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Identification of CD19$^-$B220$^+$c-Kit$^+$Flt3/Flk-2$^+$ cells as early B lymphoid precursors before pre-B-I cells in juvenile mouse bone marrow

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Abstract

The combined analysis of the expression of receptor tyrosine kinases c-Kit and Flt3/Flk-2 and of the human CD25 gene expressed as a transgene under the regulation of the mouse λ5 promoter in the bone marrow of 1-week-old mice allows us to identify three stages of B lymphocyte development before the CD19$^+$c-Kit$^+$ pre-B-I cells. Single-cell PCR analysis of the rearrangement status of the Ig heavy chain alleles allows us to order these early stages of B cell development as follows: (i) B220$^+$CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi}λ5$^-$, (ii) B220$^+$CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi}λ5$^+$ and (iii) B220$^+$CD19$^+$c-Kit^{lo}Flt3/Flk-2^{lo}λ5$^+$ before B220$^+$CD19$^+$c-Kit^{lo}Flt3/Flk-2$^-$λ5$^+$ pre-B-I cells. All these progenitors are clonable on stromal cells in the presence of IL-7 and can differentiate to CD19$^+$c-Kit$^-$ B-lineage cells. A combination of stem cell factor, Flt3 ligand and IL-7 was also able to support the proliferation and differentiation of the progenitors in a suspension culture. Furthermore, the analyses indicate that the onset of D_HJ_H rearrangements precedes the expression of the λ5 gene. These progenitor populations were characteristic of juvenile mice and could not be detected in the bone marrow of adult mice. Hence the expression pattern, and probably the function, of the receptor tyrosine kinases in early B cell differentiation appears to be different in juvenile and adult mice.

Introduction

Early B lymphopoiesis in mouse bone marrow occurs in a microenvironment in which early progenitors are in contact with stromal cells. Cell–cell contacts as well as secreted cytokines guide this early B cell development. B-lineage cells develop from pluripotent hematopoietic stem cells (HSC) which are Thy-1^{low}Sca-1$^+$ lineage marker-negative and which comprise ~0.05% of all bone marrow cells of a young mouse (for reviews, see 1,2). HSC are further subdivided into long-lived cells capable of hematopoietic reconstitution of all lineages of blood cells throughout life, short-lived cells with the same capacity and non-self-renewing pluripotent cells (3). They can be distinguished from each other by differential expression of c-Kit (4), CD34 (5), and low levels of lineage-specific markers such as CD4 and Mac-1 (6). Commitment to lymphoid-restricted development is found in cells which are lineage marker-negative Thy-1$^-$Sca-1^{lo}c-Kit^{lo}IL-7R$^+$. Hence, the common progenitors of T, B and NK cells appear to be IL-7 responsive (7).

Commitment to the B lymphocyte lineage of development is characterized by the up-regulation of expression of B-lineage related or restricted genes, such as CD45R (B220), CD19, sterile transcripts of the Ig μ heavy chain gene locus, as well as $V_{pre\text{-}B}$ and λ5 encoding the surrogate light chain (for reviews, see 8,9). Furthermore, B-lineage-committed progenitors of the mouse are clonable on stromal cells in the presence of recombinant IL-7. These long-term proliferating pro- and pre-B-I cells (for a nomenclature review see 10) can repopulate some of the B-lineage compartments, but not the T-lineage compartments of a rearrangement-deficient severe combined immunodeficient mouse (11,12). Early B-lineage committed progenitors have been found, which appear bipotential for generating myeloid/B lymphoid mixed colonies

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in vitro (13). These bipotential progenitors express a C1qRP-related glycosylated transmembrane protein recognized by the mAb AA4.1 (14).

In fact, a series of molecules expressed in and on progenitors have been employed to characterize and FACS purify early B-lineage progenitors and precursors in the bone marrow of adult mice. Initially these progenitors and precursors were found by Hardy *et al.* (15) in the fraction termed 'A' which was found to be B220 $(CD45R)^+CD43^+CD24$ [heat-stable antigen (HSA)]^{lo}BP-1^-. Four subpopulations were characterized within this fraction A by Rolink *et al.* (16), which were all found not to express the B-lineage-specific marker CD19: (i) $NK1.1^+$ precursors of NK cells, (ii) $CD4^+$ cells not developing into B-lineage cells upon transplantation or tissue culture, (iii) MHC class II^+ cells again not developing into B-lineage cells and (iv) marker-negative cells which could develop into B-lineage cells. *In vitro*, these marker-negative cells within fraction A developed into $CD19^+$ cells, which proliferated long term on stromal cells in the presence of IL-7. Thereafter, Li *et al.* (17) characterized B-lineage progenitors within Hardy's fraction A further as $CD19^-AA4.1^+$, partly $CD4^+$ cells which express components of the pre-B cell receptor (Igα, Igβ, λ5), early B-lineage related transcription factors (Pax-5, E12, E47), as well as sterile transcripts of the μ heavy chain locus.

Recently, Allman *et al.* (18) further characterized the three subpopulations of fraction A, referred as A_0, A_1 and A_2, in terms of expression of stem cell-related markers, potential to give rise to multiple lineages of hematopoietic cells and frequency of D_HJ_H-rearranged alleles. Their results suggested that fraction A_1 and A_2 sorted from adult bone marrow contains only B-lineage committed progenitors which have germline configuration of IgH alleles. Another interesting phenotype of these progenitors compared to $CD19^+$ pro-B-I or fraction 'B' cells is the absence of expression of c-Kit receptor tyrosine kinase on the surface, implying that c-Kit is once down-regulated in the course of B cell differentiation from HSC in the bone marrow of adult mice.

Although c-Kit has been implicated in proliferation of HSC (19–21), the role of c-Kit in B cell development is still controversial. Despite indications that c-Kit is an essential molecule for B cell development *in vitro* (22), it was also shown that B cells can be generated *in vivo* in the absence of c-Kit (23–25). B cell development in the bone marrow of normal adult mice was not inhibited by injection of an antagonistic anti-c-Kit mAb (19). This inconsistency could be partially due to the existence of other receptor tyrosine kinases like Flt3/Flk-2, which may compensate for the loss of c-Kit function. The ligands for c-Kit and Flt3/Flk-2, the stem cell factor (SCF) and FL, are cofactors for the growth of progenitor cells *in vitro* (26–29) (for review, see 30,31). Targeted disruption of the *flt3/flk-2* gene causes deficiency of early differentiation of B lymphocytes in mouse bone marrow (32). A severely enhanced phenotype was observed in juvenile mice when the disrupted allele was brought into the *W/W^v* background which have defects in the c-*kit* gene, suggesting that c-Kit and Flt3/Flk-2 might be involved in early B cell development in juvenile mice. The role of c-Kit and Flt3/Flk-2 in the adult bone marrow cannot be examined in the compound mice because of their lethality. However, co-injection of antagonistic mAb against c-Kit and Flt3/Flk-2 into normal adult mice did not affect B cell development in the bone marrow (33), suggesting that these receptor tyrosine kinases are indispensable for B cell development in the bone marrow of juvenile mice but not of adult mice.

In order to identify early B cell progenitors which express c-Kit and Flt3/Flk-2 in the bone marrow of juvenile mice, we combined the analysis of the expression of c-Kit, Flt3/Flk-2, B220, CD19, CD43, CD24, BP-1, NK1.1, Sca-1, CD4, AA4.1 and λ5 of surrogate light chain on sIg^- progenitors of 1-week-old mouse bone marrow with an *in vitro* analysis of their growth properties in medium containing SCF, FL and IL-7, their clonability on stromal cells in the presence of IL-7, and their capacity to differentiate to $CD19^+$c-Kit^- B-lineage cells. We identified three B-lineage progenitors which express both c-Kit and Flt3/Flk-2 before the stage of a pre-B-I cell. Single-cell PCR analysis of the rearrangement status of the IgH alleles (34) allows us to order them in their B-lineage pathway of development in juvenile mouse bone marrow.

Methods

Mice

C57BL/6 mice of various ages were purchased from Biological Research Laboratories (Füllinsdorf, Switzerland). RAG2-deficient mice obtained from Dr F. Alt (The Children's Hospital, Boston, MA) and 5′λ5–huCD25 transgenic mice developed by Dr I.-L. Mårtensson (Lund University, Lund, Sweden) (35) were bred in our animal facilities.

Antibodies

The FITC- and phycoerythrin (PE)-labeled mAb 1D3 (anti-CD19), FITC- and biotin-conjugated mAb RA3-6B2 (anti-CD45R, B220), biotin-conjugated mAb RM4-5 (anti-CD4), E13-161.7 (anti-Ly-6A/E, Sca-1) and PK136 (anti-NK1.1), and unlabeled mAb M1/70 (anti-CD11b, Mac-1) and TER-119 (anti-TER119) were purchased from PharMingen (San Diego, CA). The FITC-labeled mAb 2A3 (anti-human CD25) was purchased from Becton Dickinson (San Jose, CA). The mAb AA4.1 (anti-AA4.1), A2F10 (anti-Flt3/Flk-2) (33) and ACK4 (anti-c-Kit) were purified from hybridoma culture supernatants on a Protein G–Sepharose column (Pharmacia, Uppsala, Sweden) as recommended in the instruction manual. They were labeled with biotin, PE or allophycocyanin (APC) by standard methods.

Flow cytometry

Bone marrow cells derived from 1-week-old mice were incubated with unlabeled anti-Mac-1 and anti-TER119 mAb. Cells were then depleted of positive cells by using magnetic beads conjugated with anti-rat IgG (Dynal, Oslo, Norway) according to the instruction manual. The Mac-1^-TER119^- cells were stained with FITC-labeled anti-CD19, PE-labeled anti-Flt3/Flk-2, APC-labeled anti-c-Kit and biotin-conjugated mAb, which were revealed by streptavidin–Red613 (Gibco/BRL, Life Technologies, Paisley, UK). Stained cells were resuspended in HBSS (Gibco/BRL) containing 1% BSA and 200 nM TO-PRO-1 iodide (Molecular Probes, Eugene, OR) to exclude dead cells by gating in FL1 (FITC range). In some experiments, streptavidin–PharRed (PharMingen) and propidium iodide

(Sigma, St Louis, MO) were used in place of streptavidin–Red613 and TO-PRO-1 iodide. Cells were analyzed by a FACStar Plus (Becton Dickinson, Mountain View, CA), which was calibrated by using 2 μm microspheres (Polysciences, Warrington, PA), and data was printed out by using software Lysys II or CellQuest (Becton Dickinson). For cell sorting, bone marrow cells were stained with FITC-labeled anti-CD19 together with unlabeled anti-Mac-1 and anti-TER119. Cells were depleted of positive cells and re-stained as described above. Cell sorting was performed by a FACStar Plus . The $CD19^-B220^+$ cells were gated first, and c-Kit^{hi}Flt3/Flk-2^{lo} and c-Kit^{lo}Flt3/Flk-2^{hi} cells in this fraction were sorted by using two overlapping sorting gates. Cells that fell into the overlapping region were logically rejected during sorting so that contamination of the two close populations was minimized. Sorted cell populations were routinely reanalyzed and showed >95% purity. In some experiments, FITC (CD19)-negative cells sorted in the first round were re-stained with FITC–anti-human CD25, and positive and negative cells were re-sorted. For single-cell PCR analysis, cells were sorted into 96-well plates at the density of 1 cell/well by using an automatic cell deposition unit (ACDU).

Cell culture on a stromal cell layer

For limiting dilution assay of B cell progenitors, a ST2 cell (36) monolayer was allowed to form in 96-well plates (Falcon, Oxnard, CA). Sorted $CD19^-B220^+$c-Kit^+Flt3/Flk-2^+ cells were diluted in various concentrations and inoculated into the plates. Cells were cultured in IMDM (Gibco/BRL) supplemented with 2% FCS (Gibco/BRL), 1×non-essential amino acids, 5×10^{-5} M β-mercaptoethanol (Fluka, Buchs, Switzerland), 5 μg/ml insulin (Sigma) and 0.03% Primatone RL (Quest International, Naarden, The Netherlands) in the presence of 100 U/ml IL-7. IL-7 was derived from culture supernatant of J558L cells transfected with murine IL-7 cDNA. After 10 days of culture, outgrowth of B lymphocytes was surveyed under a microscope. In some experiments, cultured cells were harvested by gentle pipetting, stained with several mAb and analyzed by flow cytometry.

Culture with recombinant ligands

Sorted cells were inoculated into 96-well plates at the density of 3.5×10^3 cells/well containing 200 μl culture medium described above. Recombinant murine c-Kit ligand (SCF), human Flt3/Flk-2 ligand (FL) and murine IL-7 were added at a concentration of 100 ng/ml. The recombinant factors were purchased from R & D Systems Europe (Oxford, UK). After 1 week of culture, harvested cells were stained with FITC–anti-B220 and PE–anti-CD19, and analyzed by flow cytometry. For the short-term culture in the absence of ligands, 2×10^4 cells of sorted population were inoculated in a well of a 24-well plate and cultured for 24 h without the soluble ligands. Harvested cells did not retain mAb used for sorting on the surface after the culture period. Cells were re-stained with mAb and analyzed by flow cytometry.

In vitro colony formation assay

Cells were incubated in 1 ml of α-MEM (Gibco/BRL) containing 1.2% methylcellulose (Muromachi Kagaku Kogyo, Tokyo, Japan), 30% FCS (Gibco/BRL), 1% deionized BSA (Sigma), 50 μM β-mercaptoethanol, in the presence of 100 U/ml murine IL-3, 10 ng/ml murine SCF and 2 U/ml murine erythropoietin (Boehringer Mannheim, Mannheim, Germany). After 7 days of culture period, aggregates consisting of >40 cells were differentially scored as colonies.

RT-PCR

Total RNA was prepared from sorted cells using RNAzol B (Biotecx, Houston, TX). RNA was reverse transcribed by using Superscript II reverse transcriptase (Life Technologies, Gaithersburg, MD) and oligo$(dT)_{12-18}$ primer (Life Technologies) according to the instruction manual. PCR assays were performed in the reaction mixture containing 1×PCR buffer (Life Technologies), 200 μM dNTPs (Pharmacia), 2.5 mM $MgCl_2$, TaqStart antibody (Clontech, Palo Alt, CA), AmpliTaq DNA polymerase (Roche, Basel, Switzerland) and 1 μM of the following primers: β-actin, 5′-CCT AAG GCC AAC CGT GAA AAG-3′, 5′-TCT TCA TGG TGC TAG GAG CCA-3′; λ5, 5′-CTT GAG GGT CAA TGA AGC TCA GAG TA 3′, 5′-CTT GGG CTG ACC TAG GAT TG-3′; Mb-1, 5′-GCC AGG GGG TCT AGA AGC-3′, 5′-TCA CTT GGC ACC CAG TAC AA-3′; μ_0, 5′-AAC ATC TGA GTT TCT GAG GCT TGG-3′, 5′-TCA TCT GAA CCT TCA AGG ATG CTC-3′. Amplification of the cDNA was carried out with 1 cycle at 95°C for 30 s followed by 30 cycles at 95°C for 30 s, 57°C for 30 s, 72°C for 60 s and one additional cycle at 72°C for 10 min. RT-PCR products were electrophoresed through 1.5% agarose gel and transferred to Zeta-Probe GT membrane (BioRad, Hercules, CA) with 0.4 M NaOH. The membranes were hybridized with ^{32}P-labeled specific probes and washed according to the manufacturer's recommendations and exposed to X-ray films (Eastman Kodak, Rochester, NY).

Single-cell PCR for D_HJ_H rearrangement

Single-cell PCR was carried out according to the method previously described (34,37). Cells were sorted into 96-well plates containing 10 mM Tris–HCl, pH 7.4 at a density of 1 cell/well by using ACDU. Cells were treated with 1 mg/ml proteinase K (Boehringer Mannheim) at 55°C for 60 min followed by inactivation at 95°C for 10 min. DNA amplification was performed in two rounds of PCR using a Hybaid Omnigene PCR machine (Hybaid, Middlesex, UK). PCR conditions and sequences of the primers were described before (34). The first round of amplification contained two different 5′ D_H primers ($D_{FL/SP}$ and D_{Q52}) and a 5′ J_H1 primer in combination with two 3′ primers downstream of J_H2 and J_H4. The 3′ J_H2 primer largely enhanced detectability of germline alleles. In the second round of PCR, 1 μl of the first PCR amplification was reamplified with a nested 5′ J_H1 primer and the 3′ J_H2 primer for germline configuration, or nested 5′ D_H primers and either the 3′ J_H2 or J_H4 primer for D_HJ_H-rearranged alleles. The second PCR products were analyzed on 1.5% agarose gels stained with ethidium bromide. The 3′ J_H2 primer: 5′-AGG TGT CCC TAG TCC TTC ATG ACC TG-3′; the 5′ J_H1 primer (2nd round): 5′-GAG GCA GAA CAG AGA CTG TGC TAC TGG-3′.

Results

The expression of c-Kit and Flt3/Flk-2 on mouse bone marrow cells

In order to identify early B cell precursors which may co-express c-Kit and Flt3/Flk-2, we first analyzed the expression

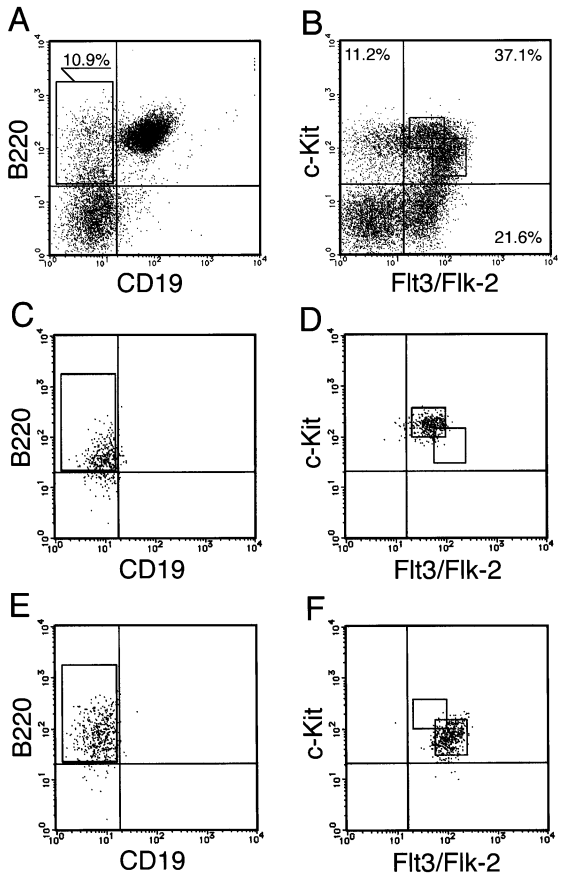


Fig. 1. FACS analysis and sorting of $B220^+CD19^-c\text{-}Kit^+Flt3/Flk\text{-}2^+$ bone marrow cells from 1-week-old normal B6 mice. (A) Cells were stained with anti-Mac-1 and anti-TER-119 mAb, and depleted of positive cells by using immunomagnetic beads. Cells were then stained with FITC–anti-CD19, PE–anti-Flt3/Flk-2, APC–anti-c-Kit and biotinylated anti-B220 (CD45R) which was revealed with Red613–streptavidin. Dead cells were excluded by staining with TO-PRO-1 iodide which was detected in FL1 (FITC range). (B) $B220^+CD19^-$ cells were gated as indicated in (A), and expression of c-Kit and Flt3/Flk-2 on this population was analyzed. Two overlapping sorting gates are shown. (C and D) Reanalysis of sorted $c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ cell fraction. (E and F) Reanalysis of sorted $c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$ cell fraction.

of c-Kit and Flt3/Flk-2 on the $CD19^-B220^+$ cell population in the bone marrow of a very young, i.e. 1-week-old, B6 mouse by using specific mAb. $CD19^-B220^+$ cells comprised 11.6 ± 2.5% of the bone marrow cells depleted of Mac-1^+ and $TER119^+$ cells (Fig. 1A). In the $CD19^-B220^+$ cell population, we detected cells which co-express c-Kit and Flt3/Flk-2 (Fig. 1B). These cells comprise 38.0 ± 2.8% of the $CD19^-B220^+$ cell population (7×10^3 cells/femur). They could be further subdivided into two fractions according to the expression level of c-Kit and Flt3/Flk-2, i.e. $CD19^-B220^+c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ and $CD19^-B220^+c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$. In this paper, we refer to these fractions as $c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ and $c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$ fractions respectively. These fractions were also detected at the same frequency in the bone marrow of age-matched RAG2-deficient mice, suggesting that rearrangements of Ig or TCR gene loci are not required for the development of these cells (data not shown).

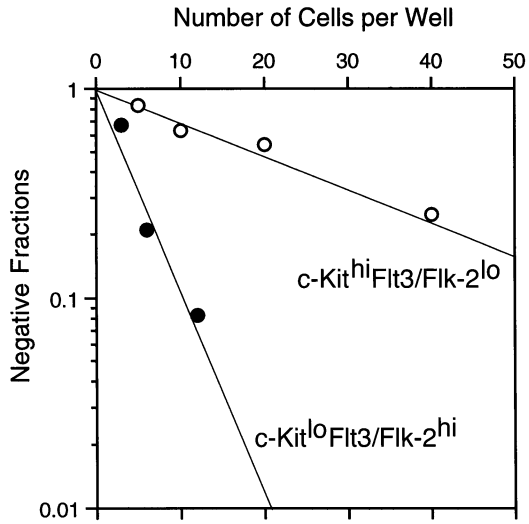


Fig. 2. Proliferative capacity of the $c\text{-}Kit^+Flt3/Flk\text{-}2^+$ cells in response to stromal cells and IL-7. The $CD19^-B220^+c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ (○) and $CD19^-B220^+c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$ (●) cells sorted from bone marrow of 1-week-old B6 mice were diluted in various concentrations and inoculated into 96-well plates which were pre-seeded with stromal cells. Cells were cultured for 10 days in the presence of IL-7 and growth of B lymphocytes was monitored under a microscope. The data points represent the fraction of wells showing no growth of B lymphocytes out of 96 wells seeded.

Proliferative capacity of the $c\text{-}Kit^+Flt3/Flk\text{-}2^+$ cells in response to stromal cells

In order to examine whether the $c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ and $c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$ fractions contain any B lymphoid progenitors, these fractions were sorted by FACS from bone marrow cells of 1-week-old B6 mice. Two overlapping gates were used for sorting to minimize possible contamination of the two close populations, as the cell sorter logically rejects cells that fall into the overlapping region (Fig. 1B). Although both populations were $B220^+$, the reanalyses of the sorted fractions revealed that the intensity of B220 fluorescence was higher in the $c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$ cells than in $c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ cells (Fig. 1C–F). The sorted cells were cultured in the presence of stromal cells and IL-7. Both fractions gave rise to proliferation of $CD19^+c\text{-}Kit^+$ as well as $CD19^+c\text{-}Kit^-$ cells which contain surface IgM^+ B cells under bulk culture conditions (data not shown).

To determine the frequency of B cell progenitors which are clonable in the presence of stromal cells and IL-7, sorted fractions were cultured under limiting dilution conditions. Figure 2 shows that around one in five cells in the $c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$ fraction gave rise to a B cell colony in this culture condition, whereas the $c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ fractions contained less B cell progenitors (one in 30 cells). FACS analysis of the colonies derived from each fraction confirmed that they consisted of $CD19^+$ B lymphocytes (data not shown).

We also examined the frequency of myeloid and erythroid progenitor cells in the fractions sorted from 1-week-old mouse bone marrow by *in vitro* colony formation analysis with recombinant cytokines. In contrast to the frequency of B cell progenitors, the $c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ fraction contained high

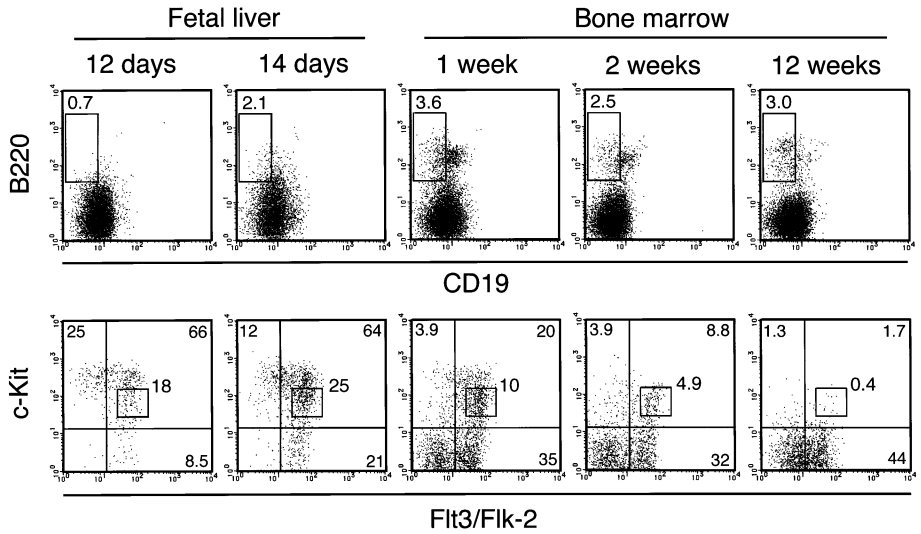


Fig. 3. Ontogenic restriction of the c-Kit^{lo}Flt3/Flk-2^{hi} cell fraction. The fetal liver and bone marrow cells of different ages were stained with FITC–anti-CD19, non-labeled anti-Mac-1 and anti-TER119 mAb, and depleted of positive cells by using immunomagnetic beads. Cells were then stained with FITC–anti-CD19, PE–anti-Flt3/Flk-2, APC–anti-c-Kit and biotinylated anti-B220 (CD45R) which was revealed with streptavidin–PharRed. Dead cells were excluded by staining with propidium iodide. B220^{+}CD19^{-} cells were gated as indicated in upper panels, and expression of c-Kit and Flt3/Flk-2 on this population was analyzed (lower panels). The numbers indicate the percentage of cells that appeared in each area.

Table 1. Colony formation of CD19^{-}B220^{+} subfractions in response to IL-3, SCF and erythropoetin

Cell fractions	No. of colonies per dish[a] (frequency)			
	CFU-M	CFU-G	CFU-GM	BFU-E[b]
c-Kit^{hi}Flt3/Flk-2^{lo}	134.0 ± 12.7 (1/4)	48.5 ± 2.1 (1/10)	32.5 ± 3.5 (1/15)	0.0 ± 0.0 (<1/500)
c-Kit^{lo}Flt3/Flk-2^{hi}	5.5 ± 1.3 (1/91)	0.0 ± 0.0 (<1/500)	1.3 ± 0.5 (1/385)	0.0 ± 0.0 (<1/500)

Bone marrow cells sorted from 1-week-old B6 mice were cultured in a semi-solid medium containing recombinant murine IL-3, SCF and erythropoetin. The inoculum size was 500 cells/dish. Colonies were differentially counted after 7 days of culture period.

[a]Mean ± SD of triplicate cultures.

[b]Lin^{-}c-Kit^{+} cells, analyzed as a positive control, gave rise to erythroid colonies at the frequency of 1/152 cells.

frequencies of myeloid progenitors (one in two to three cells), whereas the c-Kit^{lo}Flt3/Flk-2^{hi} fraction contained less myeloid progenitors (one in 70 cells) (Table 1). As B lymphoid progenitor was highly enriched in the c-Kit^{lo}Flt3/Flk-2^{hi} fraction, further analyses were mainly performed on this fraction.

Ontogenic restriction of the c-Kit^{lo}Flt3/Flk-2^{hi} cell fraction

In 1-week-old mice, B220^{+} cells were abundantly found in the CD19^{-}Mac-1^{-}TER119^{-}c-Kit^{+}Flt3/Flk-2^{+} cell population (>50% in c-Kit^{hi}Flt3/Flk-2^{lo} cells and >90% in c-Kit^{lo}Flt3/Flk-2^{hi} cells). In contrast, the same population in the bone marrow of adult mice contained few, if any, B220^{+} cells (data not shown). We detected only few B cell progenitors in the CD19^{-}c-Kit^{hi}Flt3/Flk-2^{lo} and CD19^{-}c-Kit^{lo}Flt3/Flk-2^{hi} fractions sorted from adult bone marrow (less than one in 100 cells).

To investigate time course for the development of the c-Kit^{lo}Flt3/Flk-2^{hi} cells during ontogeny, we examined presence of this fraction in the fetal liver and bone marrow of different ages by flow cytometry (Fig. 3). In the fetal liver, committed B cell precursors emerge at day 14 of gestation (38). At this time point, significant numbers of CD19^{-}B220^{+} cells were present in the fetal liver and the c-Kit^{lo}Flt3/Flk-2^{hi} cells were detectable in the B220^{+} population. During neonatal ontogeny, the c-Kit^{lo}Flt3/Flk-2^{hi} cells were consistently present in the bone marrow of 1-day-old and 1-week-old mice (Fig. 3 and data not shown). In the bone marrow of 2-week-old mice, this cell fraction decreased in number and became almost undetectable at 12 weeks of age. This result indicates that the presence of the CD19^{-}B220^{+}c-Kit^{lo}Flt3/Flk-2^{hi} fraction is restricted to the fetal liver and bone marrow of juvenile mice, and is not detectable in the adult bone marrow.

Cellular phenotype of the c-Kit^{lo}Flt3/Flk-2^{hi} fraction

The surface character of the c-Kit^{lo}Flt3/Flk-2^{hi} fraction in the bone marrow of 1-week-old mice was further examined with additional mAb (Fig. 4). Weak expression of CD4 and Sca-1 was found on half of the c-Kit^{lo}Flt3/Flk-2^{hi} cells, whereas none of them expressed NK1.1. Interestingly, about one-third of the c-Kit^{lo}Flt3/Flk-2^{hi} cells showed expression of AA4.1. The expression of AA4.1 was not detected in the c-Kit^{hi}Flt3/Flk-2^{lo}

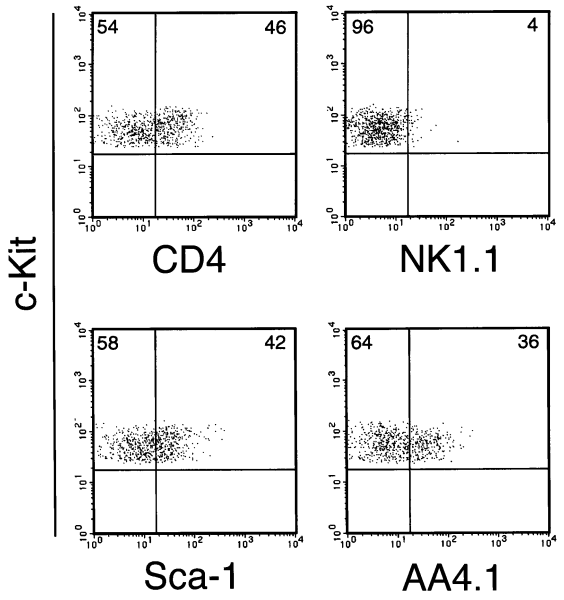


Fig. 4. Cell surface phenotype of the CD19$^-$B220$^+$c-Kit^{lo}Flt3/Flk-2^{hi} cells. Bone marrow cells from 1-week-old B6 mice were stained with FITC–anti-CD19, PE–anti-Flt3/Flk-2, APC–anti-c-Kit and biotinylated mAb with different specificity. CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi} cells were gated and re-plotted for staining of anti-c-Kit and biotinylated mAb which were revealed by streptavidin–Red613.

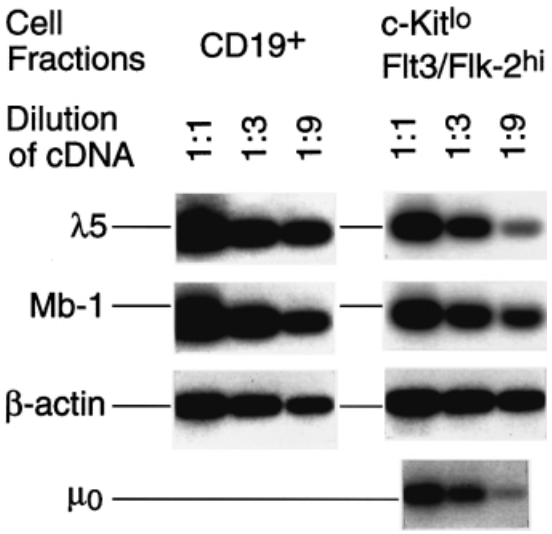


Fig. 5. Expression of mRNA for λ5, Mb-1, β-actin and μ_0 transcripts in sorted CD19$^+$ and CD19$^-$B220$^+$c-Kit^{lo}Flt3/Flk-2^{hi} cells from bone marrow of 1-week-old B6 mice. Different dilution of cDNA prepared from the sorted fractions were subjected to PCR amplification specific for β-actin, λ5, Mb-1 and μ_0 transcripts. PCR products were separated on 1.5% agarose gel and transferred to membranes and hybridized with specific probes.

fraction (data not shown). Li *et al.* reported that only the AA4.1$^+$ cells in Hardy's fraction A were able to proliferate on a stromal cell layer (17). In agreement with their result, we observed >4 times higher frequency of B cell progenitors in the c-Kit^{lo}Flt3/Flk-2^{hi}AA4.1$^+$ cells than in the c-Kit^{lo}Flt3/Flk-2^{hi}AA4.1$^-$ cells (data not shown). This allows us to estimate that 80% of the B cell progenitors in the c-Kit^{lo}Flt3/Flk-2^{hi} fraction express AA4.1.

We also analyzed the transcription of B cell-specific genes by RT-PCR (Fig. 5). In the c-Kit^{lo}Flt3/Flk-2^{hi} fraction, we detected transcripts of the *λ5* and *mb-1* genes as well as a sterile μ heavy chain transcripts (μ_0), although the expression levels of *λ5* and *mb-1* were lower than that detected in the CD19$^+$ bone marrow cells.

Progressive differentiation of c-Kit^{lo}Flt3/Flk-2^{hi} B cell progenitors

Only 10% of the CD19$^+$c-Kit$^+$ pre-B-I cells in the bone marrow of juvenile mice weakly express Flt3/Flk-2 (data not shown). This fact prompted us to investigate how the expression of the tyrosine kinase receptors changes upon differentiation of c-Kit^{lo}Flt3/Flk-2^{hi} B cell progenitors. A short-term (24 h) culture of sorted c-Kit^{lo}Flt3/Flk-2^{hi} cells in the absence of stromal cells and soluble ligands revealed that the cells rapidly down-regulate the expression of Flt3/Flk-2 but not c-Kit upon differentiation to the CD19$^+$ stage with an apparently intermediate stage of CD19$^+$c-Kit^{lo}Flt3/Flk-2^{lo} cells (Fig. 6). The frequency of B cell progenitors which are reactive to stromal cells and IL-7 in CD19$^+$c-Kit^{lo}Flt3/Flk-2^{lo} and CD19$^+$c-Kit^{lo}Flt3/Flk-2$^-$ cells freshly sorted from bone marrow of juvenile mice were comparably high (1:3 and 1:6 respectively, data not shown). In addition to the CD19$^+$c-Kit^{lo}Flt3/Flk-2^{lo} cells,

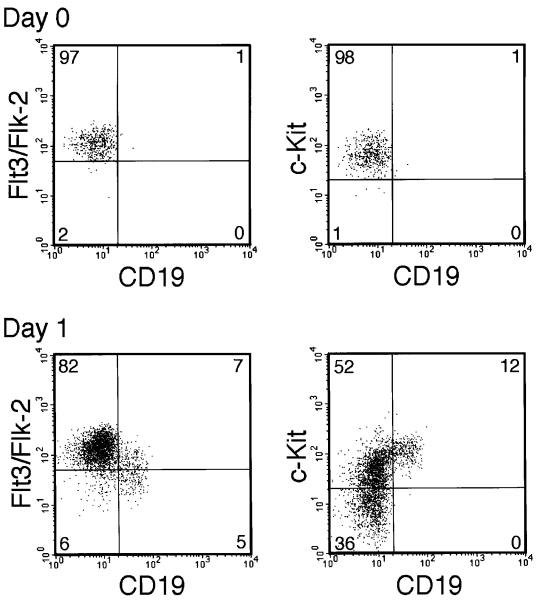


Fig. 6. Differentiation of CD19$^+$c-Kit^{lo}Flt3/Flk-2^{lo} cells from CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi} B cell progenitors. The B220$^+$CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi} cells sorted from bone marrow of 1-week-old B6 mice (upper panels) were cultured for 24 h in the absence of stromal cells and soluble ligands. Cells were re-stained with FITC–anti-CD19, PE–anti-Flt3/Flk-2 and APC–anti-c-Kit mAb, and analyzed by flow cytometry (lower panels).

another population which is CD19$^-$c-Kit$^-$Flt3/Flk-2^{hi} also appeared in the short-term culture of the c-Kit^{lo}Flt3/Flk-2^{hi} cells (Fig. 6). Although we have not further examined this population, we propose that this might represent another

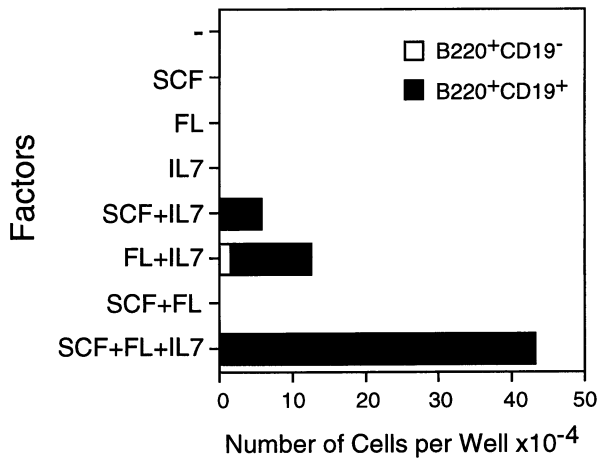


Fig. 7. Growth requirement of $B220^+CD19^-$c-Kit^{lo}Flt3/Flk-2^{hi} cells *in vitro*. $B220^+CD19^-$c-Kit^{lo}Flt3/Flk-2^{hi} cells sorted from bone marrow of 1-week-old B6 mice were inoculated into 96-well plates at the density of 3.5×10^3 cells/well. Cells were cultured for 1 week in the presence of SCF, FL and IL-7. Cultured cells were stained with FITC–anti-B220 and PE–anti-CD19 mAb, and analyzed by flow cytometry. The data represent the number of cells recovered per well.

subpopulation in fraction A, $B220^+CD19^-CD4^+$c-Kit^-Flt3/Flk-2^+ cells which do not show any progenitor activities (33).

Growth factor requirement of c-Kit^{lo}Flt3/Flk-2^{hi} B cell progenitors

We next examined growth factor requirements for the early B cell progenitors *in vitro*. The cells in the c-Kit^{lo}Flt3/Flk-2^{hi} fraction which were sorted from 1-week-old mice were cultured in the presence of recombinant soluble ligands (Fig. 7). Neither c-Kit ligand (SCF), Flt3/Flk-2 ligand (FL) nor IL-7 alone could support the growth of the cells in this fraction. On the other hand, the combined presence of IL-7 and either SCF or FL promoted the growth of $B220^+CD19^+$ cells from the same cell population. PCR analysis of the status of rearrangements in the IgH locus in the $B220^+CD19^+$ cells showed that most of the cells had D_HJ_H-rearranged IgH alleles (data not shown). These results suggest that IL-7 and one of the receptor tyrosine kinases are required for proliferative expansion and differentiation of $CD19^+$ cells from early $CD19^-$ B cell precursors *in vitro*. Recovery of the $CD19^+$ cells was highest when the cells were cultured in the presence of all three factors, indicating a synergistic effect of SCF and FL. One out of four cells in the c-Kit^{lo}Flt3/Flk-2^{hi} fraction gave rise to a colony which consists of $CD19^+$ cells in a methylcellulose culture containing SCF, FL and IL-7, and the frequency was consistent with that obtained on the stromal cell layer (data not shown).

The c-Kit^{lo}Flt3/Flk-2^{hi} B cell progenitors in 5′λ5–huCD25 transgenic mice

In order to investigate the expression of surrogate light chain in the c-Kit^{lo}Flt3/Flk-2^{hi} fraction which is enriched for B cell progenitors, we took advantage of a transgenic mouse in which the human CD25 gene (encoding the IL-2 receptor α chain) has been introduced as a transgene under the regulation of the mouse λ5 promoter. Previous analysis of these transgenic mice has shown that the human CD25 gene is expressed in parallel to surrogate light chain (35). Hence, human CD25 expression in these mice is a marker for endogenous λ5 expression. Flow cytometry analysis of bone marrow cells of 1-week-old transgenic mice showed that 25% of the cells in the c-Kit^{lo}Flt3/Flk-2^{hi} fraction expressed the huCD25 antigen (Fig. 8A).

We next subdivided the c-Kit^{lo}Flt3/Flk-2^{hi} fraction into $huCD25^+$ and $huCD25^-$ populations, and measured the frequency of stromal cell-dependent B cell precursors (Fig. 8B). One in three cells in the $huCD25^+$ population produced B cell colonies on a stromal cell layer. Surprisingly, the $huCD25^-$ population also contained significant number of B cell progenitors (one in 10 cells). This frequency suggests that at least half of B cell progenitors in the c-Kit^{lo}Flt3/Flk-2^{hi} fraction does not express the huCD25 transgene.

Colonies derived from the $huCD25^-$ population were composed of $CD19^+huCD25^+$ cells, indicating that the $huCD25^-$ progenitors are proper precursors for the B lymphoid lineage (data not shown). Interestingly, colonies derived from $huCD25^-$ cells contained, on average, 10 times more cells than colonies derived from $huCD25^+$ cells ($5.5 \pm 3.4\times10^4$ versus $0.5 \pm 0.4\times10^4$ on average of 14 colonies after 9 days of culture). This suggests that $huCD25^-$ progenitors are more immature than $huCD25^+$ progenitors and have a strong proliferative capacity.

We examined expression of the endogenous *λ5* gene in the c-Kit^{lo}Flt3/Flk-$2^{hi}huCD25^+$ and $huCD25^-$ cells by RT-PCR. λ5 transcript was observed in the $huCD25^+$ population but not in the $huCD25^-$ population, indicating the specific expression of the transgene (Fig. 8C). Thus, the results suggest that the $huCD25^-$ fraction contains early B lymphoid precursors which do not yet express λ5.

D_HJ_H gene configuration of the c-Kit^{lo}Flt3/Flk-2^{hi} fraction

Finally, we analyzed the configuration of the μ heavy chain loci of the c-Kit^{lo}Flt3/Flk-$2^{hi}huCD25^+$ and c-Kit^{lo}Flt3/Flk-$2^{hi}huCD25^-$ cells on a single-cell level by PCR. D_HJ_H-rearranged alleles were amplified in the presence of primers specific to 5′ sequences of D_{FL16}, D_{SP2}, D_{Q52} and J_H1, and 3′ sequences of J_H2 and J_H4. The result summarized in

Table 2. Single-cell PCR analysis of D_HJ_H gene configuration in the c-Kit^{lo}Flt3/Flk-$2^{hi}huCD25^+$ and c-Kit^{lo}Flt3/Flk-2^{hi}huCD25$^-$ cells

	Cell fractions	
	c-Kit^{lo}Flt3/Flk-$2^{hi}huCD25^-$	c-Kit^{lo}Flt3/Flk-$2^{hi}huCD25^+$
Total no. of cells tested	46	46
No. of cells with[a]		
DJ/DJ	0	6
DJ/–	2	12
DJ/GL	5	7
GL/–	35	14
–/–	4	7

[a]DJ/–, only one D_HJ_H-rearranged allele was detected; GL/–, only germline allele was detected; –/–, no PCR band was detected.

Table 2 shows striking differences in the number of cells with germline versus D_HJ_H-rearranged IgH alleles. In the c-Kit^{lo}Flt3/Flk-2^{hi}huCD25$^-$ fraction, 15% of the cells contained at least one D_HJ_H-rearranged allele whereas, in the c-Kit^{lo}Flt3/Flk-2^{hi}huCD25$^+$ fraction, approximately half did. By comparison, >95% of all CD19$^+$c-Kit$^+$ pre-B-I cells have both IgH alleles in D_HJ_H-rearranged configuration (34). These analyses allow us to order the B-lineage progenitors in the following sequence: (i) B220$^+$CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi}(huCD25$^-$)λ5$^-$, (ii) B220$^+$CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi}(huCD25$^+$)λ5$^+$, (iii) B220$^+$CD19$^+$c-Kit^{lo}Flt3/Flk-2^{lo}(huCD25$^+$)λ5$^+$ and (iv) B220$^+$CD19$^+$c-Kit^{lo}Flt3/Flk-2$^-$(huCD25$^+$)λ5$^+$ (pre-B-I). It was also suggested that B cell progenitors in the c-Kit^{lo}Flt3/Flk-2^{hi} fraction have already started D_HJ_H gene joining and this may take place prior to λ5 expression.

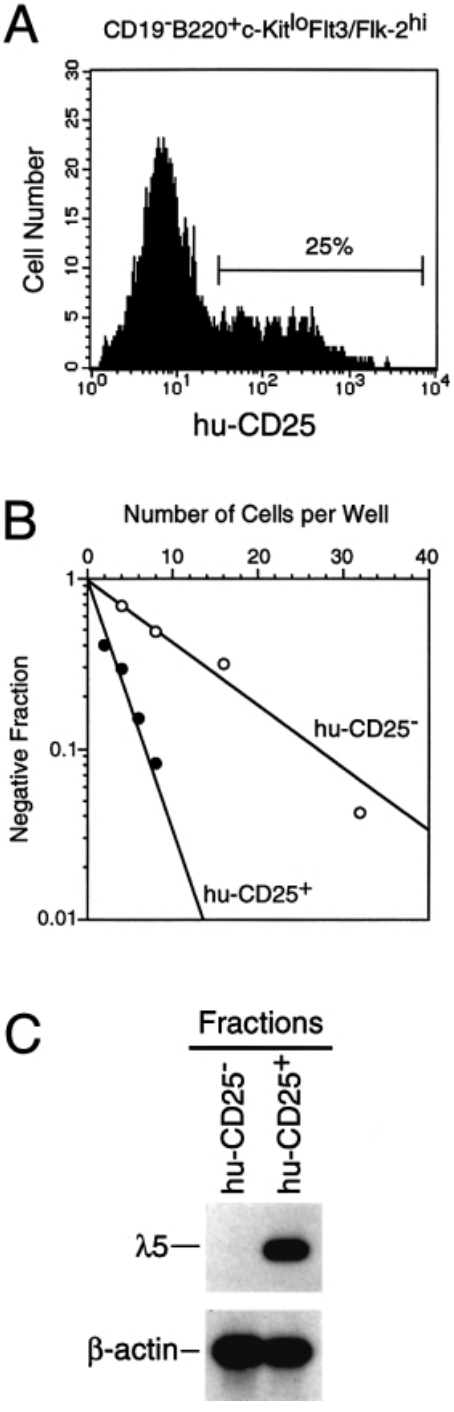
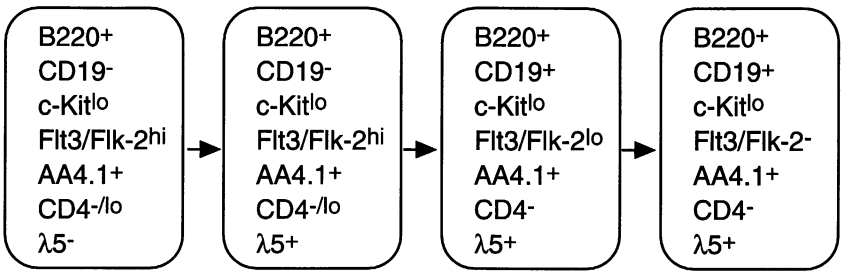


Fig. 8. B220$^+$CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi} B cell progenitors in the bone marrow of 5′λ5–huCD25 transgenic mice. (A) Expression of the human CD25 transgene in the B220$^+$CD19$^-$c-Kit^{lo}Flt3/Flk-2^{hi} cells from bone marrow of 1-week-old 5′λ5–huCD25 transgenic mice. B220$^+$CD19$^-$c-Kit^{lo}flt3/Flk-2^{hi} cells were sorted from bone marrow of the transgenic mice as described in the legend of Fig. 1. Sorted cells were re-stained with FITC–anti-huCD25 mAb and analyzed by flow cytometry. The huCD25$^+$ and huCD25$^-$ cells were re-sorted for further analyses. (B) Limiting dilution analysis of the c-Kit^{lo}Flt3/Flk-2^{hi}huCD25$^+$ (●) and c-Kit^{lo}Flt3/Flk-2^{hi}huCD25$^-$ (○) cells. The frequency of stromal cell/IL-7-dependent B cell progenitors in the sorted cells was determined as described in the legend of Fig. 2. (C) Expression of mRNA for λ5 and β-actin in sorted c-Kit^{lo}Flt3/Flk-2^{hi}huCD25$^-$ and c-Kit^{lo}Flt3/Flk-2^{hi}huCD25$^+$ cells. cDNA prepared from the sorted fractions were subjected to PCR amplification specific for β-actin and λ5 transcripts. PCR products were analyzed as described in the legend of Fig. 5.

Discussion

The B220$^+$CD19$^+$ committed B lymphoid progenitor in the bone marrow of adult mice, which is referred as the pre-B-I cell, is known to express low levels of a receptor tyrosine kinase c-Kit (39,40). The proliferation of this progenitor in tissue culture has been found to be influenced by the ligand for c-Kit, SCF (22,39,41). Flt3/Flk-2, another receptor tyrosine kinase with close structural similarities to c-Kit, has been found also to be expressed on early hematopoietic progenitors (42,43) as well as cell populations which contain B lymphoid progenitors (44,45). The ligand for Flt3/Flk-2 (FL) influences proliferation and differentiation of B-lineage cells from early hematopoietic progenitor cells *in vitro (*27). However, the role of the receptor tyrosine kinases on B lymphoid progenitor cells in bone marrow of adult mice is controversial. No deficiencies in B lymphopoiesis have been shown in the bone marrow of *W/W^v* mice that have defects in the c-*kit* gene (23). Injection of c-Kit-specific mAb into normal adult mice did not inhibit, as *in vitro* (39,40), but actually enhanced B lymphopoiesis (19). Mice defective for Flt3/Flk-2 showed only partial defects in B lymphopoiesis in adult bone marrow (32). The frequency of stromal cell-dependent B lymphoid progenitors in the B220$^+$Flt3/Flk-2$^+$ fraction sorted from adult bone marrow was almost comparable to that detected in whole B220$^+$ cells (33). Simultaneous treatment of normal adult mice with Flt3/Flk-2-specific mAb and c-Kit-specific mAb did not affect B lymphopoiesis (33). These findings suggested that the receptor tyrosine kinases have a minimal role, if any, on B cell progenitors in adult bone marrow.

The moderate defect of pro-B cells in Flt3/Flk-2-deficient mice is already observed at the age of 3 weeks (32). Interestingly, 3-week-old mice deficient for both c-Kit and Flt3/Flk-2 exhibit a more severe defect in B cell development (32). B



% in total B220+ cells*	2.4%	0.8%	1.8%	19%
Frequency of progenitors clonable on stromal cells/IL-7	1/10	1/3	1/3	1/6
Relative proliferation capacity‡	10	1	1	1
% of cells containing at least one D_HJ_H rearranged allele	15%	54%	not determined	>95%

Fig. 9. A model of early developmental stages of B cell lineage in the bone marrow of juvenile mice. *Percent of cell population in total $B220^+$ cells in the bone marrow at 1 week of age. ‡Relative clone size at day 9 of *in vitro* culture (see Results).

cell progenitors isolated from fetal liver and bone marrow of young mice were shown to proliferate *in vitro* in response to IL-7 and either of SCF and FL (26,29). Therefore, the receptor tyrosine kinases might have a distinct role on B cell precursors at different ages. In this paper, we studied the expression of the two tyrosine kinase receptors in early B lymphoid progenitor populations in the bone marrow of 1-week-old mice, collectively identified by their expression of B220 (CD45R). Our results show that the differential expression pattern of these two receptors and the λ5 component of the surrogate light chain are valuable additional markers to allow further characterization of early B-lineage progenitors in juvenile mice. All these $B220^+$ progenitors are defined as committed to B-lineage development by their capacity to develop, under the stimulatory influence of IL-7 and stromal cells (11), into clones of further differentiated $CD19^+$ B-lineage cells (Figs 2 and 8). Together with the analyses of other markers used in previous studies by other laboratories for a definition of B-lineage-committed progenitors and precursors (15,40,46) (Fig. 4), and with the help of a single-cell PCR analysis of the rearrangement status of the IgH loci in these cells (34) (Table 2), we propose a sequence of progenitors with phenotypes shown in Fig. 9.

In our scheme, $B220^+CD19^-$ B lymphoid progenitors express both c-Kit and Flt3/Flk-2. Furthermore, these progenitors respond optimally *in vitro* to the combined activities of SCF, FL and IL-7 (Fig. 7), suggesting that the two receptors act together on and in one progenitor. The expression level of c-Kit does not change until progenitors differentiate to pre-B-II cells, whereas Flt3/Flk-2 is rapidly down-regulated upon differentiation into pre-B-I cells (Fig. 6). Down-regulation of Flt3/Flk-2 upon differentiation of murine B cell precursors is consistent with the situation in human B lymphopoiesis (47), although the expression of FLT3 (CD135) on B cell progenitors persists longer in the human bone marrow where FLT3 is expressed on a part of $CD34^+CD19^+$ pre-B-I cells and $CD34^-$ $CD10^+$ pre-B-II cells (47,48).

We have assumed, and illustrated in Fig. 9, that only one line of B cell development exists in juvenile bone marrow at these early stages. It is, however, still possible that other parallel lineages of B cell progenitors exist, which co-express c-Kit and Flt3/Flk-2. Our analysis estimated that 80% of the B cell progenitors in the $c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$ fraction express AA4.1. Although this agrees with previous reports by others showing that most of early B cell progenitors are found in the $AA4.1^+$ cell fraction (13,17,18), significant number of B cell progenitors are still present in the $c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}AA4.1^-$ fraction. We also detected a fraction of $CD19^-B220^{lo}c\text{-}Kit^{hi}Flt3/Flk\text{-}2^{lo}$ cells which comprised 3% of all $B220^+$ cells (Fig. 1). These cells are again $AA4.1^-$ (data not shown), whereas one in 30 of them yielded a colony of $CD19^+$ B-lineage cells (Fig. 2). The relationship of these fractions with the major developmental pathway of early B lymphoid precursors proposed in Fig. 9 remains to be clarified.

The bone marrow of a juvenile mouse has >0.5% of all $B220^+$ cells as $c\text{-}Kit^{lo}Flt3/Flk\text{-}2^{hi}$ B-lineage progenitors which respond to stromal cells and IL-7 (Figs 1 and 2). On the other hand, the same population was hardly detected in adult mice (Fig. 3). Although it remains to be investigated whether this is a consequence of a decrease in the total number of the early progenitors with age or a change in the phenotype of early B progenitors, it should be remembered that the frequency of early precursors which are clonable on stromal cells and IL-7 has previously been shown to decrease during 8 months of life (49,50). Therefore, we consider it likely that the early B-lineage progenitors identified in this paper are found in young, but much less frequently in adult mice. This might account for the previous result that simultaneous treatment of mAb specific to c-Kit and Flt3/Flk-2 did not affect B lymphopoiesis in the bone marrow of normal adult mice (33). Changes in frequency of B lymphoid precursors in mice are reminiscent of the situation in human B lymphopoiesis where, again only young but, much less, old individuals have early progenitors in their bone marrow (48,51).

We showed that the CD19$^-$B220$^+$c-Kit^{lo}Flt3/Flk-2^{hi} cell fraction was detectable in the fetal liver by day 14 of gestation (Fig. 3). This population was present in the bone marrow of neonatal to 2 weeks-old mice but not in the adult mice (Fig. 3). This is consistent with previous report showing that pro-B cells which belong to fractions A_1 and A_2 in adult bone marrow do not express c-Kit (18). It is well known that there are several differences between adult and fetal B lymphopoiesis. Pro-B cells in the fetal liver but not in the adult bone marrow reconstitute CD5$^+$ (B1) B cells (12,52), indicating different developmental pathways of the B cell lineage during ontogeny. Terminal deoxynucleotidyl transferase is absent in the fetal liver, which results in the lack of N segment addition during V–D–J joining in fetal and neonatal life (53). More significantly, pre-B and immature IgM$^+$ B cells generated in the fetal liver initially lack MHC class II expression, whereas B-lineage cells in the adult bone marrow express class II antigen from pre-B cell stage (54,55). This finding suggested that the fetal- and adult-type B cell lineages can be distinguished by a difference in cell surface phenotype. The adult-type lineage begins to emerge in the bone marrow by 8 days of postnatal age and gradually replaces the fetal-type lineage which predominates in the bone marrow at birth (54). The fetal-type lineage is characterized by class II$^-$ pre-B and immature B cells, whereas the c-Kit^{lo}Flt3/Flk-2^{hi} fraction reported here belongs to pro-B cells (Table 2). Taking this into account, kinetics of the c-Kit^{lo}Flt3/Flk-2^{hi} fraction in juvenile bone marrow might be compatible with that of the fetal-type lineage and this may suggest that the c-Kit^{lo}Flt3/Flk-2^{hi} cells correspond to the fetal-type B cell progenitors. If this is the case, expression of the genes for the receptor tyrosine kinases is differently regulated in the process of fetal- and adult-type B cell development. Alternatively, the c-Kit^{lo}Flt3/Flk-2^{hi} fraction may represent a differentiation pathway which is not simply confined to the fetal-type lineage but also exists in the adult-type lineage. During neonatal ontogeny, rapid proliferation of B lymphoid progenitors would be required until the adult-type immune system reaches a state of homeostasis (50). We would, therefore, suggest that c-Kit and Flt3/Flk-2 might play a distinct role in the formation of a pro-B cell pool which is more prominent in neonatal mice than in adult mice.

Li *et al.* have reported that the λ*5* gene expression becomes detectable in the cells which belong to fraction A_2 before D_HJ_H gene rearrangement commences (17,18,56). This has been further confirmed by Mårtensson *et al.* (35) by using the 5′λ5-huCD25 transgenic mice. In contrast to these results, our experiments showed that the commencement of D_HJ_H rearrangement in the B lymphoid progenitors of juvenile mice precedes λ5 expression (Table 2). The λ5 expression has been found on B cell progenitors in RAG2-deficient mice (57), and D_HJ_H gene rearrangements have been shown to occur normally in B cell precursors of λ5-deficient mice (58). Taken together, D_HJ_H recombination and the λ*5* gene expression might take place independently and in different timing. The frequency of the cells containing at least one D_HJ_H-rearranged allele was comparable to that of clonable B lymphoid progenitors in the c-Kit^{lo}Flt3/Flk-2^{hi}λ5(huCD25)$^-$ and c-Kit^{lo}Flt3/Flk-2^{hi}λ5(huCD25)$^+$ fractions (Fig. 8 and Table 2). Therefore, it is likely that most of the clonable progenitors in these fractions have started D_HJ_H recombination. Allman *et al.* (18) proposed that commitment to the B lymphoid lineage occurs before D_HJ_H recombination. If this is also the case in juvenile mice, there should be some intermediate steps to be identified between the earliest diverging point to B lymphoid lineage of development and the c-Kit^{lo}Flt3/Flk-2^{hi}λ5$^-$ pro-B cell stage.

In conclusion, the differential expression of the two receptor tyrosine kinases c-Kit and Flt3/Flk-2 on early B-lineage progenitors is a valuable tool which should help to clarify the molecular controls underlying early stages of B lymphopoiesis.

Acknowledgments

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Abbreviations

ACDU	automatic cell deposition unit
APC	allophycocyanin
FL	Flt3/Flk-2 ligand
HSA	heat-stable antigen
HSC	hematopoietic stem cells
PE	phycoerythrin
SCF	stem cell factor

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