Phenotype and Function of Human Hematopoietic Cells Engrafting Immunodeficient CB17-Severe Combined Immunodeficiency Mice and Nonobese Diabetic-Severe Combined Immunodeficiency Mice After Transplantation of Human Cord Blood Mononuclear Cells

By Françoise Pflumio, Brigitte Izac, André Katz, Leonard D. Shultz, William Vainchenker, and Laure Coulombel

In an attempt to understand better the regulation of stem cell function in chimeric immunodeficient mice transplanted with human cells, and the filiation between progenitor cells identified in vitro and in vivo, we assessed the different compartments of hematopoietic progenitors found in the marrow of CB17-severe combined immunodeficiency (SCID) mice (34 mice, 9 experiments) after intravenous injection of 2 to 3 x 10⁷ cord blood mononuclear cells. On average 6.3 ± 4 × 10⁷ human cells were detected per four long bones 4 to 6 weeks after the transplant predominantly represented by granulomonocytic (CD11b⁺) and B lymphoid (CD19⁺) cells. Twenty five percent of these human cells expressed the CD34 antigen, of which 90% coexpressed the CD38 antigen and 50% the CD19 antigen. Functional assessment of primitive progenitor cells (both clonogenic and long-term culture-initiating cells [LTC-IC]) was performed after human CD34⁺ cells and CD34⁺/CD38⁻ cells have been sorted from chimeric CB17-SCID marrow 3 to 10 weeks after intravenous (IV) injection of human cells. The frequency of both colony-forming cells and LTC-IC was low (4% and 0.4%, respectively in the CD34⁺ fraction) when compared with the frequencies of cells with similar function in CD34⁺ cells from the startingcord blood mononuclear cells (26% ± 7% and 7.2% ± 5%, respectively). More surprisingly, the frequency of LTC-IC was also low in the human CD34⁺ CD38⁻ fraction sorted from chimeric mice. This observation might be partly accounted for by the expansion of the CD34⁺ CD19⁺ B-cell precursor compartment. Despite their decreased frequency and absolute numbers, the differentiation capability of these LTC-IC, assessed by their clonogenic progeny output after 5 weeks in coulture with murine stromal cells was intact when compared with that of input LTC-IC. Furthermore the ratio between clonogenic progenitor cells and LTC-IC was similar in severe combined immunodeficiency (SCID) mice studied 4 weeks after transplant and in adult marrow or cord blood suspensions. Results generated in experiments where nonobese diabetic (NOD)-SCID mice were used as recipients indicate a higher level of engraftment but no change in the distribution of clonogenic cells or LTC-IC. These results suggest that the hierarchy of hematopoietic differentiation classically defined in human hematopoeitic tissues can be reconstituted in immunodeficient SCID or NOD-SCID mice.

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existence in human hematopoietic tissues of cells with long-term reconstituting ability, but, in contrast to the mouse studies, almost nothing is known of the functional properties of human cells generating such progeny after their transplantation in immunodeficient mice. For example, it will be interesting to know whether these cells share some of the properties defined for LTC-IC identified in vitro, by analogy with a recent study showing the close relationship between mouse LTC-IC and totipotent murine cells with in vivo reconstituting ability (CRU). Establishment of in vivo methods for quantitating such human stem cells and dissecting their regulation will be needed to evaluate the reconstituting potential of cell suspensions used in human transplant procedures and to monitor the impact on this potential of manipulating these cells before their transplantation.

In this study, we characterized the different compartments of human progenitor and precursor cells detected in the marrow of CB17-severe combined immunodeficiency (SCID) and nonobese diabetic (NOD)-SCID mice transplanted with human cord blood mononuclear cells. This was done early (3 to 10 weeks) after transplantation to determine initial events occurring after migration of cells within the mouse marrow. We show that human engraftment was consistently detected, with a preferential amplification of granulomacrophagic and B lymphoid cells both at the level of differentiated and CD34+ cells. As a result, the frequencies of myeloid clonalogenic progenitor cells and LTC-IC were low, both in the CD34+ and the more immature CD34+/CD38− population, but the ratio of LTC-IC versus clonalogenic cells was 1/10, close to that observed in the input population. These results suggest that the hierarchy of hematopoietic differentiation classically defined in human hematopoietic tissues can be reconstituted in immunodeficient SCID or NOD-SCID mice. These data will serve as a basis for future experiments aimed at investigating whether selective amplification of these compartments can be modulated by regulatory molecules.

MATERIALS AND METHODS

Mice. Homozygous CB-17-scid/scid (hereafter called CB17-SCID) breeding pairs were originally obtained from Dr John Dick (Hospital for Sick Children, Toronto, Ontario, Canada). NOD-/-scid/scid (hereafter called NOD-SCID) were obtained from the Jackson Laboratory (Bar Harbor, ME) and from Dr J. Dick. Mice were housed in the animal facilities of the Institut Gustave Roussy under sterile conditions in air-filtered containers. CB17-SCID and NOD-SCID mice were given a sublethal dose of irradiation (3.5 Gy, cobalt-60 Eldorado S irradiator; AECL, Medical, Ontario, Canada) before human cell transplantation. We did not observe radiation-induced death of immunodeficient (ID) mice at this dosage. Mice were anesthetized briefly with ether during the intravenous (IV) injection of human hematopoietic cord blood mononuclear cells (CB-MNC; 0.5 to 3 × 106 cells/mouse) in the orbital vein.

Collection and fractionation of human CB-MNC. Cord blood samples from full-term newborns were collected upon informed consent of the mother at the Hospital Saint-Vincent de Paul (Paris, France). Samples were diluted 1:2 in phosphate-buffered saline (PBS) without magnesium (−Mg) and calcium (−Ca) before separation over Ficoll/Hypaque. All further manipulations of low-density CB-MNC were done in PBS-Mg-Ca except for the transplantation step where the cells were kept in α-minimal essential medium (MEM) supplemented with 10% fetal calf serum (FCS; Techgen, Leu lUs, France; α-MEM/10% FCS). Low-density CB-MNC were then either directly injected into ID mice or subjected to a standard CD34 immunomagnetic beads separation. The purity of selected cells was checked by flow cytometry and the proportion of CD34+ cells was consistently higher than 80% in the lymphocyte/blast cell window.

Harvest of marrow cell suspensions from transplanted CB17-SCID and NOD-SCID mice. Previous studies have shown that human cells preferentially seed the mouse bone marrow when transplanted in SCID mice. At indicated time points, transplanted ID mice were killed and bone marrow cells (BMC) were obtained by flushing the mouse femurs and tibiae with α-MEM/10% FCS. Nucleated cells were counted and used either for phenotypic analysis and/or assessment of hematopoietic progenitor cells. When purification of human CD34+ or CD34+/CD38− populations was performed, the cells were incubated overnight at 37°C in α-MEM/10% FCS and the CD34+ and/or CD34+/CD38− fractions were sorted the following day by cytofluorometry. Human cell engraftment was expressed as the absolute number of different cell types calculated by multiplying the total number of cells obtained from two femurs and two tibiae by the percent of human HLA-class I or CD45+ or CD34+ cells measured by flow cytometry.

Immunofluorescence and flow cytometry cell analysis and sorting. Cells were incubated with murine antihuman specific antibodies coupled to phycoerythrin (RPE) or fluorescein isothiocyanate (FITC) in 50 µl of α-MEM/10% FCS during 20 minutes, on ice. The panel of monoclonal antibodies (MoAbs) used included R-PE-Cy-5-conjugated anti-CD45 (Caltag, South San Francisco, CA) and FITC-conjugated MoAbs recognizing a polymorphic determinant on HLA-ABC (W6/32; Serotec, Besyon, France), CD34 (HPCA-2; Becton Dickinson, San Jose, CA), CD33 (WM-54; Dako, Trappes, France), CD11b (IOM1; Immunotech), CD7 (Pharmingen, San Diego, CA), CD15 (CD-1; Dako), PE-conjugated MoAbs included anti-CD34 (HPCA-2; Becton Dickinson), CD19 (Leu-12; Becton Dickinson), CD14 (IOM2; Immunotech) and glycophorin A (Glycophorin; Immunotech). Lack of cross reactivity of the mouse antihuman antibodies was tested by labeling marrow cells from untreated mice with the above MoAbs and background levels were measured using isotypic controls. Analysis was done on a FACS-Sort (Becton Dickinson). When two-color labeling was performed, compensation was set up with single stained samples. Low forward scatter elements (red cells or debris) were excluded from the analysis by gating them out and 10,000 events were collected and analyzed using the Cellquest software (Becton Dickinson). Sorting of CD34+, CD34+/CD38− and CD34+/CD38+ fractions was performed using a FACS-Vantage equipped with an argon ion laser (Innova 70-4; Coherent radiation, Palo Alto, CA) tuned to 488 nm and operating at 500 mw. A morphological gate including all the CD34+ cells was determined on two parameter histograms side scatter (SSC) versus forward scatter (FSC). Positivity or negativity for the CD34 among the CD34+ cells was determined using control cells labeled with the PE-HPCA2 and an irrelevant IgG1 MoAb. Compensation was set as described above. To initiate long-term cultures at limiting dilutions, CD34+/CD38− cells were directly sorted into 96-well tissue culture plates precoated with the appropriate feeder cells (MS5 or human bone marrow adherent cells) using an automatic cloning design unit (Becton Dickinson).

Human clonogenic progenitor assays. Cells (2.5 × 106 low-density CB-MNC, 103 CD34+ cells, 5 × 104 CD34+/CD38− cells, and 5 × 105 CD34+/CD38− cells) were plated in 0.8% methylcellulose in Iscove’s modified Dulbecco’s medium (purchased from Stem Cell Technologies, Vancouver, Canada) supplemented with 30% FCS, 1% deionized BSA (Stem Cell
Technologies), 10^{-4} mmol/L β-2 mercaptoethanol, 2 mmol/L L-glutamine. Optimal conditions for detecting human erythroid and granulocytic progenitors included rhuEPO (erythropoietin, 2 U/mL; kindly provided by AMGEN, Thousand Oaks, CA), rhuSCF (stem cell factor, 50 ng/mL; AMGEN), rhuIL-3 (100 U/mL) and rhu-G-CSF (granulocyte-colony stimulating factor, 10 ng/mL; AMGEN). These conditions were used when either low-density or sorted CD34+ CB-MNC populations were assayed. As rhuG-CSF also stimulates mouse cells, it was replaced by rhu-GM-CSF (granulocyte-macrophage colony-stimulating factor, 10 ng/mL, Immunex, Seattle, WA) in colony assays initiated with unseparated BMC from SCID mice. Scoring of colonies derived from CFU-E (colony-forming unit erythroid), mature BFU-E (burst-forming unit erythroid), immature BFU-E and CFU-GM (colony-forming unit granulocyte-macrophage) was performed using criteria previously described in detail.22

Long term cultures (LTC). Human CD34+ or CD34+CD38- flow cells sorted from CB-MNC before transplantation or from the marrow of transplanted mice were cultured at limiting dilution (1 to 10^3 cells/well) in 96-well plates precoated with unirradiated murine M55 cells in α-MEM supplemented with 12.5% FCS, 12.5% horse serum (HyClone, Logan UT) and 10^{-4} mmol/L β-mercaptoethanol as described by Croisille et al.23 In one experiment, LTC were done on 10 Gy-irradiated human BM feeders obtained by replating the adherent cells (15,000 cells/well) of standard 4 to 6 week-old long-term bone marrow cultures established as previously described by Coughlombel et al.23 Cultures were kept for 2 to 3 days at 37°C and subsequently transferred at 33°C, 5% CO2. After 5 to 6 weeks, the nonadherent and adherent cells from each well were harvested, pooled and plated in 1 mL of methylcellulose medium containing rhuSCF, rhuIL-3, rhuEPO, and rhu-G-CSF to evaluate the number of progeny clonogenic progenitors. A positive well was defined as a well in which at least one progenitor was detected. Linearity between the number of input cells and the proportion of negative wells was checked and the frequency of primitive progenitors generating clonogenic progenitors in LTC assays was calculated according to Poisson statistical analysis.24 An LTC-IC was defined in this study as a cell that gives rise to at least one clonogenic progenitor cell after 4 to 6 weeks in coculture on either human marrow-derived adherent cells or murine MS-5 cells.

RESULTS

A total of 11 separate experiments were performed where human cord blood mononuclear cells (CB-MNC) were injected into immune-deficient mice. Nine experiments used CB17-SCID recipients and three NOD-SCID recipients. In experiment 9, mononuclear cells from the same cord were injected into SCID and NOD-SCID recipients.

Levels of human cell engraftment in SCID and NOD-SCID mice following the transplantation of CB-MNC. In nine different experiments (2 to 9 mice/experiment, 9 separate samples), 0.5 to 3 × 10^7 low-density human mononuclear cells isolated from cord blood were injected intravenously into sublethally irradiated SCID mice (n = 39), and the level of human cell engraftment was measured in the bone marrow of the mice between 3 to 10 weeks posttransplant. To eliminate fluctuations in the numbers of total nucleated cells consequent to heterogeneous cell recovery in marrow cellularity a few weeks after irradiation, we expressed human cell engraftment as the absolute numbers of human cells per four long bones rather than as the proportion of total marrow cells.

In SCID mice injected with 2 × 10^7 cells (n = 34), the number of human cells (based on expression of HLA class I or CD45) recovered per four long bones varied between 0.3 and 27 × 10^6 with a mean (±standard deviation [SD]) of 6.3 ± 6 × 10^5 human cells 3 to 4 weeks after transplantation (Fig 1A). Results from two experiments where mice were analyzed at later time points (5 and 10 weeks posttransplant, compare open and closed symbols in exp 1 and 6, Fig 1A) showed that the numbers of human cells were equal to or higher than those observed early after transplant (7 ± 5 × 10^5 human cells/four long bones at 3 weeks v 22 ± 1 × 10^5 at week 5 in exp 1 and 8 ± 4 × 10^5 human cells/four long bones at week 4 v 15 ± 6 × 10^5 at 10 weeks in exp 6).

In three experiments, 13 NOD-SCID mice were injected with CB-MNC (exp 9b: three mice injected with 0.5 × 10^7 and three mice with 1 × 10^7 human CB-MNC; exp 10 and 11: seven mice injected with 2 × 10^7 human CB-MNC). In one experiment (exp 9), cells from the same cord blood were injected to five SCID mice (exp 9a) and six NOD-SCID (exp 9b). As shown in Fig 1, in this experiment, 10 times more human nucleated (Fig 1A) and CD34+ (Fig 1B) cells were recovered from NOD-SCID mice than from SCID mice. Of note, the cellularity of NOD-SCID marrow in this experiment was higher than that of SCID mice when measured early after transplantation, 21 ± 5 × 10^6 cells/four long bones in NOD-SCID mice versus 10 ± 5 × 10^6 cells/four long bones in SCID mice. This difference may be partly explained by the age and sex differences between the two strains of mice: 4-month old male NOD-SCID mice were used, but 2-month old female SCID. Higher recoveries of human nucleated cells in NOD-SCID mice has been confirmed in two additional experiments where 2-month-old NOD-SCID mice (five females and two males) were transplanted with 2 × 10^7 CB-MNC (Fig 1, exp 10 and 11). Although the levels of human cell engraftment achieved in both sets of mice were lower than those seen in exp 9b, the numbers of human cells (22 ± 4.6 × 10^5, range, 3 to 29 × 10^5) were significantly increased compared with those obtained in SCID mice engrafted with the same numbers of CB-MNC (Mann-Whitney Wilcoxon test P = .001) despite identical marrow cellularity. These results confirm recent findings published by different groups showing that NOD-SCID mice are better recipients than SCID mice.11,12,25

On average, 25% of human cord blood cells detected in SCID mice expressed the CD34 antigen (Table 1). Although the range of the percentages of CD34+ cells varied from 0.8 to 67, the variation in the proportions of CD34+ cells observed within each experiment was smaller. For example, in two different experiments, the proportion of CD34+ cells varied from 0.8% to 13% [0.8%, 4%, 6%, 12%, 13% in five recipients] and from 19% to 57% [19%, 27%, 34%, 43%, 57% in five recipients], respectively. Thus, on average 1.5 ± 1.8 × 10^5 CD34+ cells were found per four long bones in 34 mice (range, 0.1 × 10^5 to 7.5 × 10^5) (Fig 1B, Table 1). These numbers of CD34+ cells were almost equivalent to the numbers (2 ± 1.4 × 10^5) present in the input CB-MNC population injected at day 0. In view of the unknown seeding efficiency of human cells and taking into account the fact that four long bones represents 20% of the total
marrow mass in the mouse, it is most likely that a net increase in the absolute number of CD34+ cells has occurred in the mouse tissues. The proportions of CD34+ human cells were identical in NOD-SCID mice, but as expected from the above-described increase in nucleated cells, the absolute numbers of CD34+ cells recovered from NOD-SCID mice were higher than the absolute number of CD34+ cells recovered from SCID mice (Fig 1B).

**Phenotype of human cells engrafting the marrow of CB-17 SCID and NOD-SCID mice.** Cells recovered from transplanted SCID and NOD-SCID mice were stained with a panel of lineage-specific antibodies spanning the erythroid, granulocytic, B and T lymphoid lineage, and of antibodies segregating cells according to their maturation stage. Multilineage human cell engraftment occurred in every CB17-SCID and NOD-SCID mouse (Fig 2 and Table 1). Among HLA class I human cells recovered from SCID mice, CD11b+ granulomonocytic and CD19+ B lymphoid compartments were predominant representing 52% ± 22% and 50% ± 30% of human cells, respectively (Table 1). In contrast, human T cells (CD3+) were rare. The pattern was slightly different in NOD-SCID mice analyzed after CB-MNC engraftment in that a significantly lower proportion of CD11b+ cells (20%) and of CD19+ cells (28%) was observed. Human T cells were detected in one of three experiments with NOD-SCID recipients (3 of 4 mice had up to 60% CD3+ T cells), but this result almost certainly reflected unique properties of the cord blood sample because it has not been reproduced. Although our results suggest some differences in the proportions of human myeloid and B lymphoid populations engrafted in NOD-SCID versus SCID mice, additional experiments will be required to further document this trend.

A high proportion (25% and up to >50% in some experiments) of human cells present in the mouse marrow 4 to 6 weeks after transplant expressed the CD34 antigen, and most of those coexpressed CD38 (Fig 2E) as is commonly seen in adult human marrow. This pattern of expression of both CD34 and CD38 antigens was totally different from that observed on the input cells (10 CB-MNC analyzed). Most, if not all, input CD34+ CB-MNC uniformly expressed high levels of this antigen and intermediate levels of CD38 (Fig
were observed between individual mice (for example, 2.6%, progenitor cells engrafted into SCID and NOD-SCID mice. Indeed, very similar cloning efficiencies of engrafted human CD34+ cells were observed in each individual mouse BM. Indeed, very similar cloning efficiencies of engrafted human CD34+ cells were observed in SCID mice from one experiment, and 2.7%, 2.6%, 4.2% in three NOD-SCID mice injected with the same suspension), as well as between the different experimental groups, with a mean ± SD of 3.2% ± 2% (n = 19).

Progenitor cells were distributed equally between CFU-GM and BFU-E (47% and 53%, respectively), and iBFU-E represented 10% of the total BFU-E compartment. These proportions were close to those observed in normal adult marrow. Lack of amplification of the Epo-dependent CFU-E most likely reflected inefficient murine Epo concentrations. Similar cloning efficiencies and progenitors repartition were observed in the marrow of NOD-SCID mice engrafted with CB-MNC indicating that all compartments of the hematopoietic system were equally increased (Table 2) without preferential amplification of earlier cells.

In a second series of experiments, to eliminate potential interactions with murine cells, we purified human CD34+ cells and CD34+CD38- low cells from reconstituted mice and initiated colony-assays and LTC (see next section) with these sorted cells. The cloning efficiency of output human CD34+ cells was low (4% ± 1%, n = 7 experiments) as compared with that of input CD34+ CB-MNC (26% ± 7%, n = 6 experiments) (P < .001, Student’s t-test) and similar in SCID and NOD-SCID recipients (Table 3). These low cloning efficiencies were not surprising considering results from colony assays performed with unsorted cells, and the amplification of the CD34+CD19- B lymphoid compartment. The cloning efficiency of human CD34+CD38- low cells sorted from engrafted mice and plated in methylcellulose in presence of human cytokines and the murine feeder cell line MS5 was 5% to 9% and characteristic large colonies were observed. Of note, in both colony-assays initiated with either CD34+ or CD34+CD38- low cells sorted from transplanted mice, the proportion of erythroid progenitors was significantly lower than that observed in similar colony-assays performed with CD34+ fractions before transplantation (22% ± 10% vs 42% ± 16% for CD34+ progenitors and 16% ± 5% for CD34+/CD38- low cells (.05 > P > .02, Student’s t-test).

Quantification of LTC-IC present in human CD34+ and CD34+CD38- low cells purified before and after transplantation.

Table 1. Phenotype of Human Hematopoietic Cells Engrafted Into the Marrow of Immunodeficient CB17-SCID and NOD-SCID Mice Following Transplantation of Human CB-MNC

<table>
<thead>
<tr>
<th></th>
<th>% of Human HLA Class I+ or CD45+ Cells</th>
<th>% of Human CD34+ Cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>CD3+</td>
<td>CD19+</td>
</tr>
<tr>
<td>CB17-SCID</td>
<td>Mean ± SD</td>
<td>Range</td>
</tr>
<tr>
<td></td>
<td>0.5 ± 1</td>
<td>(0-2.5)</td>
</tr>
<tr>
<td>NOD-SCID</td>
<td>14 ± 3</td>
<td>(9-61)</td>
</tr>
</tbody>
</table>

CB17-SCID mice (2 to 11 experiments) were injected with 0.5 to 3 × 10⁷ human CB-MNC and analyzed 3 to 10 weeks after transplant. NOD-SCID mice (3 experiments) were injected with 0.5 to 2 × 10⁷ cells and analyzed 4 to 8 weeks after transplant. Numbers represent the mean ± SD of the proportion of either HLA class I, CD45+ (left panel) or CD34+ (right panel) cells positive for the indicated marker in all mice analyzed.

Abbreviations: ND, not done; GPA, glycophorin A; n, number of mice analyzed.
Fig 2. Phenotypic analysis of human hematopoietic cells engrafting bone marrow of CB17-SCID mice following transplantation of human CBMNC. Mice were injected intravenously with $1 \times 10^7$ (A, B, C, and E) or $2 \times 10^7$ (D, F, G, and H) human CB-MNC and their bone marrow cells were analyzed 3 to 4 weeks after transplantation. Light-density input CB-MNC (I) and cells from transplanted mice (A through H) were stained with human specific MoAbs and 10,000 events were acquired on a FACS-sort apparatus as described in the Materials and Methods. The results are expressed as the percent of total marrow cells (including both mouse and human cells) that were positive for the indicated marker. Positive gates were defined from results obtained in samples labeled with nonimmune isotype-matched IgG controls. Plots A, B, C, and E were obtained from analysis of cells from the same mouse; F and H and D and G each from a different mouse.

Table 2. Human Clonogenic Progenitors Detected in the Marrow of CB17-SCID and NOD-SCID Mice Following Transplantation of Human CB-MNC

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Recipients</th>
<th>No.*</th>
<th>Progenitor Cells/10^5 Plated Cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>CFU-E</td>
</tr>
<tr>
<td>1</td>
<td>CB17-SCID</td>
<td>3</td>
<td>36 ± 40</td>
</tr>
<tr>
<td>2</td>
<td>CB17-SCID</td>
<td>4</td>
<td>11 ± 10</td>
</tr>
<tr>
<td>3</td>
<td>CB17-SCID</td>
<td>4</td>
<td>7 ± 7</td>
</tr>
<tr>
<td>4</td>
<td>CB17-SCID</td>
<td>3</td>
<td>13 ± 11</td>
</tr>
<tr>
<td>5a</td>
<td>CB17-SCID</td>
<td>4</td>
<td>0.5 ± 0.7</td>
</tr>
<tr>
<td>5b</td>
<td>NOD-SCID</td>
<td>3</td>
<td>36 ± 8</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td>3</td>
<td>14 ± 11</td>
</tr>
</tbody>
</table>

Mice were transplanted with $3 \times 10^7$ (exp 1), $2 \times 10^7$ (exp 2, 3, 4, 6), and $0.5 \times 10^7$ (exp 5a and 5b) CB-MNC and were killed 3 to 4 weeks after transplantation. Unfractionated bone marrow cells from chimeric mice were plated in semisolid colony assays in conditions specific for the growth of human progenitor cells. Cultures were established in duplicate. Numbers refer to the mean ± SD of colonies observed in assays initiated with marrow cells from the number of mice indicated.

* Number of mice.
Human CD34+ cells were purified by flow cytometry from CB-MNC (input cells) and from the bone marrow of transplanted CB17-SCID (exp 1 through 4) and NOD-SCID (exp 5 and 6) mice 3 to 10 weeks after transplantation (output cells). Sorted cells were either plated in semisolid colony assays and/or during 5 to 6 weeks in limiting dilutions long-term coculture assays on murine stromal cells (Table 3). The frequency of LTC-IC was calculated using Poisson statistics.

Abbreviations: ND, not done.

* Average number of clonogenic progenitors produced per LTC-IC.

**Table 3. Frequency of Clonogenic Progenitor Cells and of LTC-IC in Purified Human Cord Blood CD34+ Cells Before (Input) and After (Output) Transplantation in CB17-SCID and NOD-SCID Mice**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Clonogenic Progenitor Frequency</th>
<th>LTC-IC Frequency</th>
<th>Clonogenic Progenitors/ LTC-IC</th>
<th>Weeks Posttransplant</th>
<th>Clonogenic Progenitor Frequency</th>
<th>LTC-IC Frequency</th>
<th>Clonogenic Progenitors/ LTC-IC</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1/5</td>
<td>1/40</td>
<td>28</td>
<td>ND</td>
<td>ND</td>
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<td>ND</td>
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<td>ND</td>
<td>1/8</td>
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<td>3</td>
<td>1/16</td>
<td>1/350</td>
<td>40</td>
</tr>
<tr>
<td>3</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4</td>
<td>1/28</td>
<td>1/350</td>
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<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>10</td>
<td>1/28</td>
<td>1/280</td>
<td>25</td>
</tr>
<tr>
<td>5</td>
<td>1/3</td>
<td>1/15</td>
<td>38</td>
<td>3</td>
<td>1/38</td>
<td>1/125</td>
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<tr>
<td>6</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>4</td>
<td>1/37</td>
<td>1/500</td>
<td>10</td>
</tr>
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**DISCUSSION**

Evidence has been recently provided that human cells intravenously injected into immunodeficient mice colonize the marrow cavities and function during several months in the mouse environment.7,13,14 However, the biological properties of the subset of input cells responsible for engraftment have not been defined, and the different compartments of progenitor cells detected after engraftment have rarely been quantified. In this study, we characterized the phenotype and functions of human cells present in the marrow of both SCID and NOD-SCID mice intravenously injected with human mononuclear cells from cord blood. We precisely quantitated not only mature cells, but also both clonogenic progenitor cells (detected in colony assays) and a more immature compartment of LTC-IC (detected in coculture assays on murine stromal cells) in the marrow of recipient mice 3 to 10 weeks after transplantation. The analysis was performed early after transplantation to guarantee development of all progenitor cells that would have seeded the marrow. This was predicted from previous in vitro studies from this12,22 and other laboratories11,27,28 showing that clonogenic and LTC-IC progenitors survive and differentiate in 4 weeks in a murine environment. However, at later time...
development, early progenitor cells might be compromised in the absence of human cytokines.

Frequencies and absolute numbers of both clonogenic progenitors and LTC-ICs were low in the CD34+ and CD34+CD38−low fractions when compared with frequencies of these cells in the starting CD34+ CB-MNC fraction or from data published for adult marrow cells of equivalent phenotype.12,24,29,30 A possible explanation is that progenitors were diluted by the amplification of the CD34+CD19+ B lymphoid compartment, which represented usually over 50% of CD34+ cells. However, this does not account for the low frequency of LTC-ICs in the CD34+CD38−low fraction (<1/200) unless a subfraction of nonmyeloid primitive cells is also amplified in this fraction, which will have to be determined by performing a more detailed phenotypic analysis of the transplanted cells. As LTC-IC were barely detectable in the CD38+ fraction, there was no argument for a discrepancy between phenotype and function in the transplanted mice.

Nevertheless, comparison of the numbers of clonogenic progenitors and LTC-ICs showed some interesting features: first, the ratio of LTC-IC versus clonogenic progenitors in the engrafted mouse, either by comparing frequencies (on average 1 of 30 clonogenic and 1 of 300 LTC-IC in the CD34+ fraction) or absolute numbers (200 to 500 LTC-IC and 2,000 to 5,000 clonogenic cells per four long bones), was 1 of 10. This ratio was similar in the starting cord blood CD34+ population and suggests that from a functional point of view, the initial steps of hematopoiesis proceed harmoniously up to the progenitor stage in the chimeric SCID mice engrafted with human cord blood cells. This was not totally unexpected considering previous results from this and other laboratories that clearly showed that adult marrow and cord blood LTC-IC can develop in a murine environment in the absence of an exogenous source of human cytokines31,32 and confirms and extends a preliminary report on the presence of CD34+ Thy1+ LTC-IC in the marrow of SCID mice engrafted with human bone marrow cells in the absence of human cytokines.19

Second, the differentiation capability of CD34+ or CD34+CD38−low LTC-IC present in the marrow of reconstituted mice, measured by the production of clonogenic progenitors in the 5-week coculture assay on MS-5, was similar (20 to 30 clonogenic progenitors per LTC-IC) to that of LTC-IC in the starting cord blood and close to previously published data for LTC-IC from human marrow when cultured in similar conditions, ie, with the same feeder cells.13

Even though the progenitor output was very heterogeneous, which has been previously reported,24 40% of the wells initiated with 1 LTC-IC can be estimated to contain more than 10 clonogenic progenitors and 10% more than 100 in our culture conditions, when cells are cocultured on the MS-5 murine stromal cells. Whether or not these high proliferative-potential LTC-IC were present in the input cells and survived in the mouse marrow or were generated by a more primitive ancestor is as yet unknown, but might be elucidated by studying the turnover of these cells by cell cycle analysis. In any case, this observation clearly suggests that very primitive progenitor cells with the potential to produce myeloid cells for extended periods of time might engraft in these chimeric mice. Even though absolute numbers of total LTC-IC probably decline during the graft period, this does not exclude that a subpopulation of these, in fact, proliferated and contributed to the output after transplantation as has been recently shown in vitro.70

Finally, it should be mentioned that these numbers were certainly underestimated because all calculations were performed for four long bones, which represents 20% of the total marrow mass and because the seeding efficiency of human cells in SCID mouse tissues is unknown.

If the LTC-IC progenitor cell transition seems preserved, this was not true downstream, and amplification of the more
mature compartments was compromised. This was obvious by the decrease in CFU-E or GPA+ cells, but was also indicated by an abnormally high proportion of CD34+ cells (25% in SCID and 30% in NOD-SCID) within human nucleated cells. This observation was anticipated because many murine cytokines do not cross the barrier species. Although human cytokines might be secreted endogenously by some CB-MNC injected in the mouse, as recently documented by measuring human cytokine levels in the serum of mice transplanted with mobilized peripheral blood cells,13 the levels and combinations might not be appropriate for an optimum development of mature myeloid human cells. However, a striking feature was the amplification of the B-cell compartment, both CD34+CD19+ and CD34+CD19+ cells at least in the CB17-SCID mice.

Predominance of human B CD19+ cells in SCID mice reconstituted with fetal tissues, but not adult tissues,7,10 has been previously reported independent of the transplant procedure, intravenous injection of donor cells,13,15 or micoinjection in a human fetal bone shaft.6 Our phenotypic analysis of CD34+ cells present after 4 weeks in the SCID marrow extend these data and underlines the predominance of a CD34+CD19+ population that did not preexist in initial cord blood samples, as less than 1% of CD34+ cells coexpress CD19 in our experiment, and some studies have reported that cells with this phenotype were lacking.4 Further work will be required to find out whether such a preferential amplification of B precursor cells is a property inherent to cord blood cells or is facilitated by the relative depletion of myeloid cells in a murine environment, an observation which has been largely confirmed in vitro where conditions for optimal B and myeloid development are exclusive.26,32 The genetic background on the recipient mice might also influence the phenotype of engrafted cells as suggested by our finding of a lower proportion of CD19+ cells in the NOD-SCID recipients. However, because the variability in the proportion of cells with different phenotypes was high between experiments, this observation will have to be confirmed in more recipients. Our first experiments clearly confirm that conclusions raised with data generated in SCID mice will also apply to NOD-SCID mice. This is important as NOD-SCID recipients will most likely replace CB17-SCID mice in reconstitution experiments, as they favor a much higher level of engraftment19,20 and will facilitate experiments aimed at determining the reconstitution potential of purified cells only available in small numbers.

Finally, if the ability of this mouse model to discriminate qualitative patterns of hematopoiesis has been proven,25 the high variability encountered in the recoveries of nucleated human cells (Fig 1A) and the distribution of phenotypes (Table 1) may question its sensitivity to evaluate quantitative alterations in hematopoietic development. Importantly, however, dispersion was seen essentially in the compartment of mature cells and not in the CD34+ cell compartment, and the frequencies of colony-forming cells and LTC-IC within the CD34+ progenitor compartment were very reproducible between experiments. Although this variability was undoubtedly partly donor-dependent, it was also probably accounted for in our study by the compromised conditions for terminal differentiation of human precursors and will improve in mice given adequate concentrations of human cytokines. Further improvement will also come from the establishment in this chimeric model of quantitative assays equivalent to the CRU (competitive repopulating unit) assay used to quantify murine cells capable of reconstituting and sustaining long-term hematopoiesis in vivo.94 Despite these limitations inherent to any assay using human cells, the same hierarchy of progenitors that has been defined from in vitro studies was consistently reproduced in the immunodeficient mouse. This has important implications and will help to get more insights into the characterization of regulatory molecules such as cytokines or stromal-derived activities regulating in vivo the function of early progenitor cells.

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Phenotype and function of human hematopoietic cells engrafting immune-deficient CB17-severe combined immunodeficiency mice and nonobese diabetic-severe combined immunodeficiency mice after transplantation of human cord blood mononuclear cells

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