Evidence for Engraftment of Donor-Type Multipotent CD34+ Cells in a Patient With Selective T-Lymphocyte Reconstitution After Bone Marrow Transplantation for B⁻SCID

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Severe combined immunodeficiencies (SCID), a heterogeneous group of disorders of infancy, are fatal without treatment directed at immunologic reconstitution. Allogeneic bone marrow transplantation (BMT), which is such a treatment presents some unique features in SCID, especially when T-lymphocyte–depleted HLA haploidentical allografts are used. Donor-type T lymphopoiesis, less often B lymphopoiesis, develops, whereas myelopoiesis remains the recipient-type. Little is known about the engrafting cells in this peculiar lymphohematopoietic chimerism and the pathophysiology of the frequent failure of B-lymphocyte reconstitution. To address these issues, we purified CD34+ BM cells from a patient with selective T-lymphocyte reconstitution after HLA haploidentical BMT for B⁻SCID. Phenotypic analysis of CD34+ cells was performed by flow cytometry, and functional studies of donor- and recipient-type CD34+ cells were performed in vitro. Donor-type CD34+ cells, constituting ~2% of the CD34+ cells, were detected; both CD34+HLA-DR⁻ cells and CD34+ cells coexpressing B-(CD10 and CD19) and T-(CD2 and CD7) lymphocyte–associated cell surface molecules. Donor-type CD34+ cells coexpressing myeloid-associated molecules (CD13, CD14, CD15, and CD33) were undetectable. However, donor-type CD34+ myeloid progenitors could be shown in functional assays. Recipient-type CD34+ cells coexpressing B- and T-lymphocyte– as well as myeloid-associated molecules were detected, but recipient-type CD34+ cells could not be driven into T-lymphocyte differentiation in vitro. These findings provide evidence for engraftment of multipotent stem cells in our patient with B⁻SCID. Furthermore, the failure of B-lymphocyte reconstitution cannot be explained by lack of donor-type B-lymphocyte progenitors. Donor-type B lymphopoiesis and myelopoiesis are prevented by an unidentified mechanism.
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S EVERE COMBINED immunodeficiencies (SCID) form a heterogeneous group of genetic disorders of infancy characterized by profound deficits of both numbers and functional capacities of B and T lymphocytes. Without treatment directed at immunologic reconstitution, typical forms of SCID are fatal, usually within the first year of life.[1,2] A number of variants of the disorder can be distinguished, and the molecular basis of the immunologic abnormality has been resolved in several variants.[2]

Allogeneic bone marrow transplantation (BMT) offers a curative treatment for SCID. Because of the profound immunodeficiency and the failure to mount an effective alloresponse, BMT has traditionally been performed without cytoablative conditioning. Using HLA-identical family donors, complete immunologic reconstitution is usually the result, with long-term survival rates surpassing 90% in more recent series.[3-5] In the absence of an HLA-identical donor, BMT from mismatched donors such as haploidentical parents has become the treatment of choice. To prevent graft-versus-host disease (GVHD), these marrow grafts have been depleted of mature T lymphocytes.[6,7] The outcome of HLA-haploidentical, T-lymphocyte–depleted BMT has been less favorable, with a survival rate of 60%.[2] Reconstitution of T-lymphocyte immunity develops slowly and frequently may completely fail to develop. In sharp contrast to HLA-identical BMT, humoral immunity frequently remains deficient and the failure has been shown to be closely related to lack of mature donor-type B lymphocytes.[2,5,8] Accordingly, cytoablative conditioning is now advocated in HLA-nonidentical BMT for SCID.[2,9]

Allogeneic BMT without cytoablative conditioning for SCID presents a unique state of lymphohematopoietic chimerism with development of donor-type T lymphocytes and, less often, B lymphocytes, whereas post-BMT myelopoiesis remains of the recipient-type. It has been unclear whether this peculiar chimerism is also present at the stem cell level. We report here on an 11-year-old girl who received a paternal T-lymphocyte–depleted BMT without cytoablative conditioning at the age of 6 months for B⁻SCID, an autosomal SCID characterized by the absence of both B and T lymphocytes. A stable reconstitution of T-lymphocyte immunity was accomplished, but humoral immunity remained impaired with absence of circulating B lymphocytes and absence of detectable levels of self-sustained Igs. When investigated at the level of CD34+ cells 10 years post-BMT, we found evidence for BM engraftment of multipotent donor-type CD34+ cells. Interestingly, recipient-type CD34+ cells with phenotypic characteristics of B- and T-lymphocyte progenitors were demonstrable, consistent with a recently proposed pathophysiologic mechanism underlying the failure of lymphoid development in B⁻SCID.

MATERIALS AND METHODS

Patient characteristics. The patient was an 11-year-old girl. B⁻SCID was diagnosed at the age of 3 months using clinical and laboratory criteria defined by the World Health Organization classification.[10] The patient lacked B and T lymphocytes, but natural killer (NK) cells were present and functional. Adenosine deaminase and purine nucleotide phosphorylase levels were normal. There was no evidence of transplacental engraftment of maternal T lympho-

cytes.[11,12] Without cytoablative conditioning, the patient (HLA-A2,26; B38,51; DR6,7) received a T-lymphocyte–depleted[7] BM graft from her father (HLA-A26,26; B38,38; DR6,6) at the age of 6 months. She thrived normally without any signs of acute or chronic GVHD. T-lymphocyte immunity was reconstituted by donor-type cells,[11,13] but humoral immunity remained impaired with absence of circulating B lymphocytes and self-sustained serum Ig levels, necessitating regular Ig substitution.[11] Because the patient and her family were due to return to their native country, where regular Ig substitution cannot be guaranteed, a second BMT from the same donor after cytoablative conditioning was scheduled, aiming at complete immunologic reconstitution. While the patient was under general anaesthesia, autologous BM was harvested to serve as back-up in case of graft failure after the second BMT.

Cell preparation. BM (100 mL) from the patient was processed for in vitro studies together with peripheral blood from the recipient and donor. CD34+ cells were isolated with Dynabeads M-450 (Dynal, Oslo, Norway) coupled with a monoclonal antibody (MoAb) against CD34 (BI-3C5) from mononuclear cells prepared from the BM sample by density gradient centrifugation (1.077 g/mL; Lymphoprep; Nycomed Pharma, Oslo, Norway).[14,15] Host and donor peripheral blood mononuclear cells were prepared by density gradient centrifugation.

Antibodies. The following MoAbs were used for flow cytometry: fluorescein isothiocyanate (FITC)-labeled Leu-5b (CD2), Leu-4 (CD3), Leu-9 (CD7), anti-Calla (CD10), Leu-12 (CD19), Leu-16 (CD20), Leu-11a (CD16), Leu-M1 (CD15), anti-HPCA-2 (CD34), anti-HLA-DR, and anti-TCR-α/β-1 were purchased from Becton Dickinson Immunocytometry Systems (BDIS; San Jose, CA) and My-4 and My-9 were purchased from Coulter Immunology (Hialeah, FL). Phycoerythrin (PE)-labeled anti-HPCA-2, Leu-M9 (CD33), Leu-M7 (CD13), and Leu-19 (CD56) were purchased from BDIS. Simultest Control γ_1/γ_2(IgG1 FITC + IgG2a PE) was purchased from BDIS. Anti-CD3 (101.1) was obtained from Novo Research Institute (Copenhagen, Denmark). Anti-HLA-A2 (ITI-8G12) was developed in our own laboratory by G. Gaudernack. Fluoresceinated goat antimouse IgG1 and IgM polyclonal antibodies were purchased from Southern Biotechnology Associates, Inc (Birmingham, AL).

Flow cytometry and cell sorting. Cell staining was performed according to the manufacturer's instructions. Flow cytometry analyses were performed on a FACScan with the FACScan Research Software (BDIS). The degree of forward and orthogonal light scatter and the presence of two fluorescence signals were determined for each cell.

Cell sorting of immunomagnetically purified CD34+ BM cells was performed on an Epics Elite (Coulter Electronics, Hialeah, FL). A sort gate within a dual parameter cytogram of forward against orthogonal light scatter was drawn. A second and third amorphous gate was drawn on the two-color cytograms. Sort equations were set to positively sort cells satisfying both gates, ie, CD34+HLA-A2+ and CD34+HLA-A2− cells, respectively.

Culture assays. Cultures for T-lymphocyte differentiation were prepared as previously described.[16] In short, CD34+HLA-A2+ and CD34+HLA-A2− cells were plated on HLA-A2–mismatched adherent thymic stromal cells, 1,000 cells/well in 24-well tissue culture plates (Costar, Cambridge, MA) in 1 mL Iscove's modified Dulbecco's medium (IMDM; Bio Whittaker, Walkersville, MA) with 10% (vol/vol) fetal calf serum (FCS; GIBCO, Paisley, UK), glutamine (2 mmol/L; GIBCO), 10% (vol/vol) thymic stromal cell supernatant, c-*kit* ligand (2 U/mL; Genetics Institute, Cambridge, MA), penicillin (60 μg/mL), and streptomycin (100 μg/mL). Subsequently, the cultures were fed by carefully removing half the supernatant and replacing it with fresh supplemented medium twice weekly for 4 weeks. Nonadherent cells were harvested, enumerated, and phenotyped by flow cytometry.

Cultures for myeloid differentiation were prepared as described elsewhere.[17] In short, CD34+HLA-A2+ and CD34+HLA-A2− cells were plated in 200 μL IMDM with 10% FCS, glutamine (2 mmol/L), penicillin, streptomycin, c-*kit* ligand (2 U/mL), interleukin-3 (IL-3; 80 U/mL), and granulocyte-macrophage colony-stimulating factor (GM-CSF; 80 U/mL) at a concentration of 1,000 cells/well in 96-well U-bottomed tissue culture plates (Costar). Subsequently, the cultures were fed by removing half the supernatant and replacing it with fresh supplemented medium twice weekly for 3 and 4 weeks, respectively. The cells were harvested, enumerated, and analyzed by flow cytometry and light microscopy of May-Grünwald-Giemsa-stained cytospin slides.

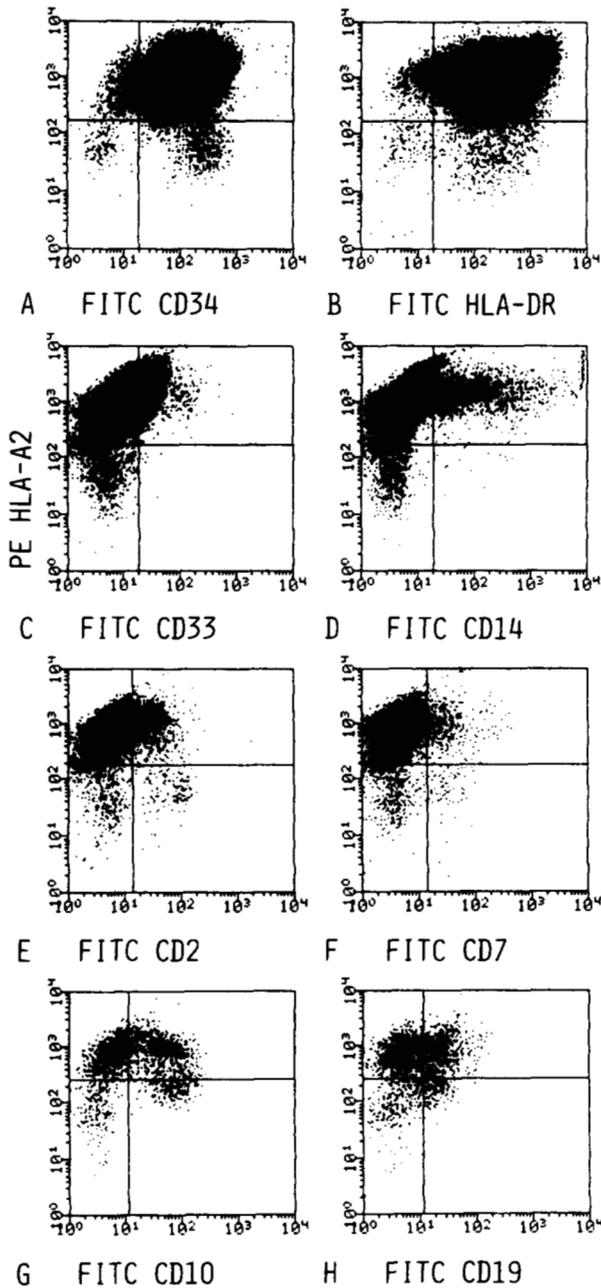All cultures were kept in a humidified atmosphere of 5% CO_2 in air at 37°C.

RESULTS

Selective restoration of functional T lymphocytes. Flow cytometric analysis of peripheral blood from the patient confirmed previous findings[11] of phenotypically normal donor-type T lymphocytes, no detectable B lymphocytes, and recipient-type NK and myeloid cells (data not shown). The donor-type T lymphocytes were functionally normal, as described in detail elsewhere.[11,13]

Characteristics of CD34+ cells. The immunomagnetically isolated BM cells contained more than 98% CD34+ cells. Purified CD34+ cells were analyzed by flow cytometry for expression of HLA-A2, HLA-DR, and lineage-associated cell surface molecules. Both recipient-(HLA-A2+) and donor-type (HLA-A2−) CD34+ cells were found (Fig 1A). The latter subset constituted ~2% of all CD34+ cells. There was no donor-unique allele that could be shown on the CD34+ cells, at least by flow cytometry. Accordingly, the donor-type cells were defined by the absence rather than the presence of a unique HLA allele, HLA-A2. To evaluate the validity of defining the origin of CD34+ cells by the presence or absence of expression of HLA class I molecules, a flow cytometric comparison of HLA-A2 expression on CD34+ cells from an HLA-A2+ and an HLA-A2− individual was undertaken. HLA-A2 was found to be expressed rather uniformly on CD34+ cells from the HLA-A2+ individual, whereas CD34+ cells from the HLA-A2− individual were shown to be HLA-A2− (Fig 2).

Recipient-type CD34+ cells were found to coexpress the myeloid-associated molecules CD13 (81%), CD14 (2%), CD15 (24%), and CD33 (83%); the B-lymphocyte–associated molecules CD10 (5%) and CD19 (3%); and T-lymphocyte–associated molecules CD2 (6%) and CD7 (2%) (Fig 1C through H). The proportions of recipient-type CD34+ cells expressing CD2 and CD7 were comparable to, whereas the proportions of recipient-type CD34+ cells expressing CD10 and CD19 were lower, than what we have reported in healthy adults.[18] Donor-type CD34+ cells expressing B-(~50%) and T-(~20%) lymphocyte–associated molecules were detectable (Fig 1E through H). Donor-type CD34+ cells expressing myeloid-associated molecules were undetectable (Fig 1C and D). Finally, donor-as well as recipient-type CD34+HLA-DR− cells were shown in low numbers (Fig 1B).

Myeloid cultures. Recipient- and donor-type CD34+ cells were cultured in liquid culture in the presence of c-*kit* ligand, GM-CSF, and IL-3. Recipient-type CD34+ cells

A FITC CD34   B FITC HLA-DR

C FITC CD33   D FITC CD14

E FITC CD2   F FITC CD7

G FITC CD10   H FITC CD19

Fig 1. Flow cytometric analysis of CD34⁺ BM cells obtained 10 years after HLA-haploidentical BMT for B⁻SCID. Purified CD34⁺ BM cells were stained with anti–HLA-A2 (IgM), followed by PE-labeled anti-IgM Ab and FITC-labeled anti-CD34, anti–HLA-DR, anti-CD33, anti-CD13, anti-CD14, anti-CD15, anti-CD2, anti-CD7, anti-CD10, and anti-CD19 MoAbs. The expression of HLA-A2 by CD34⁺ cells is shown in (A), by HLA-DR⁺ cells in (B), by CD33⁺ cells in (C), by CD14⁺ cells in (D), by CD2⁺ cells in (E), by CD7⁺ cells in (F), by CD10⁺ cells in (G), and by CD19⁺ cells in (H). The expression of HLA-A2 by CD13⁺ and CD15⁺ is not shown, but demonstrates absence of HLA-A2⁻CD13⁺ and HLA-A2⁻CD15⁺ cells. Cells stained with FITC- and PE-labeled irrelevant MoAbs served as negative control.
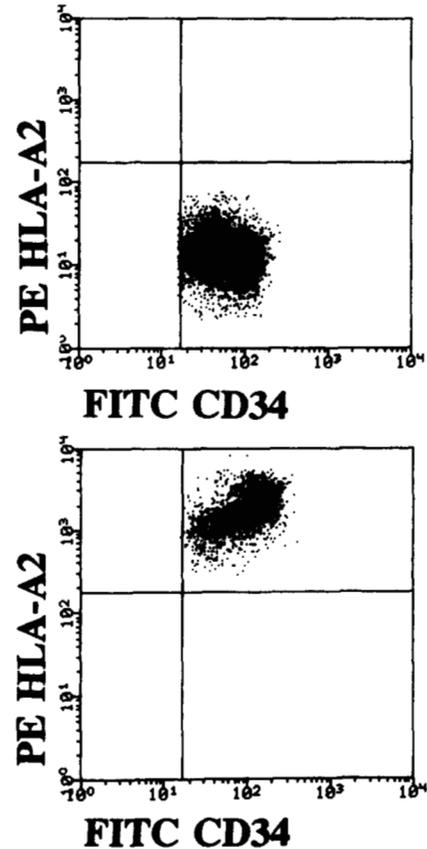
Fig 2. Flow cytometric analysis of expression of HLA class I cell surface molecules on CD34⁺. Purified CD34⁺ cells from an HLA-A2⁻ (upper cytogram) and an HLA-A2⁺ (lower cytogram) individual were stained with anti–HLA-A2 MoAb, followed by FITC-labeled anti-CD34 MoAb and PE-labeled anti-IgM Ab. Cells stained with FITC- and PE-labeled irrelevant MoAbs served as negative control.

showed a 700-fold increase in cell number in 3 weeks and gave rise to a morphologically (data not shown) and phenotypically myeloid progeny (Fig 3). Donor-type CD34⁺ cells proliferated less vigorously, showing a 20-fold increase in cell number in 4 weeks. Cells emerging after culture of donor-type CD34⁺ cells displayed the HLA-A2⁻ phenotype, and they were shown to be of the myeloid lineage by their expression of CD14 and CD15 (Fig 3). These markers as well as CD13 and CD33 were not detectable on donor-type CD34⁺ cells before culture. As shown, extensive in vitro culture, in the presence of the cytokines indicated, did not significantly modulate the expression of HLA-A2. All cells emerging after culture of recipient-type CD34⁺ cells expressed HLA-A2, whereas cells emerging in the cultures initiated by CD34⁺HLA-A2⁻ (donor-type) cells displayed the HLA-A2⁻ phenotype (Fig 3).

T-lymphocyte cultures. Recipient-type CD34⁺ cells were cultured on thymic stromal cells, proliferated well, and gave rise to a myeloid progeny without any detectable cells of the T-lymphoid lineage. Control cultures of CD34⁺ BM cells from healthy adults on thymic stromal cells prepared from the same thymus donor always generated mature T
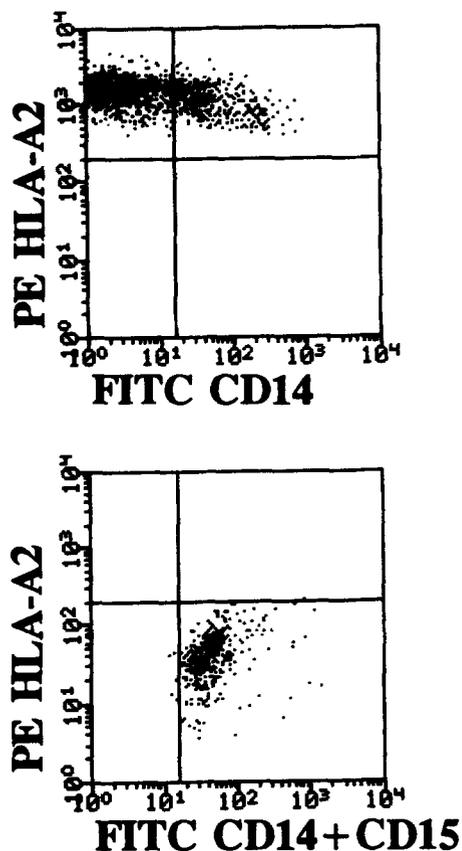
Fig 3. Flow cytometric analysis of cells recovered from myeloid cultures. CD34+ cells of recipient-type (upper cytogram) and donor-type were cultured in the presence of GM-CSF, IL-3, and c-kit ligand. Cells recovered from these cultures were stained with anti-HLA-A2 MoAb, followed by FITC-labeled anti-CD14 and anti-CD15 MoAbs and PE-labeled anti-IgM Ab. Cultured cells stained with FITC- and PE-labeled irrelevant MoAbs served as negative control.

lymphocytes in addition to myeloid cells.[16] Because of a lack of a sufficient number of HLA-A2⁻CD34+ cells, the T-lymphocyte differentiation assay was unsuccessful for donor-type CD34+ cells.

DISCUSSION

To our knowledge, this is the first time lymphohematopoietic chimerism at the level of CD34+ cells has been shown after BMT for SCID. The phenotypic and functional studies of purified CD34+ BM cells from an 11-year-old girl with selective T-lymphocyte reconstitution after HLA-haploidentical BMT for B⁻SCID presented in this report provide evidence for engraftment of donor-type CD34+ cells. The presence in the recipient 10 years after BMT of donor-type CD34+HLA-DR⁻ cells, of donor-type CD34+ cells expressing B- and T-lymphocyte–associated cell surface molecules, and of donor-type CD34+ cells capable of generating a myeloid progeny in vitro indicates that the cells responsible for engraftment were multipotent stem cells.

In this study, because of the lack of a donor-unique allele that could positively identify donor-type cells by flow cytom-

etry, donor-type cells were defined by the absence of the HLA-A2 allele. Hence, it may be argued that the CD34+HLA-A2⁻ cells detected and subsequently interpreted to be of donor-type, actually were recipient-type cells failing to express HLA-A2 cell surface molecules detectable by the methods applied. To address this issue, expression of HLA-A2, as detected by the anti–HLA-A2 MoAb ITI-8G12 in flow cytometry, were analyzed on CD34+ cells purified from an HLA-A2+ and an HLA-A2⁻ individual. We were unable to detect, even in low numbers, CD34+ cells from the HLA-A2+ individual that apparently failed to express HLA-A2, and CD34+ cells from the HLA-A2⁻ individual that apparently expressed HLA-A2 (Fig 2). Thus, we consider the detection of CD34+HLA-A2⁻ cells as valid evidence for the presence of donor-type CD34+ cells in our patient. Furthermore, we cite the demonstration of donor-type myeloid progenitors in functional assays as one piece of evidence for engraftment of multipotent stem cells. Once again, identifying cells as donor-type relies on the absence of the HLA-A2 allele. Expression of HLA-A2 cell surface molecules might be modulated by in vitro culture. However, when analyzing cells from cultures initiated by CD34+HLA-A2+ cells, we found HLA-A2 to be expressed uniformly on all cells (Fig 3). For this reason, we consider it unlikely that the HLA-A2⁻ cells recovered from the cultures initiated by CD34+HLA-A2⁻ cells (Fig 3) actually represented outgrowth of contaminating recipient-type cells failing to express detectable HLA-A2 after extensive in vitro culture.

The flow cytometric analysis disclosing the presence of donor-type B- and T-lymphocyte prprogenitors, but an apparent absence of donor-type myeloid progenitors in our patient, could indicate engraftment of common lymphoid progenitor cells.[19] However, the detection, by functional assays, of donor-type myeloid progenitors and the detection of donor-type CD34+HLA-DR⁻ cells strongly favors engraftment of multipotent stem cells to be the case in our patient, and that donor-type B lymphopoiesis and myelopoiesis are prevented in vivo. This finding is in contrast with the notion that, when selective T-lymphocyte reconstitution is the result of BMT for SCID, this results from direct migration of donor lymphohematopoietic stem cells to the thymus, where they differentiate into T lymphocytes.[2,20] The demonstration of donor-type CD34+ BM cells 10 years after BMT is incompatible with such an explanation being operative in our patient with B⁻SCID.

Our data indicating engraftment of multipotent lymphohematopoietic stem cells pose the question of how donor-type B-lymphoid and myeloid development is prevented. In our patient, ~2% of the CD34+ cells were of the donor-type. This finding might suggest that lack of mature donor-type B lymphocytes and myeloid cells were caused by inadequacy of "space" within the BM microenvironment[8] and/ or engraftment of a low number of CD34+ cells. However, neither of these possiblities could account for the difference in B-lymphocyte reconstitution observed in HLA-identical and -nonidentical BMT for SCID.[2] Although donor-type myeloid progenitors could be detected in functional assays, the lack of phenotypically defined[18] donor-type myeloid progenitors in the presence of phenotypically defined donor-type B-

and T-lymphocyte progenitors may indicate that different mechanisms are responsible for arresting donor-type myeloid and B-lymphoid development in our patient.

The pathophysiology underlying the failure of lymphoid development in B⁻SCID is not totally unraveled. However, recent experimental data indicate a functional defect in rearranging the genes of the antigen receptor variable regions.[21] This abnormality is analogous to the abnormality described in a mouse model of SCID[22-27] in which early B-lymphocyte precursors (pro-B cells) in the absence of more mature cells of the B-lymphoid lineage have been shown recently.[28] We show here that early B-and T-lymphocyte precursors can be found in human B⁻SCID. Furthermore, recipient-type CD34⁺ cells were unable to generate T lymphocytes in a "normal," in vitro thymic microenvironment. These findings are compatible with the notion that a functional defect in rearranging the genes encoding the antigen variable regions is responsible for the lack of mature B and T lymphocytes in B⁻SCID.

In conclusion, our results provide evidence for BM engraftment of multipotent stem cells in patients with selective reconstitution of T-lymphocyte immunity after BMT for B⁻SCID. The lack of mature donor-type B lymphocytes and myeloid cells cannot be accounted for by the lack of donor-type B-lymphoid and myeloid progenitors, but seems to be related to a yet unidentified mechanism preventing maturation along these lineages. The detection of recipient-type B- and T-lymphocyte progenitors is consistent with a pathophysiology in B⁻SCID attributed to defective rearrangement of gene segments coding for antigen receptors, resulting in abortion of early B and T lymphocytes during differentiation.

ACKNOWLEDGMENT

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