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Relatively Normal Human Lymphopoiesis but Rapid Turnover of Newly Formed B Cells in Transplanted Nonobese Diabetic/SCID Mice

Maria Isabel D. Rossi, 2* Kay L. Medina, 2* Karla Garrett, * Grant Kolar, † Phillip C. Comp, ‡ Leonard D. Shultz, § J. Donald Capra, † Patrick Wilson, † Arthur Schipul, ¶ and Paul W. Kincade 3*

Human B lineage lymphocyte precursors in chimeric nonobese diabetic/SCID mice transplanted with umbilical cord blood cells were directly compared with those present in normal bone marrow. All precursor subsets were represented and in nearly normal proportions. Cell cycle activity and population dynamics were investigated by staining for the Ki-67 nuclear Ag as well as by incorporation experiments using 5-bromo-2′-deoxyuridine. Again, this revealed that human B lymphopoiesis in chimeras parallels that in normal marrow with respect to replication and progression through the lineage. Moreover, sequencing of Ig gene rearrangement products showed that a diverse repertoire of V_H genes was utilized by the newly formed lymphocytes but there was no evidence for somatic hypermutation. The newly formed B cells frequently acquired the CD5 Ag and had a short life span in the periphery. Thus, all molecular requirements for normal B lymphocyte formation are present in nonobese diabetic/SCID mice, but additional factors are needed for recruitment of B cells into a fully mature, long-lived pool. The model can now be exploited to learn about species restricted and conserved environmental cues for human B lymphocyte production. The Journal of Immunology, 2001, 167: 3033–3042.

Many questions remain about molecular mechanisms responsible for formation and maintenance of the humoral immune system and it is unclear whether all findings made in experimental animals can be extrapolated to humans. Gene targeting experiments and natural mutations suggest that IL-7 is more important for B cell formation in mice than it is in humans (1–4). On the other hand, consequences of mutations in the Btk tyrosine kinase gene are more severe for humans than for mice (5, 6). Observations of that kind provide impetus for animal models that would efficiently support formation of human B cells from hematopoietic stem cells and nonobese diabetic (NOD) 5/SCID mice are extremely interesting in that context (7–9). Many laboratories have shown that B cells are formed in these animals from transplanted human stem cells (8, 10–12), but it remains unclear how closely this engraftment reflects normal steady-state conditions. For example, it remains uncertain if a stable equilibrium is attained where a pool of quiescent donor stem cells is established, if these stem cells continuously generate early lymphocyte precursors, and if the precursors progress through a normal sequence of proliferation and differentiation steps. Alternatively, human B cells might only be transiently produced as a wave of differentiation from the engrafted precursors. Various checkpoints and quality control events have been identified from animal studies, but we do not know whether they pertain to this model. For example, human lymphocytes might survive in chimeric mice without regard for the presence and/or specificity of Ag receptors, whereas this is a rigorously controlled process under normal circumstances. Finally, no information is available about the diversity of B cells in this model and all could result from clonal expansion of a small number of precursors.

A thorough understanding and validation of the NOD/SCID chimera model should be invaluable for many purposes. A common environment would be very useful for testing the differentiation potential of stem cells from fetal, adult, and aged marrow sources. Stem cells in some strains of mice undergo age-related changes and this important issue begs study with respect to human stem cells (13). Molecules within bone marrow and spleen serve as positive and negative regulators for the production, maintenance, and function of B cells. Some that await identification might be effectively studied in the NOD/SCID model on the basis of species specificity, by genetic manipulation, or by use of neutralizing Abs. By exploitation of techniques developed for animal studies, new insight should be obtained into the kinetics of production and turnover of human B cells.

Experimental animal studies suggest that B lymphopoiesis occurring during fetal life may differ in important ways from that which occurs in adult marrow. A different repertoire of Ab variable region genes is used and the B cells that express them differ from adult B cells in other respects (14, 15). The more limited information available suggests this may also be the case for human B cells (16), but we have little idea of its basis in either species.

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4 Abbreviations used in this paper: NOD, nonobese diabetic; APC, allophycocyanin; BrdU, 5-bromo-2′-deoxyuridine; EBF, early B cell factor; s, surface.
Again, it would be ideal to compare the fate of stem cells from embryonic and adult sources in a common environment. B cells in animals and humans are known to be heterogeneous in other ways. Some of this diversity may result from functional specialization and it has even been proposed that there are separate lineages of B cell differentiation (17). Alternatively, heterogeneity of B cells may result from encounters with Ag and participation in immune responses (18–20).

Our study was designed to learn whether the normal stages of human B lymphopoiesis are represented in chimeric NOD/SCID mice and that indeed appears to be the case. Precursor-product ratios compared favorably to those present in fetal and adult marrow samples, suggesting a relatively normal progression through this sequence. Furthermore, typical patterns of Ig gene rearrangements were documented. We conclude that the model provides exceptional opportunities for investigation of human B cell formation. The short survival time of newly formed B cells raises questions about requirements for their maintenance in peripheral lymphoid tissues.

Materials and Methods

Animals

NOD/LtSz-scid/scid (NOD/SCID) mice were obtained from a breeding colony established at the Laboratory Animal Facility at the Oklahoma Medical Research Foundation (Oklahoma City, OK) from breeding pairs kindly provided by Dr. L. D. Schultz (The Jackson Laboratory, Bar Harbor, ME). Animals were housed in a restricted barrier facility maintained in Medical Research Foundation (Oklahoma City, OK) from breeding pairs colony established at the Laboratory Animal Facility at the Oklahoma Cellgro, Herndon, VA). The mononuclear cell fraction from cord blood from Southern Biotechnology Associates (Birmingham, AL). The biotin-conjugated Abs for flow cytometry were provided by Dr. F. Finkelman (University of Cincinnati Medical Center, Cincinnati, OH). The M25 Ab that neutralizes murine and human IL-7 was generously provided by Zupo et al. (22). To reveal BrdU, after fixation and permeabilization, the cells were incubated in 1 ml of 0.15 M NaCl saline containing 4.2 mM MgCl2, 10 μM HCl, and 100 U of DNase (Sigma) for 30 min at 25°C. The cells were washed twice with PBS containing 3% FBS and 0.01% sodium azide with the staining buffer, and incubated for 15 min on ice with the second-step reagent. Streptavidin Red 613 was used to reveal the biotinylated Abs. The cells were then prepared for intracellular staining with fixation by 1% paraformaldehyde in PBS and permeabilized with 70% ethanol at −20°C for >30 min and washed twice with the staining buffer. The cells were incubated with anti-human IgM Ab for 30 min at room temperature and then stained with mouse Ig (polyclonal) before proceeding to the manufacturer’s instructions. To establish the proliferative fraction, cells were incubated with the Ab anti-ki-67 (mib-1) for 1 h on ice, as described by Zupo et al. (22). To reveal BrdU, after fixation and permeabilization, the cells were incubated in 1 ml of 0.15 M NaCl saline containing 4.2 mM MgCl2, 10 μM HCl, and 100 U of DNase (Sigma) for 30 min at 25°C. The cells were washed twice with PBS contain-

BrdU administration

BrdU (Sigma) was administered to mice in the drinking water at a concentration of 1 mg/ml for different periods of time. This dose was based on previous studies (21). The water bottle containing the BrdU solution was protected from light and changed every 2–3 days throughout the time course of the experiment. Alternatively, in short-term experiments, BrdU was injected i.p. (1 mg/mouse) in PBS before the addition of BrdU to the drinking water.

Intracellular and surface staining for FACS analysis

Four-color immunofluorescence analysis was used for the identification of the different B cell precursor populations. Single-cell suspensions from bone marrow, spleen, and the peritoneal cavity were pooled for 10 min on ice with unconjugated mouse Ig or mouse serum. The cells were incubated for 15–30 min on ice with directly conjugated or biotinylated Abs specific for human cell surface Ags. Cells were washed twice with PBS containing 3% FBS and 0.01% sodium azide (staining buffer), and incubated for 15 min on ice with the second-step reagent. Streptavidin Red 613 was used to reveal the biotinylated Abs. The cells were then prepared for intracellular staining with fixation by 1% paraformaldehyde in PBS and permeabilized with 70% ethanol at −20°C for >30 min and washed twice with the staining buffer. The cells were incubated with anti-human IgM Ab for 30 min at room temperature and then stained with mouse Ig (polyclonal) before proceeding to the manufacturer’s instructions. To establish the proliferative fraction, cells were incubated with the Ab anti-ki-67 (mib-1) for 1 h on ice, as described by Zupo et al. (22). To reveal BrdU, after fixation and permeabilization, the cells were incubated in 1 ml of 0.15 M NaCl saline containing 4.2 mM MgCl2, 10 μM HCl, and 100 U of DNase (Sigma) for 30 min at 25°C. The cells were washed twice with PBS contain-

Cell sorting

Bone marrow and spleen suspensions recovered from transplanted NOD/SCID mice were incubated with anti-human CD5-PE and anti-human IgM-APC. Alternatively, bone marrow cell suspensions were stained with anti-human CD5-FITC, anti-human CD3-PET, and anti-human IgM-APC. Single CD19+ IgM+ or IgM+ CD5− or IgM− CD5+ cells were sorted into each 96-well PCR plate using a MoFlo (Cytometry, Fort Collins, CO) cell sorter with an automatic cell deposition unit. Bulk sorting of human CD34+ hematopoietic cell subsets were conducted after depletion of mouse lineage- positive cells from NOD/SCID mouse bone marrow. Cells were incubated with purified mAbs to Mac-1/1M7/70, Gr-1/1G6; B220/RA-3, and Ter119 (FACSCANTO) mouse IgM (BD Pharmingen) before proceeding to the manufacturer’s instructions. To establish the proliferative fraction, cells were incubated with the Ab anti-ki-67 (mib-1) for 1 h on ice, as described by Zupo et al. (22). To reveal BrdU, after fixation and permeabilization, the cells were incubated in 1 ml of 0.15 M NaCl saline containing 4.2 mM MgCl2, 10 μM HCl, and 100 U of DNase (Sigma) for 30 min at 25°C. The cells were washed twice with PBS contain-

RT-PCR analyses of lymphoid genes

Sorted cells were put in TRIzol reagent (Life Technologies) and total RNA was extracted following the manufacturer’s instructions. Total RNA or mRNA was treated with DNase I to remove genomic DNA. RT-PCR analyses of lymphoid genes were performed using oligo(dT) and Moloney murine leukemia virus reverse transcriptase (Life Technologies) following standard protocols. Semiquantitative PCR was done to measure relative differences in transcript levels of target cDNAs against levels of the reference gene GAPDH. The PCR was done in 100 μl containing 1× PCR buffer (Takara Biomedicals, Osaka, Japan), 1.5 mM MgCl2, 200 μM dATP, dGTP, and dTTP, 100 μM dCTP, and 50 μM of each primer. Samples were overlaid with mineral oil. For quantification, 0.5 μl of [α-32P]dCTP (Amersham, Arlington Heights, IL) was included in each reaction tube. Samples were denatured in a DNA thermocycler (PerkinElmer, Norwalk, CT) for 10 min at 95°C. To increase specificity, 2.5 U of Taq DNA polymerase (Takara Biomedicals) was added to each sample during this initial denaturation. Samples were then cycled for 1 min at 94°C, 1-min annealing at 60°C, and 1-min extension at 72°C. Aliquots were removed at cycles 25, 28, 31, and 34 for GAPDH and cycles 32, 35, and 38 for all others to ensure that PCR remained within the exponential range of amplification. Aliquots (5 μl) were denatured in a formamide loading

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buffer and applied to a 6% polyacrylamide gel containing 7 M urea. Incorporation of $[^{35}S]$dCTP into PCR product bands was quantified from dry gels using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA). Primers were as follows: GAPDH sense: 5’-TCTCAGAGGCAGTCTGGTC-3’; 475-bp expected product, recombinase-activating gene (RAG) sense: 5’-CCTAGAGCTTTAGCTGCTAGAG-3’; RAG1 antisense: 5’-AGGCGG CTAATGCTAGCTTACTCTC-3’; 681-bp product, RAG2 sense: 5’-CATGTTTAGG CTCTTCCAGGCT-3’; 422-bp product, 5’-CCCGGCCTAC CGTCAGGCC-3’; EBF antisense: 5’-TGACTGAGACGACGAGGAGT ACTAGT-3’; 621-bp product, early B cell factor (EBF) sense: 5’-CCGGGC TCACCTTGGAGAACCGAC-3’; EBF antisense: 5’-CAGGGATGCAT GTTCCCCAGT-3’; 638-bp product.

**Single-cell PCR for V$	extsubscript{H}$ gene analysis**

Single cells were directly deposited into 5 $\mu$L of an alkaline lysing solution (200 mM KOH/50 mM DTT) in 96-well plates and subsequently neutralized with 5 $\mu$L of neutralization solution (900 mM Tris-HCl (pH 9.0), 300 mM KCl, and 200 mM HCl) (24). Genomic amplification was performed using 2 $\mu$L of a random 15-mer primer, 400 $\mu$L of dNTP Mix (Roche, Basel, Switzerland), 5 U of Taq DNA polymerase (Promega, Madison, WI), 1.5 mM MgCl$_2$, $5x$ Taq polymerase buffer (final concentration 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100; Promega) in a final volume of 25 $\mu$L. This mixture was subjected to the cycling conditions described in Ref. 24. Five microliters of this material was then added to an initial gene-specific reaction mix containing 100 $\mu$L of dNTP mix, 5 U of Taq DNA polymerase, 1.5 mM MgCl$_2$, $5x$ Taq polymerase buffer (final concentration 50 mM KCl, 10 mM Tris-HCl (pH 9.0), and 0.1% Triton X-100), and 500 ng of each internal primer (24) sealed in wax. Cycling conditions included an initial 4-min incubation at 95°C followed by 1 cycle of 1 min at 94°C, 1 min at the lowest primer annealing temperature, and 1 min at 72°C. Thirty-nine subsequent cycles were performed where incubation at the primer annealing temperature was decreased by 30 s and a final extension incubation was performed for 10 min at 72°C. A second gene-specific PCR using 10 $\mu$L of the product of the first gene-specific PCR was performed using the same reaction mix added to an internal set of PCR primers (24) sealed in wax. Cycling conditions were performed as described above for the first gene-specific PCR amplification.

**Cloning and sequencing of Ig V$	extsubscript{H}$A genes**

More than 2000 cells were collected for each lymphocyte population and mRNA was isolated using an oligo(dT) system (Ambion). Reverse transcription was performed using the Superscript III (Invitrogen, San Diego, CA) containing 500 ng of each cDNA-dilution-PCR product (8) was performed with the same V$	extsubscript{H}$A primer set used for the single-cell PCR experiments detailed above and a $\mu$H chain constant region-specific primer (5’-CTGCAGGCATTGAGGAC-3’). Reaction conditions were the same as described above for the first gene-specific, single-cell analyses except that total cycles were reduced to 25 rounds of amplification. PCR products were agarose gel purified (Qiagen, Valencia, CA) and transformed into E. coli using a PCR-Blunt cloning kit (Invitrogen, San Diego, CA). Successful transformations were identified by blue-white screening and picked for overnight cultures from which plasmid DNA was prepared (QIAprep Spin Miniprep kit; Qiagen) and sequenced on an Applied Biosystems 377 fluorescence automated DNA sequencer (Applied Biosystems, Foster City, CA). Sequences were identified using the DNAPLOT search components of the VBASE (http://www.mrc-cpe.cam.ac.uk/imt-doc/, coordinated by I. M. Tomlinson, Medical Research Council Biosystems, Foster City, CA). Sequences were identified using the VBASE (http://www.mrc-cpe.cam.ac.uk/imt-doc/, coordinated by I. M. Tomlinson, Medical Research Council Biosystems, Foster City, CA).

**Results**

**Engraftment and normal differentiation of human cells in NOD/SCID bone marrow**

It has been previously shown that engraftment of SCID and NOD/SCID mice requires conditioning treatment (25, 26). Our preliminary experiments revealed that survival and human cell chimerism were optimal with a low dose (100 cGy) of $^{137}$Cs irradiation, and we confirmed that the majority of cells belonged to the B lymphocyte lineage (8, 11, 12, 27). Human CD45$^+$ cells represented 39.4 $\pm$ 17.6% of total bone marrow cells in these animals and 76.7 $\pm$ 7.1% of them expressed CD19. In contrast, we found that an average of 5.9 $\pm$ 3.9% of the CD45$^+$ cells in normal adult marrow specimens was CD19$^+$. Preferential development of B lineage cells was also apparent at the CD34$^+$ stage where 57.7 $\pm$ 7.0% of the human population expressed CD19. Additional analysis revealed that 79.8 $\pm$ 6.9% were TdT$^+$, 12.2 $\pm$ 6.9% were TdT$^+$ CD10$^-$ CD19$^-$, and 6.6 $\pm$ 2.4% were TdT$^+$ CD10$^+$ CD19$^+$. The importance of IL-7 to the development of human lymphocytes was assessed by injection of the M25 monoclonal Ab capable of neutralizing both murine and human cytokines. Human CD45$^+$ cells represented 41.8 $\pm$ 13.5% of total bone marrow cells in treated animals and 76.3 $\pm$ 7% of them were also CD19$^+$.

Human B lymphopoiesis has been described in terms of sequential gain and loss of differentiation markers, and we used flow cytometry to resolve various subsets in the chimeric bone marrow. Expression of TdT is an early lymphoid lineage milestone and TdT$^+$ cells were readily detected among the early CD34$^+$ CD10$^+$ CD19$^-$ fraction (Fig. 1, A and B). CD34$^+$ CD10$^+$ CD19$^+$ cells that are potential common lymphoid progenitors (28) represented another easily resolved category (Fig. 1, A and B), and RT-PCR analysis of sorted marrow cells (Fig. 1, E–G) showed that four lymphocyte-associated genes were markedly up-regulated at that stage (Fig. 1D). As with their counterparts in normal human marrow (Refs. 29 and 30 and data not shown), the CD34$^+$ TdT$^+$ CD19$^+$ pro-B cells uniformly displayed CD10 (Fig. 1B).

Pre-B cells in chimeric bone marrow were resolved as the cytoplasmic $\mu$ H chain$^+$ surface $\kappa$ or $\lambda$ fraction of CD19$^+$ lymphocytes and further subdivided according to size (Fig. 2). It can also be seen that most, but not all of the pre-B cells had down-regulated CD34. The final steps in B lymphopoiesis are marked by acquisition of Ig L chains, followed by surface (s) IgD at the immature and naive B cell stages. Although both of these human lymphocyte subsets were present in chimeric mice, slgM$^+$ slgD$^-$ CD24$^+$ CD38$^-$ memory B cells that recirculate through normal adult marrow (31) were conspicuously absent (Fig. 3A). The B cells in chimeric bones closely resembled those normally present in fetal marrow with respect to CD24 density along with display of CD38, CD10, and CD43 Ags (Fig. 3A and data not shown). In addition, they were uniformly small (data not shown). Small pre-B cells were overrepresented among the CD19$^+$ fraction of donor cells as compared with those normally found in fetal and adult human bone marrow (Fig. 3B). However, this difference was not significantly different. Otherwise, ratios between B lineage lymphoid compartments were remarkably like those in normal human marrow. This represents the first direct comparison of human lymphopoiesis in the animal model to normal fetal and adult human marrow. We conclude that human hematopoietic cells preferentially expand and differentiate along the B lymphocyte lineage in NOD/SCID mice. Although small pre-B cells are elevated and memory B cells are absent, the entire series of human B lineage differentiation is otherwise represented in near-normal proportions.

**Acquisition of CD5 by human B cells in chimeric animals**

As expected from the marrow analysis, chimeric mice lacked a fully mature B cell population. Expression of TdT is an early lymphoid lineage milestone and TdT$^+$ cells were readily detected among the early CD34$^+$ CD10$^+$ CD19$^-$ fraction (Fig. 1, A and B). CD34$^+$ CD10$^+$ CD19$^+$ cells that are potential common lymphoid progenitors (28) represented another easily resolved category (Fig. 1, A and B), and RT-PCR analysis of sorted marrow cells (Fig. 1, E–G) showed that four lymphocyte-associated genes were markedly up-regulated at that stage (Fig. 1D). As with their counterparts in normal human marrow (Refs. 29 and 30 and data not shown), the CD34$^+$ TdT$^+$ CD19$^+$ pro-B cells uniformly displayed CD10 (Fig. 1B).

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cells in NOD/SCID mice also express the CD5 Ag. We found that B cells in chimeric bones closely resembled those in human fetal marrow in this respect and that 70.8 ± 10.5% of the B cells in spleen were CD5⁺ (Fig. 4). CD5⁺ B cells were also recovered from the peritoneal cavity of the mice, and particularly when the extent of chimerism was high (data not shown). CD5⁺ B cells are conspicuous in normal umbilical cord blood and we compared them to B cells that arose in transplanted mice (Fig. 4, E–H). Although CD5⁺ B cells from both sources were CD23⁻ and CD11b⁻ (data not shown), those recovered from mice expressed more CD10 and CD43 Ags, and densities of sIgD were lower. Thus, impressive numbers of human B cells are produced in NOD/SCID mice and they resemble normal neonatal B cells in some, but not all respects.

Proliferation of human lympho-hemopoietic cells in chimeric marrow

Until now, the extensive replication required for blood cell formation could only be satisfactorily investigated in experimental animals. Moreover, it was difficult to fully appreciate population dynamics in individual samples of human bone marrow. Proliferation and turnover of lymphocyte populations have been extensively studied in mice and rats following BrdU administration (32–35). Therefore, human pre-B cells in chimeric marrow were discriminated by the presence of cytoplasmic μ H chains and absence of surface Ig L chains before simultaneous analysis of BrdU incorporation and presence of the Ki-67 Ag (Fig. 5A). There was remarkably close agreement between these two indices of proliferation. That is, all of the large pre-B and 61% of the small pre-B cells were mitotically active. The agreement between the two methods made it possible to directly compare hemopoietic cell expansion in transplanted animals to that in freshly obtained normal specimens (Fig. 5B). Proliferation of pro-B and pre-B cell compartments most closely resembled that in adult human marrow.

The experiments were then expanded to include all stages of lymphopoiesis. Hemopoietic stem cell activity in normal human bone marrow is known to be associated with cells that are CD34⁺CD38⁻low (36). Most of these cells in human fetal and adult bone marrow as well as those in cord blood are quiescent (36, 37). As others have found (8, 10), very small numbers (0.5 ± 0.5%) of human CD34⁺ cells in transplanted mice are CD38⁻low (Fig. 6A, left panel). In striking contrast to the situation in adult and even fetal human bone marrow, most of these cells in chimeric mice appeared to be actively proliferating when examined 7–10 wk after transplantation (Fig. 6A, middle panel). We separately analyzed CD34⁺ cells that totally lacked CD38 and ones that had a very low density of this marker (Fig. 6A, middle and right panels). Approximately 70% in each of these subsets expressed the Ki-67 nuclear Ag associated with proliferating cells. Similar results were obtained when animals were observed 20 wk after transplantation. Since most of the human CD34⁺ cells in NOD/SCID mice expressed CD19 and these pro-B cells are rapidly cycling...
BrdU incorporation was analyzed in CD34/H11001 cells that lack CD19. Virtually all CD34/H11001 CD19/H11002 cells were labeled after 1 wk of continuous administration (Fig. 6B). These findings demonstrate that while typical patterns of lymphocyte precursor proliferation are seen in transplanted NOD/SCID mice, a pool of quiescent CD34/H11001 CD38/H11002 cells normally found in human bone marrow is not established.

**Turnover of human lymphoid cells in NOD/SCID mice**

We then administered BrdU to NOD/SCID mice with transplants to study population dynamics for human lymphoid cells. As might be expected from the Ki-67 staining described above, large pre-B cells had the fastest turnover and half were labeled in 3 h (Fig. 7A). This would be compatible with a very high mitotic index and short cell cycle time. Large pre-B cells are thought to derive from the cycling pro-B cell compartment and we found that pro-B cells were also rapidly labeled. We determined a 50% renewal time of 54 h for small pre-B cells and 105 h for sIgM/H11001 B cells. Thus, there was an interval of 18 h between the last proliferating compartment and newly formed B cells. Although culture studies and patterns of tumor marker expression have previously suggested a probable sequence of differentiation, these findings represent the first kinetic analysis of human B lymphopoiesis in a marrow environment.

These findings are compatible with the results of animal studies in that the acquisition of sIg occurs without further cell division as small pre-B become B cells within bone marrow (38). Only 3.6 ± 1.9% of human B cells in the chimeric spleens expressed even low levels of Ki-67, suggesting there was little if any replication in that site. However, we found progressively increasing BrdU incorporation for cells recovered from the spleen (Fig. 7B). Indeed, the data were best described with a straight line ($r^2 = 0.97$), indicating that peripheral cells were homogeneous and relatively short-lived. The calculated 50% renewal time for these B cells was between 7 and 8 days. Thus, although NOD/SCID marrow supports extensive production of human B cells, the newly formed lymphocytes fail to enter a long-lived pool.

**Ig V H gene utilization in chimeric mice**

All of the above findings suggest that human B lymphocytes are actively produced in chimeric NOD/SCID mice, but do not exclude the possibility of oligoclonal expansion. Furthermore, our understanding of human CD5/H11001 B cells is incomplete. Therefore, we used molecular techniques to investigate the status of the Ig V H gene.
rearrangements in these lymphocytes. Single-cell PCR was conducted as an initial screen to determine whether a normal fraction of Ig gene rearrangements utilized the V<sub>H</sub>4 family. The 34% frequency we obtained is typical for B cells in normal umbilical cord blood (39). Therefore, we focused our analysis on the distribution of rearrangements involving this well-characterized V<sub>H</sub> family. Using a single set of primers, Ig transcripts were cloned and sequenced from pooled bone marrow and spleen cells (see Materials and Methods). A total of 247 separate and distinct V<sub>H</sub> fragments were sequenced, easily identified, and assigned to one of the known V<sub>H</sub>4 genes or to a less frequently used gene, V<sub>H</sub>4-30-4. As shown in Fig. 8, a normally diverse population of B lymphocytes was produced in these chimeric animals. Indeed, the range of 27–33% observed for V<sub>H</sub>4-34 is consistent with previous analyses of freshly isolated cord blood B cells (39). Utilization of three specific gene segments, V<sub>H</sub>4-34, V<sub>H</sub>4-28, and V<sub>H</sub>4-30-4 was remarkably constant across the five lymphocyte populations analyzed. Use of V<sub>H</sub>4-39 decreased with the exit of B cells from the marrow. There was an interesting trend for increased utilization of V<sub>H</sub>4-59 and, to a lesser extent, V<sub>H</sub>4-04 with CD5 acquisition. A reciprocal pattern, i.e., reduced usage by CD5<sup>+</sup> B cells, was found for V<sub>H</sub>4-61. As might be expected from the absence of T lymphocytes in these animals, we found no evidence for somatic hypermutation in these V<sub>H</sub>4 genes.

These rearrangement products were further analyzed with respect to J<sub>H</sub> gene segment utilization and HCDR3 lengths. The J<sub>H</sub>4 segment is most commonly used by normal fetal (40) and cord blood B cells (41), as was the case for 58% of the lymphocytes analyzed from transplanted mice. The average of the mean HCDR3 lengths for the population was 15.2 ± 0.7 codons and falls within limits previously described for cord blood and term infants (42, 43). Each junction represented a unique sequence and there was clear evidence for N nucleotide insertion, i.e., TdT activity. Thus, these data show that NOD/SCID bone marrow supports all aspects of human Ig gene rearrangement, generating a normally diverse population of B cells.

Discussion

The NOD/SCID model is now being extensively used as a functional assay for human stem cells. However, it also has great potential for understanding the normal steps, environmental requirements, and kinetics of B lymphopoiesis. The aim of this initial study was to determine how closely the model reflects the events that occur within normal fetal and adult bone marrow. We will conclude that the entire differentiation series from stem cells to immature B cells is represented. Relative numbers of cells at each stage and proliferative expansion compare favorably to that within normal human marrow. Moreover, a diverse population of Ag receptor genes is utilized. However, the newly formed B cells do not appear to enter the long-lived pool.

Cord blood cells are becoming widely used and are uniquely effective for clinical transplantation (44). Although it has been shown that a quiescent CD34<sup>+</sup>CD38<sup>−</sup> subset of umbilical cord blood actually engrafts NOD/SCID mice, undefined accessory cells improve the efficiency (7, 8, 10, 45). In preliminary experiments, we confirmed that was also the case with our transplantation protocol and observed no chimerism in mice injected with the CD34<sup>+</sup> fraction of cord blood cells. Stem cells from this source are much more effective in this experimental model than ones harvested from either fetal or adult marrow (Refs. 26, 46, and 47 and our unpublished observations). For all of these reasons, the entire mononuclear fraction of cord blood was used in this initial study of human B lymphopoiesis.

As others have found with this model (8, 11, 12, 27), human hematopoietic cells preferentially expand within the B lymphocyte lineage. However, this is the first detailed investigation of all stages of B lymphopoiesis with a direct comparison to the replication and differentiation events that occur within normal marrow. Stem cells with long-term repopulating potential are thought to be nonreplicating and part of the CD34<sup>+</sup>CD38<sup>−</sup> fractions of human marrow or cord blood (7, 10, 48). Furthermore, this nonproliferating fraction contains the SCID mouse repopulating cells (49, 50). Although donor type cells with this phenotype were readily identified in murine bone marrow, they appeared not to be quiescent (Fig. 6). The time required for transplanted stem cells to exit the cell cycle in a human marrow environment is not known and it is therefore unclear whether our findings reflect species differences in control over stem cell replication. However, the inability to establish a quiescent stem cell pool could account for the finding that chimerism eventually declines in transplanted NOD/SCID mice and an exhaustion of human stem cell activity is thought to occur in these animals (26, 50, 51). TGF-β, LIF, and macrophage-inflammatory protein 1α are among factors found to induce and/or promote hematopoietic cell quiescence (52, 53). Since TGF-β is conserved between mice and humans, some other species-specific factor may be limiting in the recipient animals. Although not within the scope of our study, the NOD/SCID model might be manipulated to learn more about mechanisms that control stem cell quiescence and self-renewal.

Commitment to the B lymphocyte pathway is a gradual process and early precursors may express genes associated with other blood cell lineages (54, 55). TdT is an extremely useful marker for early precursors may express genes associated with other blood cell lineages (54, 55). TdT is an extremely useful marker for early lymphocyte precursors in grafted mice closely resembles that in normal adult marrow and Ki-67 staining provides a valid assessment. Cells expressing human CD34 and CD19 Ags were gated in bone marrow (BM) harvested 7–10 wk after transplantation and resolved into pre-B and pre-B cells as shown in Fig. 2. A. Flow cytometry of BrdU<sup>+</sup> pre-B cells after 3 days of BrdU treatment is shown in comparison to Ki-67 staining to evaluate the latter as an index of proliferation. B. Ki-67<sup>+</sup> cells in chimeras are compared with those in freshly isolated normal marrow specimens. Data represent the mean results from the analysis of 18 animals ± SD. FSC, Forward scatter.
have the potential for differentiation in T, B, NK, and dendritic lineages, but reduced ability to generate myeloid progenitors. Although a sharp boundary for commitment to lymphoid lineages has not been identified, EBF and Pax5 are essential transcription factors and the latter effectively suppresses alternative differentiation fates (55). The RAG proteins are required for Ig gene recombination and it has been previously shown that D-J rearrangement products are first detectable at the CD34+CD10+CD19+ stage (57, 58). Therefore, the NOD/SCID chimera model could facilitate high-resolution analysis of the earliest categories of human lymphocyte precursors and investigation of relationships between them.

Although there are many means to assess lymphopoietic activity in animal models, methods for evaluation of human marrow are limited. The chimeric mouse system provides an opportunity to develop and validate procedures that are useful for human studies. In this study, we directly compared BrdU incorporation and Ki-67 staining as indices for proliferative activity and found good agreement between the two approaches. This is important in showing that analysis of single human specimens can be generalized. This
concordance also made it possible for us to compare mitotic activity in chimeric mice to that within freshly isolated fetal and adult marrow. Percentages of Ki-67+ human cells among most B lineage subsets were remarkably similar in the three environments. This accords with our finding of similar sized populations of early and late stage B lineage precursors and presumably means that human B lymphopoiesis is normal in NOD/SCID marrow. An increased proportion of small pre-B cells in fetal bone marrow expressed Ki67, suggesting that the final stages of B lymphopoiesis are associated with greater proliferation than during adult life. Ig L chain gene rearrangement, receptor editing, and selection of the B cell repertoire all occur during this critical stage and fetal vs adult differences in cellular expansion merit further study.

A continuous BrdU-labeling protocol was used to assess population dynamics in chimeric bone marrow and spleens. Slightly more rapid incorporation of BrdU into large pre-B cells than pro-B cells would be consistent with a short cycle time, and 50% of the large pre-B cells were BrdU+ in just 3 h. Animal studies indicate that a large fraction of these cells would be in S + G2 + M phases of the cell cycle at any moment in time (59, 60). Small nondividing pre-B and B cells are sequentially spawned from these replicating precursors with 50% turnover times of 54 and 105 h, respectively. We found an 18-h interval between the last dividing cells and acquisition of sIgM. This compares favorably with a value of 12 h reported for rats (35). Pulse-chase protocols could be used to obtain more detailed information about the timing and sequence of progression through the B lymphocyte lineage in bone marrow.

This NOD/SCID system provides an opportunity to experimentally manipulate the bone marrow environment and learn the molecular requirements for maintaining such early lymphocyte precursor populations. We injected engrafted NOD/SCID mice with a neutralizing Ab to IL-7 and found no influence on human B lymphopoiesis. This is consistent with other findings indicating that IL-7 is much less important for B lymphocyte formation in humans than it is in mice (1, 2, 4). Furthermore, administration of IL-7 did not improve B cell production in NOD/SCID mice and along with Flt3 ligand actually inhibited emergence of human B cell formation (27). This model might be exploited to learn what other cytokine(s) sustains the survival, proliferation, and differentiation of human lymphocyte precursors.

Whereas <20% of the B cells in normal human blood express CD5 (61, 62), most of the B cells recovered from transplanted mice had this marker. The origin of CD5+ B cells has been extensively studied in mice and remains controversial (17, 62). According to one model, CD5+ (B1) B cells arise via an independent differentiation pathway during embryonic life and coexist with conventional (B2) B cells that are produced within bone marrow (17). Many human fetal B cells express CD5 and this characteristic is conceivably intrinsic to cord blood stem cells. However, in preliminary studies, CD5+ human B cells were also formed in NOD/SCID mice transplanted with adult bone marrow stem cells. This is consistent with findings that CD5+ B cells predominate in the early phase of B cell regeneration after human bone marrow transplantation (63, 64). The CD5+ B cells that arose in chimeric NOD/SCID marrow differed from those in fresh cord blood samples with respect to CD10 and CD43 Ags (Fig. 5B).

Display of CD5 on B cells has also been said to result from the specificity and density of surface Ag receptors (19, 20). That is, low-level recognition of self-Ags in the absence of T cell help may cause any B lymphocytes to acquire CD5 (18, 62). There are virtually no T cells in the transplanted mice and no information was available about the specificity of B cell Ag receptors. For those and other reasons, it was important to determine which Ig V \_H gene families are expressed in the NOD/SCID model.

Molecular analysis of the V \_H transcripts provided several important insights into the model. First, the single-cell PCR studies, although limited, clearly showed that percentages of V \_H4-bearing cells were similar to many previous reports of fetal (40) and cord blood repertoires (39). That is, the transplanted cells used the full range of the V \_H repertoire. This led to a more in-depth study of the V \_H4 family.

Analysis of these transcripts resulted in five major conclusions. 1) The utilization of the individual V \_H4 gene segments was approximately the same as has been reported extensively elsewhere for both fetal (40) and cord blood (39) human B-lymphocytes. 2) Human B cells utilized the V \_H4-39 gene segment less frequently after exiting the bone marrow. Similarly, upon acquisition of the CD5 marker, the lymphocytes increased their utilization of V \_H4-59 and, to a lesser extent, V \_H4-40. Reciprocally, the use of V \_H4-61 decreased upon acquisition of CD5. These observations suggest that the lymphocyte population in these chimeric mice is in a dynamic state, with cells being selected at certain points. 3) HCDR3 lengths as well as the presence of N nucleotides are similar to those described for human cord blood and term infants (40, 42, 43). This shows that the TiIB expressed by lymphocyte precursors was functional in the murine environment. 4) \_Ig utilization was also typical of that seen in fetal and cord blood (40, 41). 5) As might be predicted by the absence of T cells in this model, there was no evidence of somatic hypermutation.

Interestingly, V \_H4-34 was consistently used at the same ratio to other gene segments by all of the lymphocyte populations studied. This member of the V \_H4 gene family is extensively used for B cell Ag receptors with specificity for RBC and B lymphocyte carbohydrate Ags (65–70). Ongoing studies will reveal whether similar patterns develop when mice are transplanted with adult human stem cells and this information may bear on the issue of what drives CD5 expression. All of these observations indicate that human lymphocyte progenitors rearrange and express Ig genes to generate immature B cells with a diverse repertoire of Ag receptors. The unique CDR3s obtained for each sequence rule out the possibility of paucipal expansion of small numbers of human B cells. The results accord with the continuous production of large
numbers of dividing lymphocytes from actively proliferating precursors.

A linear rate of BrdU incorporation into spleen B cells was one of the most striking findings of this study. Ki-67 staining revealed almost no dividing cells in that site. Therefore there must be continuous replacement by B cells from the bone marrow. This accords with the immaturity of human B cells with respect to CD24 density, presence of CD38, and display of CD10. Such newly formed lymphocytes normally represent a small fraction of rodent spleens, and a majority of the B cells are long-lived (21, 23, 32).

There is little information about the normal fate and life span of CD5+ B cells in humans and most of the lymphocytes in chimeric mice expressed this marker. However, peritoneal CD5+ B cells in mice are exceptionally long-lived (72). Furthermore, CD5 expression is common among chronic lymphocytic leukemias and the presence of somatic hypermutations in their Ig genes would be consistent with participation in immune responses (62).

Entry of newly formed B cells into the long-lived pool and continued survival normally require expression of an Ag receptor and one that is not strongly reactive with self-Ags (73, 74). It is theoretically possible that some ligand for the Ag receptor is species specific and thus lacking in the NOD/SCID environment. More likely, a species-specific cytokine(s) normally confers a long life span to B cells by attracting them to nurturing cells in peripheral lymphoid tissues (75). Finally, T lineage lymphocytes are absent from the immunodeficient mice and might contribute to life/death decisions made by newly formed B cells (76, 77).

These findings indicate that in most respects the NOD/SCID model is representative of B lymphopoiesis in normal human marrow and provide a basis for many other kinds of experimentation. For example, hormones and cytokines can be manipulated in these animals to determine their role in human B lymphocyte formation and potential consequences of therapies can be assessed. However, the full complement of mature B cells is not generated, suggesting a role for unknown, species-specific maturation factors. The cellular source of such molecules might be determined by transplantation of mature T, dendritic, NK, or other accessory cells in these animals. As another approach, IFNs and other species-specific cytokines might be administered. Identification of a human B cell maturation factor(s) might be informative about generation of the diverse repertoire of Ab specificities and suggest new ways to augment the humoral immune system. NOD/SCID mice might also provide a controlled environment for comparing the differentiation potential of different categories of lymphocyte precursors or ones isolated from different organs.

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