Characterization and Differentiation of an Early Murine Yolk Sac-Derived IL-7-Independent Pre-Pro-B Cell Line

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Characterization and Differentiation of an Early Murine Yolk Sac-Derived IL-7-Independent Pre-Pro-B Cell Line 1

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We describe a unique, stable pre-pro-B cell line (YS-PPB) derived from AA4.1 + yolk sac cells from day 10 mouse embryos. This cell line, discovered fortuitously during the course of studies of in vitro B cell differentiation, is independent of IL-7 supplementation for long term expansion in vitro. YS-PPB cells as well as clonal sublines expressed AA4.1, CD43, B220, Sca-1, CD19, heat stable antigen, MHC class I, IL-7R, and FcγR, but did not express cytoplasmic μ-chain, surface IgM (sIgM), or MHC class II molecules. PCR analysis showed that the cells expressed TdT, A5, and RAG-1 genes, but that their Ig genes were still in germline configuration. The cell line was dependent on direct contact with S17 stromal cells for growth, but, in contrast to bone marrow stem cells, required no additional growth factors for maintenance and expansion. When stimulated with IL-7 and LPS, YS-PPB cells and cells from all tested clonal sublines differentiated into sIgM + B cells in vitro. Irradiated mice reconstituted with YS-PPB cells yielded spleens containing 38% sIgM + donor-derived B cells, demonstrating that YS-PPB cells, although stably arrested in development at the boundary between pre-pro-B and pro-B stages of B cell differentiation, still retain their competence to differentiate into mature, Ig-producing B cells when transferred to a normal host environment. Thus, this new cell line can provide a reproducible source of B cell precursors arrested at that critical time in B cell differentiation when the machinery for Ig gene rearrangement is in place but rearrangement has not yet occurred. The Journal of Immunology, 1998, 161: 1284–1291.

The yolk sac is the first site of hemopoiesis during mammalian development (1). Although in situ differentiation of hemopoietic stem cells within the yolk sac appears to be limited primarily to erythropoiesis, stem cells obtained from the yolk sac have been shown capable of differentiating into all hemopoietic cell lineages when presented with an appropriate microenvironment either in vivo or in vitro (2-4).

Although the first hemopoietic stem cells can be identified both intraembryonically and in the yolk sac as early as day 7.5, the first B cell lineage commitment appears not to occur until days 11 to 12, when pro-B cells can be detected in the fetal liver (5, 6). The primary source of these cells has not been established, but it is believed that they originate extrinsically from the intraembryonic aorta/gonad/mesonephros or AGM region, from the blood islands of the extra-embryonic yolk sac, or from both (2, 7, 8).

B cell development in vitro, and presumably in vivo, is dependent on direct physical association between hemopoietic stem cells and the surrounding stromal cells that may include fibroblasts, adventitial reticular cells, epithelial cells, and endothelial cells (9). Cell-cell interactions, largely as yet undefined but partly mediated by released growth factors, generate the growth and differentiation signals that promote progression along B cell developmental pathways. The progressive stages of B cell development that result from these interactions can be defined by sequential rearrangements of Ig loci, surface expression of various stage-specific markers, distinctive growth factor requirements, and the acquisition of functional specializations (10-12).

The complexity of B cell development makes difficult a detailed in vivo analysis of these developmental events. Our knowledge of these processes has been appreciably expanded through the study of established, long term, stable B lymphocyte culture systems. These early stage B-lineage cells have been obtained exclusively from fetal liver and bone marrow. Our purpose has been to study B cell development in the mouse embryonic yolk sac at a stage preceding the time that B-lineage cells can be detected in the fetal liver, with the promising possibility that we might be able to detect new and possibly unique early stages in B cell development.

During the course of our studies, we detected an aberrant culture in which early yolk sac hemopoietic stem cells (AA4.1 +; nonadherent; density, 1.077), explanted onto a bone marrow stromal cell (S17) feeder layer, resulted in the formation of a large number of uniform, small lymphocytes, and that these cells proliferated readily while maintaining their appearance on transfer to new S17 feeder layers. We show that these cells, which we describe in terms of their cell surface Ags, Ig gene status, and response to growth factors, appeared to be arrested at the boundary between pre-pro-B cells and pro-B cells, and they could subsequently be induced to give rise to mature B cell lineages both in vitro and in vivo.

Materials and Methods

Mice

BALB/cAu (Ly5.1) and C57BL/Au (Ly5.2; originally from E. A. Boyse) mice were generated in our own colony. The appearance of the vaginal plug was designated day 0 of gestation. Four- to 6-wk-old C57BL/Au (Ly5.2) mice were used in reconstitution experiments.

Antibodies

The following Abs specific for surface markers were used: CD43, CD34, FITC-CD44, phycoerythrin-heat stable antigen (HSA), FITC-CD3, FITC-H-2K β/β/H-2La, FITC-sIgM (μ chain), FITC-CD5, FITC-CD19, and goat

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3 Abbreviations used in this paper: HSA, heat stable antigen; sIgM, surface immunoglobulin M; YS, yolk sac; YS-pre-pro-B, yolk sac-derived early progenitor B cells.
anti-rat or mouse IgG-FITC from PharMingen (San Diego, CA); FITC-CD4, phycoerythrin-CD8, and FITC-Thy-1.2 from Becton Dickinson (Mountain View, CA); anti-murine c-kit (ACK-2) from Life Technologies (Gaithersburg, MD); AA4.1 Ab from J. P. Mckearn (Searle/Monsanto, St. Louis, MO); Biotin-Sca-1 from I. L. Weissman (Stanford University, Stanford, CA); FITC-avidin from Tago (Burlingame, CA); Joro37.5 from R. P. Leder (The Children's Cancer Center, Houston, TX); B220 from P. Kincade (Oklahoma Medical Research Institute), anti-Mac-1 from the American Type Culture Collection (Manassas, VA); FITC anti-mIL-7R from M. Sandor (University of Wisconsin-Madison); anti-Ly5.1 and anti-Ly5.2 from R. R. Hardy (University of Pennsylvania School of Medicine); goat anti-mouse polyclonal Ab from PharMingen (San Diego, CA); and anti-mouse polyclonal Ig (IgG, IgA, IgM) conjugated with alkaline phosphatase from Sigma (St. Louis, MO).

Isolation of AA4.1+ yolk sac cells

Single cell suspensions were prepared from yolk sacs isolated from day 10 BALB/c (Ly5.1) mouse embryos. AA4.1+ yolk sac cells were isolated according to previously published methods (2). In brief, yolk sac cells were separated using a discontinuous gradient of Percoll (Sigma; 1.054, 1.066, and 1.077 g/ml) in 5 ml of 0.05 M Tris-HCl/0.15 M NaCl, pH 9.5, at room temperature for 1 h. After washing and blocking with PBS and 1% FBS, cells were layered onto Ab-coated plates in 5 ml of RPMI 1640/5% FBS and incubated at 4°C for 1 h. Nonadherent cells were removed, and plates were washed 8 to 10 times with PBS/5% FBS. The remaining adherent cells were then collected by forceful pipetting. The cell suspension, as determined by flow cytometry, was shown to contain >95% AA4.1+ cells.

Establishment of the pre-pro-B cell line (YS-PPB) and clonal sublines

The enriched AA4.1+ cells were dispensed onto a subconfluent layer of irradiated (2500 rad, \(^{137}\text{Cs}\) ) S17 bone marrow stromal cells in 96-well plates (two or three cells per well) at limiting dilution and cultured in the absence of growth factors. Half the medium was replaced every 5 days. Fifteen days later, mixed colonies consisting of different cells based on cell size and morphology were present in the cocultures. Individual mixed colonies were removed, transferred onto fresh irradiated S17 monolayers in 24-well plates for further cultivation, and passaged every 5 days. Although mixed colonies generally gave rise to more mixed colonies or differentiated into various myeloid lineages, a culture was identified that eventually consisted of a uniform population of small lymphocytes. This culture was further expanded and passaged repeatedly to establish a stable cell line (designated YS-PPB). For cloning of the cells, 96-well plates were prepared with near-confluent, irradiated S17 stromal cells. Single yolk sac cells from the fifth passage were then deposited into each well by cell sorting in flow FACStar™ Plus, Becton Dickinson, Mountain View, CA. The cells were incubated at 37°C in 5% CO\(_2\) for 1 h, after which nonadherent cells were collected. Polystyrene dishes (Falcon 1001, Falcon Plastic, Oxnard, CA) were precoated with protein G-purified mAb AA4.1, and incubated at 4°C for 1 h. Nonadherent cells were removed, and plates were checked for cultures showing positive growth of clones. Clones were amplified by RT-PCR using second-step reagents for 30 min where required, washed, and then resuspended in FACS buffer (PBS/1% BSA). Flow cytometric analysis was conducted using a FACSScan instrument (Becton Dickinson) and LYSYS-II or PC-LYSYS analysis software.

Immunofluorescence detection of cytoplasmic Ig \(\mu\)-chain

Cytopears were prepared using a Shandon Cytoprobe centrifuge (Shandon, Pittsburgh, PA), air-dried, and fixed (13). After washing, the slides were blooted to remove excess buffer and placed in a humidified chamber. About 10 \(\mu\)l of FITC-conjugated goat anti-mouse IgM (\(\mu\)-chain) Abs (Caltag, San Francisco, CA) was placed on the cell smears. Slides were removed from the chamber after 30 to 40 min and washed in PBS/0.1% sodium azide. A coverslip was mounted on the stained, washed cytopears with Fluormount G (Southern Biotechnologies, Birmingham, AL). Slides were observed under an immunofluorescence microscope and scored for FITC staining.

RT-PCR assay

Total RNA was extracted from 0.5 to \(1\times10^5\) cells including uncloned cultured cells, clonal YS pre-pro-B cells, S17 cells, and spleen cells from adult BALB/c mice using TRIzol reagent (Life Technologies) according to the procedure recommended by the manufacturer. (158-base, \(5\)-AAAAAGACTGACAGGCGAT TCTTAC-3') and for \(\gamma\)D-1 rearrangement (158-base, \(5\)-CAGTCACACTGCGACAG-3') were amplified by RT-PCR using \(TdT\), \(\alpha\), and \(R\gamma\)1 primers (synthesized by Biotechnology Center, University of Wisconsin, Madison), using primer sets exactly as previously reported (14, 15), and subjected to 32 PCR cycles (2400 GeneAmp Thermal Cycler, Perkin-Elmer, Norwalk, CT). Each cycle consisted of denaturing at 94°C for 50 s, annealing at 53°C for 60 s, and polymerizing at 72°C for 70 s. Amplification of the \(\beta\)-actin gene using the previously described (16) standard primer set (synthesized by Biotechnology Center, University of Wisconsin, Madison) was used throughout as a control for template integrity and for normalization of data to a constitutively expressed transcript.

Genomic DNA isolation and PCR assay

For PCR assay for Ig gene configuration, the genomic DNA was extracted from the same cell populations as those above using the QIAamp Blood Kit (Qiagen, Chatsworth, CA) according to the manufacturer’s directions. DNA quantity and quality were determined by OD scanning. PCR oligonucleotides described previously (12, 17), including specific primers for D-J\(_\gamma\)_rearrangement (DSF, \(5\)-GGGGACACCTTTGGTGUCGAGG(T)ATC TTCATCTGTG-3') and \(\alpha\)-chain (\(5\)-AAAAAGACTGACAGGCGAT TCTTAC-3') and for \(\gamma\)D-1 rearrangement (158-base, \(5\)-CAGTCACACTGCGACAG-3') were used to amplify the \(\beta\)-actin primer set (same as above). Two micrograms of DNA from each sample was used for PCR amplification. Conditions for PCR were 1 min of denaturation at 94.5°C, 30 s of annealing at 70°C, and 1.3 min of polymerization at 72°C. This cycle was repeated 32 times.

Hybridization analysis

Twenty microliters of each PCR reaction product were separated by electrophoresis in a 1.5% agarose gel and transferred to nylon membranes (GeneScreen Plus, Boston, MA) by vacuum transfer using VacuumGene equipment (Bio-Rad, Hercules, CA). The membranes were rinsed in 2 × SSC, UV cross-linked, prehybridized for 1 h at 55°C in 0.25 M Na\(_2\)PO\(_4\) (pH 7.2) containing 1 mM EDTA and 7% SDS, then hybridized in the same solution as that used for the prehybridization but containing 3 pmol/ml biotinylated oligonucleotide probe including \(5\)-AAATAGCAGACAGGCGAT TCTTAC-3' (18). \(TdT\) (\(5\)-ATTCTCTCTGGTGTG GCATAC-3') (19), \(\alpha\)-chain (\(5\)-ACCCAAATCGGGGCTTAGGAT-3') (15), \(R\gamma\)-1 (\(5\)-TCTGTTGGCTGCTTTTACA-3') (19), and \(\beta\)-actin (\(5\)- TTGGATTTGTGACAGCAAT-3'). (16) were hybridization probes as a control for the specificity of the oligonucleotide probes. After washing, the hybridized probes were detected by using a Southern-Light test kit (Biotex, Life Technologies). Kodak XAR15 film exposures (Eastman Kodak, Rochester, NY) were used to detect the chemiluminescent signals.

Cloning and sequencing

The bands of interest (putative D-J\(_\gamma\)_ rearrangements or possible primer dimers) were excised and purified from 1.5% agarose gels, and then cloned directly with a TOPO TA Cloning kit (Invitrogen, San Diego, CA). Plasmid DNA was prepared from white, kanamycin-resistant colonies using the S.N.A.P. MiniPrep kit (Invitrogen) and were sequenced with M13 primers (Invitrogen).
ELISAs

Limiting dilution cultures were established by plating clonal and uncloned YS-pre-pro-B cells at a specified concentration (24 or 36 replicates/cell concentration) on irradiated S17 cell feeder layers in 96-well plates with IL-7 at 0.1 ng/ml and LPS (25 μg/ml). Supernatants were collected after 12 to 14 days of culture. A double-sandwich ELISA was conducted as described previously for determination of the total Ig in culture supernatants (20). Briefly, immuno-plates were coated with 200 μl of the appropriate dilution of purified anti-mouse polyclonal Ab overnight at 4°C. After washing, 200 μl of serial dilutions of mixed IgM, IgG, and IgA standards (Sigma) in PBS/0.05% Tween 20 were added to the plates to generate a standard curve. Dilutions of each culture supernatant were assayed in parallel. After 2 h at room temperature, plates were blocked with 2% BSA in PBS at 37°C for 2 h followed by washing. Anti-mouse IgM/IgG/IgA (1/1000) coupled to alkaline phosphatase (Sigma) was applied and incubated and then cultured on irradiated S17 stromal cells. Wright-Giemsa staining of YS-PPB cells (magnification, ×160). B, Wright-Giemsa staining of YS-PPB cells (magnification, ×320).

B cell differentiation in vivo

The induction of differentiation of YS-PPB cells into sIgM+ B lymphocytes was conducted in 35-mm dishes containing irradiated S17 stromal cells and both IL-7 (0.1 ng/ml) and LPS (25 μg/ml, Sigma). Following 5 to 6 days of culture, 1 ml of fresh medium was added to each dish. After 12 to 14 days of culture, sIgM+/B220− cells were identified by flow cytometric analysis.

Reconstitution of irradiated mice

For repopulation of lymphoid-lineage compartments of irradiated C57BL/6 Ly5.1+ mice (1200 rad, split dose, 3-4 h interval, 166 rad/min), YS-PPB clone 2 cells (BALB/c Ly5.1+, 5 × 10^6 cells/mouse) were injected i.v. into recipient animals 4 to 6 h after irradiation. Radioprotection was provided by injection of 2 × 10^5 host-syngeneic C57BL/6 Ly5.2+ bone marrow cells. Recipient animals injected with BALB/c bone marrow cells (Ly5.1+, 5 × 10^6 cells/mouse) or PBS only were used as positive and negative controls. Twelve to sixteen weeks post-transplantation, lymphoid repopulation of spleens was assessed by flow cytometric analysis.

Results

Establishment of early yolk sac-derived pre-pro-B cell (YS-pre-pro-B) clones

AA4.1+ yolk sac cells (AA4.1+ cells, >95% determined by flow cytometry) were enriched by plastic adherence, differential centrifugation, and immunocytoadherence as described in Materials and Methods and then cultured on irradiated S17 stromal cells without the addition of exogenous growth factors. Half of the medium was replaced at 5-day intervals. After day 15 of culture, the formation of mixed colonies (4.2%) was observed under low power magnification. The round nonadherent cells were gently recovered from individual mixed colonies and then transferred onto new irradiated S17 feeder layers in 24-well plates for further expansion. About five passages later, the cells in one culture appeared to be small and lymphoid-like, while macrophage-like cells were absent. For cloning of these cells, single cells were deposited onto S17 cells in 96-well plates by FACS sorting. We found that with this protocol, 24.7% cloning efficiency could be obtained in 768 cultures conducted (cloning frequency, about one of four cells). From 189 clones, 13 clones with high proliferation potential (>5 × 10^5 cells/well) were selected for further expansion on stromal cells and were established as long term proliferating clonal sublines. Uncloned cells and four of these clones (clones 1, 2, 7, and 11) were selected at random for further expansion and characterization. Figure 1A shows the morphology of YS-PPB B cells grown on S17 stromal cells. Wright-Giemsa staining of these cells is shown in Figure 1B.

Table 1. Expression of surface markers on YS-pre-pro-B cellsa

<table>
<thead>
<tr>
<th>Surface Ag</th>
<th>Uncloned</th>
<th>Clone 1</th>
<th>Clone 2</th>
<th>Clone 7</th>
<th>Clone 11</th>
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<td>+</td>
<td>+</td>
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</table>

a Nonadherent cells were removed from the S17 feeder layers, washed in PBS/5% FBS, and then stained with a panel of Ab against cell surface antigens. Flow cytometric data were obtained for cells gated on lymphoid-like cells on the basis of low forward and side scatter signals. Cells were considered to be positive (+) or negative (−) if they were >98% positive or >98% negative for the indicated surface markers.
IL-7R, MHC class I, and B220, but sIgM, MHC II, CD5, and CD44 were not detected on the uncloned cells or any of the clonal sublines. None of the cells expressed cytoplasmic Ig heavy chain (\(\mu\)-chain) proteins or surface markers associated with pre-T (Joro37.5), mature T lymphocytes (Thy1.2, CD3, CD4, and CD8), or myeloid lineage (Mac-1) cells. As shown in Figure 2, however, expression of c-Kit varied with the different clones. Clones 7 and 11 were uniformly c-Kit\(^+\) and were present on a subpopulation of the parent line as were clones 1 and 2 (37-69%). The observed phenotypes suggest that the yolk sac-derived, long term cultured cells have been committed to an early stage of B cell development.

Ig gene status

We examined the rearrangement-associated genes TdT, \(\lambda\)5, and RAG-1 using RT-PCR. As shown in Figure 3, all clonal sublines as well as the uncloned parental cells expressed TdT, \(\lambda\)5, and RAG-1. These genes have been shown to be expressed in early B cells such as pro-B cells (10, 21) and pre-B cells (22). In contrast, mRNA for these same genes was not detected in mature splenic cells or S17 cells used as controls. To further confirm the developmental staging of the cells, IgH gene rearrangements were evaluated using PCR amplification analysis. As shown in Figure 4, DSF primer together with a primer complementary to a segment of DNA 3' of \(J_{H4}\) could amplify a ladder of rearranged DJ\(_{H4}\) products. In positive control spleen cells, four bands of the sizes expected for rearrangements to the four \(J_{H}\) elements were detected with the \(J_{H4}\) probe. Although no DJ\(_{H1-3}\) rearrangements was detectable, a band seen in the pre-pro-B cell line and its clones was close in size to the DJ\(_{H4}\) rearrangement seen in splenic cells.

To ascertain whether these bands represented DJ\(_{H4}\) rearrangements or possible primer dimers, sequence analysis illustrated that whereas the putative Ig rearrangement bands obtained from spleen cells (three clones) contained the expected DJ\(_{H4}\) and \(J_{H4}\) segments, the bands (nine clones) seen in pre-pro-B cells were primer-dimers rather than rearranged DJ\(_{H4}\) (data not shown). IgH V-D-J rearrangements were undetectable in any of the yolk sac-derived pre-pro-B cells, in contrast to the positive control spleen cells, where these were readily detected.

Characterization of clonal cell growth

To determine whether contact between YS-PPB and stromal cells was mandatory, YS-PPB cells were removed from coculture with S17 stromal cells and replated on S17 cell monolayers (positive control), in a diffusion chamber where the cells were prevented from direct contact with the stromal cells, or in medium only. Cell recovery under these different culture conditions was measured after day 3 of culture. No cells were recovered in either transfilter cultures or in cultures grown in the absence of stromal cells (Figs. 5 and 6).

The role of soluble IL-7 in the growth of YS-PPB cells was assessed by removing the cells from the stromal layers and replating them in either medium alone or medium supplemented with IL-7. Net growth was determined by viable cell counts after 3 days. Figure 5 shows that no proliferation was observed under these conditions. However, in the presence of stroma the proliferation of uncloned cells and clone 7 was augmented by IL-7, while...
clones 1, 2, and 11 were unresponsive to IL-7 under these conditions (Fig. 5) despite the fact that they also expressed IL-7R. The results obtained by cell counting were in agreement with those obtained by measuring [3H]TdR incorporation (data not shown). The growth of uncloned or clone 7 YS-pre-pro-B cells could not, however, be achieved by combining IL-7 with S17-conditioned medium (data not shown), indicating that the growth of these cells was directly dependent on signals mediated by direct contact with stromal cells and/or membrane-bound growth factors produced by these stromal cells.

**Frequency of Ig-producing B cell precursors**

The uncloned YS-PPB cells as well as the four cloned sublines were tested for their potential to produce IgGs following stimulation in vitro. The frequency of Ig-producing B cell precursors was determined by limiting dilution analysis as described in Materials and Methods. When cultured on S17 cells, all clonal sublines as well as the uncloned cells were able to respond to dual stimulation by IL-7 and LPS to produce IgGs, but none of them was able to produce Ig either on the stroma alone or with stimulation by IL-7 or LPS alone. Among these cells, the frequency of Ig-producing cells ranged from a high of 1 in 425 in clone 1 to a low of 1 in 2680 in clone 11 (Fig. 7).

**In vitro and in vivo differentiation of YS-pre-pro-B cells**

The fact that YS-PPB cells and the clonal sublines were capable of producing IgGs in response to IL-7 and LPS suggested that these cells have the potential to differentiate into IgG-producing mature B cells. We therefore cultured cells for a longer period of time (12-14 days stimulation with LPS plus IL-7) and analyzed the resultant cells by flow cytometry. The results shown in Figure 8 indicate that both the uncloned and clonal cells could be induced to differentiate into IgM⁺ B cells, although there were differences in the percentage (11-33.7%) of IgM⁺ cells between them. Once again, however, neither IL-7 nor LPS alone had this effect on the differentiation of the pre-pro-B cells (data not shown). In addition, expression of MHC II Ag could be detected on the tested cells (data not shown).

For in vivo differentiation, YS-PPB cells (clone 2) were tested for reconstituting capacity of them by transferring them into irradiated Ly5.2⁺ mice. Three months after cell injection, splenic cells free of erythrocytes were prepared from recipients and stained for B cell markers. As shown in Figure 9, nearly all (>95%) of the donor-derived Ly5.1⁺ lymphocytes expressed B220 and IgM (Fig. 9D). Furthermore, donor-derived B220 and IgM-positive cells (Ly5.1⁺) represented 71 and 54%, respectively, of the total population of B220 and IgM-positive cells (Fig. 9C); the remaining B220 and IgM-bearing cells were of host origin (Fig. 9E).

**Discussion**

Our studies have described a long term, stable, pre-pro-B cell line that originated from the primitive hemopoietic stem cells (AA4.1⁺; nonadherent; density, <1.077) found in the mouse 10-day old embryonic yolk sac. This cell line as well as several clonal...
sublines derived from it required only a subjacent feeder layer of S17 stromal cells, was not dependent on extraneous addition of growth factors, and could be expanded and maintained for >1 yr. Even after 1 yr, during which the cell line maintained the germline Ig gene configuration, the cells retained the capacity for B cell maturation. This was shown for both the YS-pre-pro-B cell line and several of the YS-pre-pro-B cell clones and could be observed in 2-wk cultures grown on S17 stromal cells and stimulated by IL-7 and LPS as well as following cell transfer into irradiated, histoincompatible, allogeneic mice. In contrast, whereas both freshly isolated and in vitro propagated AA4.1-yolk sac stem cells readily differentiate into T cells when grown in combination with stem cell-depleted embryonic thymus rudiments (2, 4, 23), neither the parental YS-PPB cell

**FIGURE 8.** In vitro differentiation of YS-pre-pro-B cells. YS-PPB cells were cultured with S17 stromal cells in the presence of IL-7 (0.1 ng/ml) and LPS (25 μg/ml). Twelve to fourteen days later, nonadherent cells were collected and analyzed for expression of B cell markers. A, Before stimulation. B, After stimulation.

**FIGURE 9.** In vivo differentiation of YS-pre-pro-B cells. For in vivo reconstitution, clone 2 YS-PPB (BALB/c; Ly5.1) cells (5 × 10⁶/mouse) along with 2 × 10⁶ C57BL/6 bone marrow cells were injected i.v. into lethally irradiated C57BL/6 Ly5.2⁺ mice. The presence of donor-derived Ly5.1⁺ B cells in spleen of recipients was determined by flow cytometric analysis after 3 mo. A, Lymphocyte gate based on light scatter. B, Gated drawn to identify Ly5.1⁺ (donor) and Ly5.1⁻ (host) cells. C, B220 and slgM staining of total lymphocyte population. D, B220 and slgM staining of donor cell population. E, B220 and slgM staining of host cell population.
We also considered the possibility that the isolated AA4.1 with its potentiating effect on proliferation of early B cells. IL-7, as assessed by bioassays or PCR (31, 32), although S17 we (unpublished observations) and others have been unable to deuced IL-7 required for growth of YS-pre-pro-B cells. However, cells from their extraembryonic site. That the dependence on specific growth factors represents a developmenal stage, and for this reason it should prove to be valuable in the study of B cell differentiation.

The fact that our yolk sac cell line could proliferate independent of IL-7 contrasts with previous reports of early B cell progenitors isolated from other embryonic sites. For example, in addition to yolk sac, fetal liver-derived pro-B cells required IL-7 for maintenance and growth in long term culture (27). Furthermore, it has previously been shown that S17 cells alone are insufficient to induce proliferation of the precursor AA4.1 12-day fetal liver cells, which proliferate only when S17 cells are combined with IL-7 (28). Human fetal bone marrow pro-B cells also require IL-7 as well as human primary stroma to promote cell expansion and optimal growth (29, 30). Our observations support the argument that the dependence on specific growth factors represents a developmental event that occurs subsequent to emigration of yolk sac cells from their extraembryonic site.

We examined the possibility that our S17 cells actually produced IL-7 required for growth of YS-pre-pro-B cells. However, we (unpublished observations) and others have been unable to detect IL-7, as assessed by bioassays or PCR (31, 32), although S17 cells do produce the ligand for the c-Kit receptor (c-Kit ligand) (31) with its potentiating effect on proliferation of early B cells. We also considered the possibility that the isolated AA4.1 yolk sac cells were contaminated with yolk sac stromal cells that produce IL-7 to support the growth of pre-pro-B cells, but such cells could not be detected by flow cytometric analysis of the starting cell population. Our results lead to the suggestion that the signals mediated by interactions between S17 stromal cells and the most primitive yolk sac hemopoietic stem cells are required for the development and the maintenance of yolk sac-derived IL-7-independent pre-pro-B cells. The critical signals could be triggered via membrane-bound factors produced by S17 stromal cells. Alternatively, this direct contact between YS-pre-pro-B cells and S17 cells may induce the production of essential, but not yet identified, factors by the stromal cells, and it is these factors that, in turn, support the growth of yolk sac-derived pre-pro-B cells. Some evidence in support of this latter possibility has been reported (33, 34). Our finding that clones 1, 2, and 11 subsequently failed to respond to IL-7, whereas the other clones became responsive suggests that clones 1, 2, and 11 represented a more primitive pre-pro-B cell population. However, it should be pointed out that all of our YS-pre-pro-B cells expressed IL-7R, so the fact that clones 1, 2, and 11 were unable to grow in response to IL-7 could reflect a block downstream of the IL-7R that renders the cells nonresponsive to IL-7 (35).

A recent report by Cumano et al. (36) that B cell precursors can be identified in the day 7.5 to 8 mouse embryo within the region destined to become the aorta/gonad/mesonephros has led to the suggestion that all hemopoietic stem cell precursors originate in a unique population of stable pre-pro-B cells further strengthens that belief, because these cells can be expanded in long term culture, yet are subsequently able to proliferate and differentiate in vivo. Our report emphasizes the fact that not all early pro-B cells are alike, and that there may well be not only definitive points in the

### Table II. Comparison of pre-pro-B and pro-B bone marrow cells with the yolk sac pre-pro-B cell line described in this report

<table>
<thead>
<tr>
<th>Cells</th>
<th>Surface Molecule</th>
<th>Gene Expression</th>
<th>Growth Dependence</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>AA4.1</td>
<td>CD43</td>
<td>B220</td>
</tr>
<tr>
<td>Bone marrow*</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lymphoid progenitor</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Pre-pro-B</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Fr.A1</td>
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<td>+</td>
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<td>Fr.A2</td>
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<td>+</td>
<td>+</td>
</tr>
<tr>
<td>YS-pre-pro-B</td>
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<td>+</td>
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<tr>
<td>Bone marrow* pro-B</td>
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<td>+</td>
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<td>+</td>
</tr>
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* From Refs. 10, 12, and 24.
References


