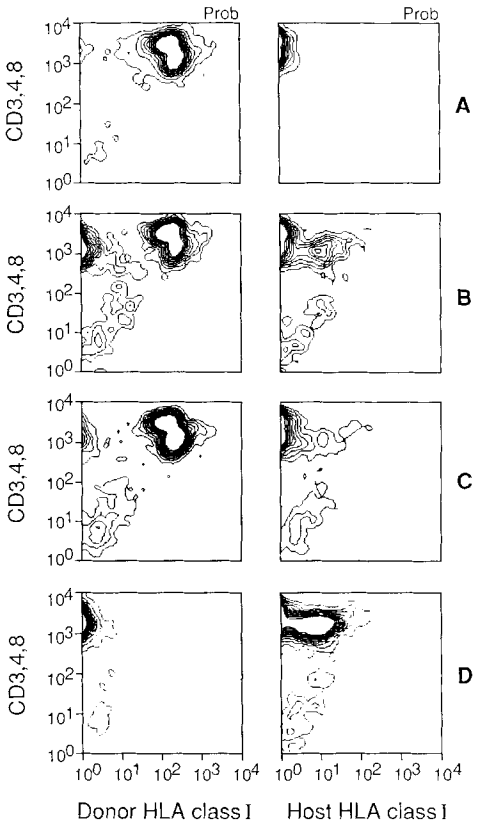

Figure 2. T cell reconstitution in the human fetal thymus after in vitro intrathymic transfer of (*A*) 10^4, (*B*) 10^3, (*C*) 10^2 CD34+ cells sorted from fetal liver, and engraftment for 3 mo into SCID mice. As a control, *D* shows a case of unsuccessful reconstitution following transfer of 10^2 cells. The thymus was 21 wk old and CD34+ cells were sorted from a 14.5-wk liver.

engraftment and differentiation of 10^4 CD34+ cells prompted an attempt to repopulate the thymus with lower numbers of these precursors. As shown in Fig. 2, complete T cell repopulation was observed after supplying the thymus with 10^3 and even 10^2 CD34+ cells, the lowest number assayed so far.

To visualize the tissue distribution of the cells generated by the microinjected precursor cell population, sections from thymus grafts fully reconstituted with 10^3 CD34+ cells were stained with mAbs specific for either host or donor class I HLA antigens. Host class I molecules were restricted to epithelial cells in the cortex and at the corticomedullary junction, and of cells with a dendritic morphology in the medulla where, by contrast, no stained lymphocytes were present (Fig. 3). The donor-specific anti-class I reagent stained almost exclusively packed lymphocytes in the medullary region. Whether cortical thymocytes were all donor derived or included resurgent host cells could not be determined in the present experimental system.

These results show that at least a fraction of the CD34+ cell population present in the fetal liver and bone marrow has the ability to differentiate into thymic lymphocytes when experimentally introduced into the thymic microenvironment.

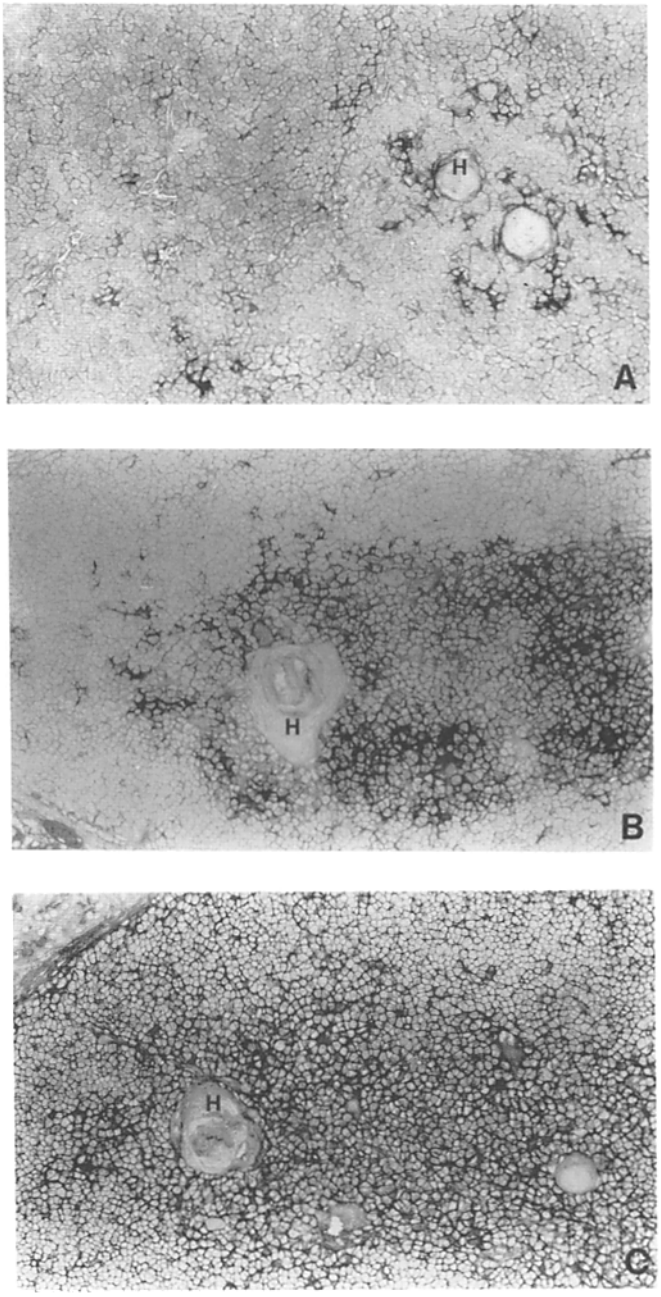

Figure 3. Immunohistochemical analysis of human fetal thymus hematopoietic reconstitution after in vitro transfer of 10^3 fetal bone marrow CD34+ cells and 4-mo engraftment in a SCID mouse. (*A*) Staining by host-specific anti-class I antibody. Epithelial cells in the cortex and medulla are stained. The medullary region, identifiable by the presence of Hassall's corpuscles (*H*), is devoid of labeled lymphocytes but contains dendritic-like cells of host origin. (*B*) Staining by donor-specific anti-class I antibody is mostly restricted to densely packed medullary lymphocytes. (*C*) Staining by a monomorphic anti-class I antibody restores the cortical and medullary class I antigen distribution of a normal thymus.

Human CD34+ cells are phenotypically heterogenous and can be subdivided into several subsets coexpressing, among others, the myeloid-specific CD33, B cell-specific CD19 and CD10, and T cell-restricted CD7 lineage markers. Interest-

ingly, human in vitro colony-forming cells can be distinguished from their precursors since only the former express CD33, whereas all of them are $CD34^+$ (12). CD7 is believed to be expressed by extrathymic pre-T cells (13, 14). The above-described thymic reconstitution assay should allow the definition of the subsets of human bone marrow cells that can give rise to thymic colonization and T cell differentiation, using techniques that defined at least two subsets of mouse bone marrow cells with this potential (15).

Due to the unavoidable variability of human material, differences are noted in the kinetics and extent of the T cell repopulation observed after in vitro intrathymic transfer of precursor cells. Also, in some cases, precursor cells sorted from one given donor were unable to reconstitute a particular thymus, at any number of transferred cells, suggesting that allogeneic reconstitution may be dependent upon yet unknown compatibility requirements. These constraints may be similar to those genetically defined factors enforcing host resistance to donor hematolymphoid transplants, even in irradiated hosts (16). However, despite these limitations, >40 successful T cell repopulations have been observed after $CD34^+$ cell transfer.

In animal models, experimental chimeras have been instrumental at elucidating the complex cell interactions involved in the ontogeny of the thymus (17). Defining the ability of potential progenitors to differentiate into T lymphocytes is an important step towards the characterization and purification of human hematopoietic stem cells, as well as in the study of positive selection (MHC restriction) and negative selection (self-tolerance) of developing human thymocytes from genotypically defined precursors in contact with genotypically defined thymic stroma. Also, this system may allow studies towards the elucidation of potential human bone marrow targets of pathogenic human lymphotropic viruses such as HIV, HTLV, and EBV.

We thank the following people from SyStemix for their contribution to this work: Nancy Mori and Carrie Garneau for expert technical assistance; Shelly Heimfeld, Yukoh Aihara, and Hideto Kaneshima for helpful discussions; Dennis Sasaki for assistance with the FACS®; the SyStemix animal facility for providing SCID, yet healthy, mice; and Nita Chapman for preparation of the manuscript.

Address correspondence to Bruno Péault, SyStemix, Inc., 3400 W. Bayshore Road, Palo Alto, CA 94303.

Received for publication 1 March 1991 and in revised form 5 August 1991.

References

1. Whitlock, C., K. Denis, D. Robertson, and O. Witte. 1985. In vitro analysis of murine B-cell development. *Annu. Rev. Immunol.* 3:213.
2. Toogood, I.R.G., T.M. Dexter, T.D. Allen, T. Suda, and L.G. Lajtha. 1980. The development of a liquid culture system for the growth of human bone marrow cultures. *Leuk. Res.* 4:449.
3. Ezine, S., I. Weissman, and R. Rouse. 1984. Bone marrow cells give rise to distinct clones within the thymus. *Nature (Lond.).* 309:629.
4. Goldschneider, I., K.L. Komschlies, and D.L. Greiner. 1986. Studies of thymocytopoiesis in rats and mice. I. Kinetics of appearance of thymocytes using a direct intrathymic adoptive transfer assay for thymocyte precursors. *J. Exp. Med.* 163:1.
5. Guidos, C.J., I.L. Weissman, and B.J. Adkins. 1989. Developmental potential of $CD4^-8^-$ thymocytes. Peripheral progeny include mature $CD4^-8^-$ T cells bearing $\alpha\ \beta$ T cell receptor. *J. Immunol.* 142:3773.
6. McCune, J.M., R. Namikawa, R. Kaneshima, L.D. Schultz, M. Lieberman, and I.L. Weissman. 1988. The SCID-hu mouse: murine model for the analysis of human hematolymphoid differentiation and function. *Science (Wash. DC).* 241:1632.
7. Civin, C.I., L.C. Strauss, C. Brovall, M.J. Fackler, J.F. Schwartz, and J.H. Shaper. 1984. Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells. *J. Immunol.* 133:157.
8. Andrews, R.G., J.W. Singer, and I.D. Bernstein. 1986. Monoclonal antibody 12-8 recognizes a 115 kd molecule present on both unipotent and multipotent colony-forming cells and their precursors. *Blood.* 67:842.
9. Lu, L., D. Walker, H.E. Broxmeyer, R. Hoffman, W. Hu, and E.J. Walker. 1987. Characterization of adult human marrow hematopoietic progenitors highly enriched by two-color cell sorting with MY-10 and major histocompatibility class II monoclonal antibodies. *J. Immunol.* 139:1823.
10. Brandt, J., N. Baird, L. Lu, E. Srour, and R.J. Hoffman. 1988. Characterization of a human hematopoietic progenitor cell capable of forming blast cell containing colonies in vitro. *Clin. Invest.* 82:1017.
11. Robinson, J.H., and R.K. Jordan. 1983. Thymus in vitro. *Immunol. Today.* 4:41.
12. Andrews, R.G., J.W. Singer, and I.D. Bernstein. 1989. Precursors of colony-forming cells in humans can be distinguished from colony-forming cells by expression of the CD33 and CD34 anitgens and light scatter properties. *J. Exp. Med.* 169:1721.
13. Haynes, B.F., M.E. Martin, H.H. Kay, and J.J. Kurtzberg. 1988. Early events in human T cell ontogeny. Phenotypic characterization and immunohistologic localization of T cell precursors in early human fetal tissues. *J. Exp. Med.* 168:1061.
14. Bertho, J.M., M.D. Mossalayi, A.H. Dalloul, G. Mouterde, and P. Debré. 1990. Isolation of an early T-cell precursor (CFU-TL) from human bone marrow. *Blood.* 75:1064.
15. Spangrude, G.J., J. Klein, S. Heimfeld, Y. Aihara, and I.L. Weissman. 1989. Two monoclonal antibodies identify thymic-repopulating cells in mouse bone marrow. *J. Immunol.* 142:425.
16. Cudkowickz, G., and J.H. Stimpfling. 1965. Hybrid resistance controlled by H-2 region: correction of data. *Science. (Wash. DC)* 147:1056.
17. Le Douarin, N.M. 1978. Ontogeny of hematopoietic organs studied in avian embryo interspecific chimeras. *In* Differentiation of Normal and Neoplastic Hematopoietic Cells. Cold Spring Harbor Laboratory. Cold Spring Harbor, NY. 5–31.