

Clonal lymphoid progenitor cell lines expressing the *BCR/ABL* oncogene retain full differentiative function

(B-cell development/stem cell/oncogenic transformation/chronic myeloid leukemia)

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Communicated by Irving L. Weissman, December 26, 1989 (received for review November 29, 1989)

ABSTRACT The early stages of hematopoiesis have been difficult to study due to problems in obtaining homogeneous populations of progenitor cells that retain both self-renewal and differentiative capacities. We have developed an *in vitro* system in which transformation of murine bone-marrow cells with the *BCR/ABL* oncogene, a gene associated with stem-cell leukemias, leads to the outgrowth of clonal lines that have an early lymphoid progenitor cell phenotype. The progenitor cells retain immunoglobulin heavy and light chain genes in a germ-line configuration. These cells give rise *in vitro* to pre-B cells that have diverse diversity-joining (D–J) region rearrangements, and on transfer to mice with severe combined immune deficiency, differentiate to surface IgM⁺, immunoglobulin-secreting B cells that respond to T-cell help and function in an antigen-specific fashion. Although their growth is stimulated by *BCR/ABL*, the progenitor cells depend for continued growth on a stromal cell-derived soluble factor distinct from the pre-B-cell growth factor, interleukin 7. These findings show that *BCR/ABL* can promote proliferation of an early hematopoietic progenitor cell without preventing its differentiation. This system provides a means of studying the complete B-cell developmental process from clonal progenitor cell to end-stage plasma cell.

B lymphopoiesis occurs in the bone marrow of adult mammals, originating from a pluripotent hematopoietic stem cell (1, 2). The first descendent of the stem cell identified as committed to the B lineage is the pro-B cell. This cell type is characterized by its lack of rearrangement at the immunoglobulin heavy and light chain loci and lack of expression of the lineage-specific surface antigens B220, MAC-1, and Thy-1 (3, 4). The pro-B cell has been thought to differentiate into mature, functional B cells by a series of sequential steps (5, 6). These stages are defined by rearrangement of diversity (D) and joining (J) region segments on at least one allele of the immunoglobulin heavy-chain locus (immature pre-B stage), productive variable (V)–DJ region joining and cytoplasmic IgM expression (pre-B cell), and finally production of functional immunoglobulin light chain and surface expression of IgM, IgD, and Ia molecules (mature B cell).

Due to difficulties in maintaining in culture early hematopoietic progenitors that retain both their self-renewal and differentiative capacities, very little is known about the signaling mechanisms that commit those cells to the B lineage and/or allow B-cell progenitors to progress from one stage to the next. One approach to this problem has been to manipulate long-term bone-marrow culture conditions to selectively expand B-cell progenitors (7). Another approach has been to immortalize the progenitor cells by virus transformation (8–10). Most of this work has used Abelson murine leukemia virus as the transforming agent. A target of *v-abl*

appears to be a B220⁺, Thy-1 B progenitor (10), but most lines obtained after transformation are of the pre-B-cell phenotype (11). Although these lines retain some differentiative potential based on their ability to undergo light chain rearrangement after transformation, efficient differentiation to mature, functional B cells has not been shown.

Several lines of evidence suggest that transformation by the P210 chimeric protein product of the *BCR/ABL* oncogene may provide a means to stimulate early progenitor cell growth without arresting differentiation. This gene product is found in >90% of patients with chronic myeloid leukemia and results from a translocation that juxtaposes portions of the *BCR* gene on chromosome 22 near the second exon of *c-ABL* on chromosome 9 (12). The *BCR/ABL* translocation appears to occur in a pluripotent stem cell. In the chronic phase of the disease, predominantly myeloid proliferation occurs, although the lymphoid lineage is also affected (12). Importantly, differentiation is not completely blocked. *In vitro*, P210-expressing hematopoietic cells and fibroblasts have altered growth characteristics but are not fully transformed (11).

By modifying conditions established by Whitlock and Witte (13) for long-term bone-marrow culture and by using the *BCR/ABL* oncogene as a transforming agent, we have derived clonal lymphoid progenitor lines that retain their differentiative capacity. These cells are more immature than populations maintained in other modified long-term bone-marrow cultures, and addition of exogenous cytokines is not required to maintain an immature phenotype. This system should be useful for studying the molecular mechanisms that regulate B-cell lymphopoiesis.

MATERIALS AND METHODS

Mice and Culture Conditions. BALB/c mice and CB.17 mice homozygous for the mutation for severe combined immune deficiency (SCID mice) (14) were bred and maintained in our colony at the University of California—Los Angeles. Single-cell suspensions of bone-marrow cells isolated from the femurs and tibias of 3- to 4-week-old BALB/c mice were obtained and infected with *BCR/ABL*-expressing virus (15). The cells were then washed and plated at 1×10^6 cells per ml in RPMI 1640 medium supplemented with 5% fetal calf serum and 50 μ M 2-mercaptoethanol onto preestablished confluent layers of the stromal cell line S17 (16) in 6-cm Petri dishes (5 ml per dish). The cultures were maintained by replacing 75% of the medium twice weekly and by seeding onto new S17 monolayers every 3–4 weeks. The construction of the pJW-RX retroviral construct that contains the complete P210 *BCR/ABL* gene product in the vector

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Abbreviations: LBMC, lymphoid bone-marrow culture; LPC, lymphoid progenitor culture; SCID, severe combined immune deficiency; TNP, 2,4,6-trinitrophenyl; IL-7, interleukin 7.

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pMV6tkneo and the production of viral stocks have been described (15).

Protein and DNA Analyses. Preparation of lysates, immunoprecipitation, autophosphorylation with [γ - 32 P]ATP, and PAGE of P210 protein from cultured cells was as described (17) with rabbit anti-abl sera pEX-2,3 (17, 18). High-molecular-weight DNA was prepared and analyzed as described (19). All probes were labeled with [α - 32 P]ATP by Random Primed DNA Labeling (Boehringer Mannheim) and had specific activities of $\approx 1 \times 10^9$ cpm/ μ g of DNA. Descriptions of specific probes and restriction enzyme digests are given in the figure legends.

SCID Mice Reconstitution and Analysis. SCID mice, 4–6 weeks of age, were reconstituted by i.v. transfer of $1\text{--}5 \times 10^6$ cultured cells. All mice were given 3 Gy of γ irradiation 18–24 hr before injection (20). For experiments that examined responsiveness to T-cell help, 2×10^6 fresh BAB-14 thymocytes that had been passed over nylon wool were included in the inoculum. Antigen-specific responsiveness was determined by immunizing animals i.p. with 70 μ g of 2,4,6-trinitrophenyl (TNP)-Ficoll 4–6 weeks after reconstitution. The animals were boosted 2 weeks later and bled after an additional 7 days. The percentage of surface IgM⁺ cells present in the spleens of reconstituted SCID mice was determined by using fluorescein isothiocyanate-conjugated goat anti-mouse IgM (Fisher Biotech, Orangeburg, NY). Cells were analyzed on an Epics C fluorescence-activated cell sorter (Coulter Electronics). Total IgM and IgG and TNP-specific antibodies present in the serum of reconstituted SCID mice were determined by solid-phase ELISA, as described (21).

RESULTS

Establishment of Clonal Cell Lines with a Progenitor Cell Phenotype. Lymphoid bone-marrow cultures (LBMC) established under conditions derived by Whitlock and Witte (13) consist of two phases: (i) an adherent layer that provides the lymphokines and cell-cell interactions required for lymphopoiesis and B-cell growth, and (ii) a nonadherent population composed of predominantly pre-B cells but also progenitor cells capable of repopulating the B-lymphocyte compartment of immunodeficient mice (21, 22). To isolate clonal populations of the progenitor cells, LBMC conditions were modified in two ways (Fig. 1). (i) The cells were plated onto the clonal stromal line S17 because it has been shown to form a contact-inhibited, confluent adherent layer capable of supporting early B-cell precursors (16). In these cultures, a bone marrow-derived, heterogeneous adherent layer does not form. (ii) The bone-marrow cells were infected with a retroviral construct containing the P210 *BCR/ABL* gene before plating on S17 stroma.

Most cells in cultures established on S17 stroma [referred to as lymphoid progenitor culture (LPC)] had not undergone rearrangement at the immunoglobulin heavy chain locus (Fig. 2A). This contrasts markedly with P210-expressing bone-marrow cultures established on a heterogeneous adherent layer that have rearranged immunoglobulin heavy chain but not light chain alleles, characteristic of early pre-B cells (15). κ and T-cell receptor β chain genes were also in germ-line configuration in the S17-derived LPC (data not shown). Phenotypically, the LPC cell appeared similar to a B progenitor recently found to compose 1–2% of normal bone marrow (10). Approximately 80% of the cells in the cultures expressed the B-lineage markers B220 (53–98%) and BP-1 (60–83%) (data not shown). The cells were negative for the myeloid surface antigen MAC-1 but expressed low levels of Thy-1. No myeloid/erythroid stem cells or granulocyte/macrophage precursors were detectable in the cultures as

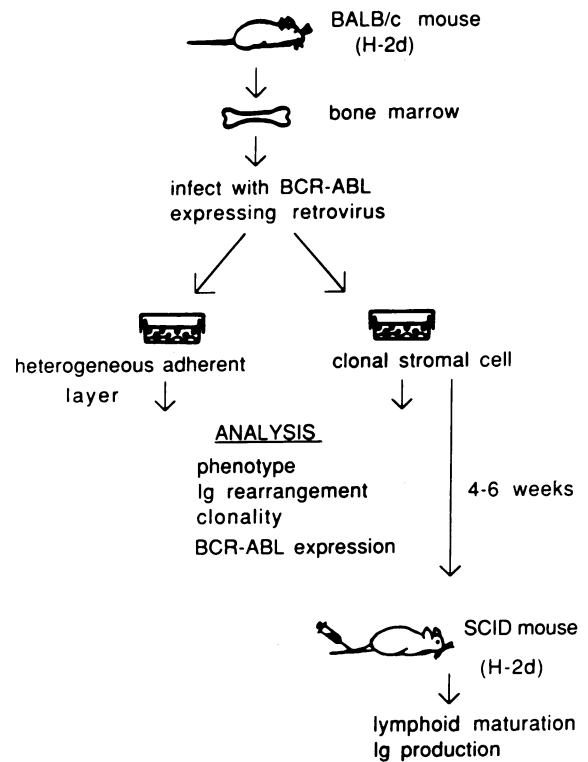


FIG. 1. Protocol for the generation and functional analysis of lymphoid progenitor cells.

measured by spleen colony (24) or *in vitro* colony forming assays (25), respectively (data not shown).

All cultures contained one predominant site of viral integration, indicating that the cultures contained the retroviral genome and that the cells were clonally derived (Fig. 2B). The cell lines could also be shown to have an integrated copy of the *BCR/ABL* gene and to express high levels of ABL mRNA of retroviral genome length [≈ 9.5 kilobases (kb)] (data not shown). All P210-infected cultures expressed P210 protein tyrosine kinase activity as measured by immunoprecipitation-autophosphorylation (Fig. 2C). The kinase activity was shown to result from P210 protein expression in >98% of the cells in most cultures as determined by immunoperoxidase staining (data not shown).

Lymphoid Progenitor Cells Do Not Respond to Interleukin 7 (IL-7). Cultures established on S17 stroma in the absence of *BCR/ABL* were also of an early lymphoid progenitor cell phenotype and could be maintained long-term *in vitro* (data not shown). P210-expressing LPCs, however, displayed a 5- to 10-fold increase in cell density compared with uninfected cells. The *BCR/ABL*-immortalized lines, similar to uninfected cultures, remained dependent on the S17 stroma for growth for >3 months (Table 1) and, therefore, were distinguishable from hematopoietic cells transformed with *v-abl* (13, 26), *src* (27), or *myc/ras* (19), which usually become growth-factor independent after several weeks.

LPC could be maintained in 50% S17 supernatant, suggesting that the role of the S17 stroma was at least partially mediated by soluble factors (Table 1). IL-7 has been shown to be important for pre-B-cell growth (30–32). Production by the S17 stromal line of IL-7 or its mRNA was undetectable by bioassay and polymerase chain reaction (data not shown). In addition, the LPCs could not be maintained in IL-7-containing cell-free supernatants (Table 1). This contrasts to a pre-B-cell clone, clone 4 (28), which increased 10-fold in cell number over a 5-day period under the same conditions.

P210-Expressing LPCs Undergo Immunoglobulin Rearrangements *in Vitro*. We observed that the cells in the culture

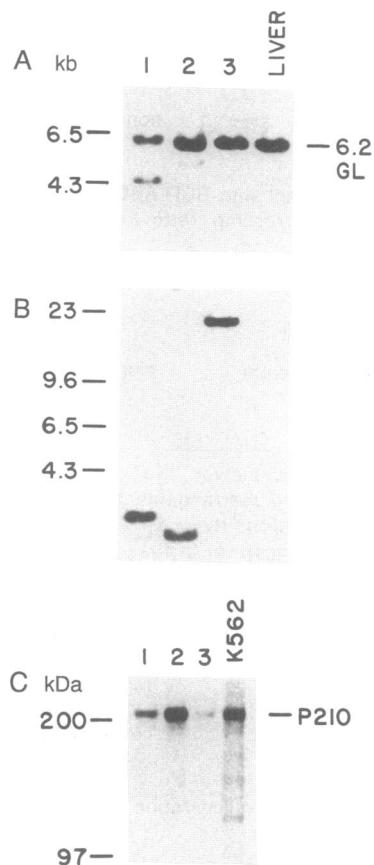


FIG. 2. Clonal outgrowths of bone-marrow cultures infected with *BCR/ABL* retain germ-line (GL) immunoglobulin. High-molecular-weight DNA was isolated from BALB/c liver or P210-infected LPC lines (lanes 1–3) 3–4 weeks after establishment. Ten micrograms of DNA was digested with *EcoRI*, electrophoresed through 0.8% agarose gel, transferred to nitrocellulose, and probed with 1.8-kb heavy chain J fragment (23) (A) or 1.2-kb *neo* probe (15) (B). Size markers are from *HindIII* digested λ bacteriophage DNA. (C) P210 autokinase assay: lysates of 2×10^6 progenitor cells from individual cultures (lanes 1–3) or from the human chronic myeloid leukemia-derived line K-562 were immunoprecipitated for tyrosine autophosphorylation assay (17) with site-directed antisera to the second exon of *ABL* (18). Samples were analyzed by NaDodSO₄/7% PAGE.

spontaneously underwent rearrangement at the immunoglobulin heavy chain locus over time, giving rise to immature pre-B cells with diverse D–J rearrangements (Fig. 3). The same viral integration site was detected in the cultures at all time points examined, showing that the early pre-B cells were derived from the progenitor cell. Only after >2 months or an estimated 30 doublings did the cultures become dominated by a single clone that had undergone rearrangement at both alleles. The cells were cytoplasmic μ^- , and κ alleles remained in a germ-line configuration (data not shown).

Transfer of LPC Cells to SCID Mice Promotes Differentiation to Surface IgM⁺ and Immunoglobulin-Secreting B Cells. We transplanted the *BCR/ABL*-expressing LPC cells into C.B17 SCID mice because differentiation of nontransformed, cultured progenitor cells to mature B cells requires transfer to an *in vivo* environment (21, 22). SCID mice are devoid of cellular and humoral immune function and are congenic with LPC cells of BALB/c origin (14). Thirty to 60 days after transfer of 1×10^6 LPC cells, the number of splenocytes in the LPC-reconstituted SCID mice was, on average, 5 times higher than in unreconstituted SCID mice and was comparable to normal BALB/c mice or SCID mice reconstituted with LBM (Table 2). Thirty to 50% of the splenocytes in LPC-reconstituted mice were B220⁺. We confirmed that

Table 1. IL-7 fails to support the growth of LPCs

Culture condition	Cells, no. $\times 10^{-5}$ per ml	
	LPC	Pre-B cell
Medium	0.9	0.3
S17 adherent layer	9.3	3.7
S17 supernatant		
50%	4.5	1.0
10%	2.6	0.5
2%	0.7	0.0
IL-7		
10%	0.7	ND
1%	0.2	9.6
0.1%	0.9	ND

P210-expressing LPC cells used in the assay were harvested from cultures 4–6 weeks after initiation. The pre-B-cell line was the cytoplasmic IgM⁺ line, clone 4, which was derived from LBM (28). Cells were washed and plated at 1×10^5 cells per ml; cell number was determined on day 5. The source of IL-7 was an IL-7-containing COS cell supernatant (29). S17 supernatant was collected from a confluent S17 stromal layer 24 hr after change of medium. ND, not determined.

these cells were of donor origin by site of viral integration and by a restriction polymorphism in the μ -heavy chain gene that distinguishes donor BALB/c from host C.B17 cells (33) (data not shown). Approximately 10% of the cells in the spleens of LPC-reconstituted SCID mice (10^7 of 10^8 cells) were surface IgM⁺ (Table 2).

Immunoglobulin, predominantly of the IgM isotype, was detected by ELISA in the serum of LPC-reconstituted SCID mice 30 days after transfer (Fig. 4). The level of serum immunoglobulin was 20–50 times greater than the background levels in unreconstituted SCID mice. Although 10-fold less than in control BALB/c mice, the level appears to correlate with the 5- to 10-fold lower number of surface IgM⁺ cells present in the spleens of the reconstituted animals relative to normal mice (Table 2). To exclude the possibility that the serum immunoglobulin was produced by cells that did not contain the *BCR/ABL/tk-neo* construct, additional experiments were conducted in which the LPCs were passaged in medium containing G418 for 2 weeks before transfer *in vivo*. No decrease in serum immunoglobulin was detected when G418-selected cells were compared with nonselected cells (Fig. 4).

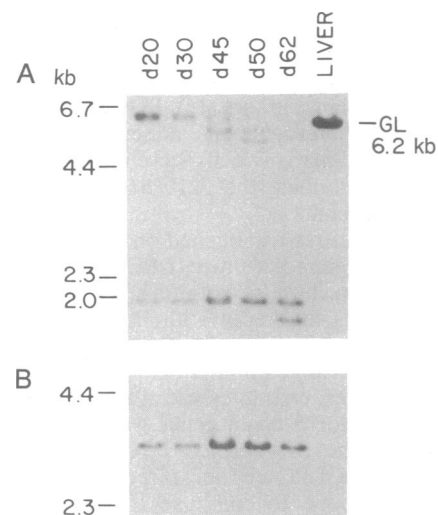


FIG. 3. LPCs undergo immunoglobulin rearrangement *in vitro*. High-molecular-weight DNA was isolated from LPCs at various times (d20–d62) after establishment and analyzed by Southern blotting for heavy chain D–J rearrangement (A) and *neo* integration (B) as described in Fig. 2. GL, germ line.

Table 2. Functional B-lymphocyte reconstitution of SCID mice by P210-expressing LPC

	Cells per spleen, no. $\times 10^{-6}$	Surface IgM ⁺ , %	Anti-TNP-Ficoll*	
			IgM	IgG
BALB/c	104 (100–108)	61 (58–63)	12,750 (7,500–18,000)	1375 (1250–1500)
SCID	18 (16–21)	<2	<50	<50
SCID + LBMC	90 (89–92)	18 (13–25)	1,750 (1250–2250)	150 (50–250)
SCID + <i>BCR/ABL</i> LPC	92 (42–153)	9 (1–14)	600 (100–1250)	<50

Mice were analyzed 8–10 weeks after reconstitution. Data represent the average of 2–4 mice in control groups and 8–10 mice in experimental groups. Ranges are given in parentheses.

*Anti-TNP titers are expressed as the reciprocal of the serum dilution to give 50% maximum response in an ELISA.

The lack of serum IgG in the LPC-reconstituted mice suggested that either a functional T-cell compartment was not reconstituted in these mice or that the progenitor cells gave rise to cells unable to undergo isotype switch. To test this, nylon wool-passed thymocytes were included in the inoculum as a source of T cells. Serum IgM levels in SCID mice receiving LPC and thymocytes were comparable with SCID mice receiving LPC alone (Fig. 4). However, mice receiving LPC and thymocytes displayed a substantial increase in serum IgG levels. All subclasses of IgG were detected (data not shown). SCID mice receiving thymocytes alone did not have detectable serum immunoglobulin (data not shown).

P210-Expressing LPCs Give Rise to Antigen-Responsive B Cells. LPC-reconstituted SCID mice were primed and boosted with the antigen TNP-Ficoll 30 days after transfer. TNP-Ficoll was chosen because it does not require T-cell help to elicit an antibody response (34). Specific anti-TNP antibodies were present in the serum as measured by ELISA (Table 2) or by hemagglutination of TNP-sheep erythrocytes 7 days after boosting (data not shown). The anti-TNP titer in the LPC-reconstituted SCID mice was approximately half that expected based on the number of IgM⁺ cells and total serum immunoglobulin level when compared with BALB/c mice or SCID mice reconstituted with LBMC cells.

DISCUSSION

We describe an experimental system that promotes outgrowth of clonal lymphoid progenitor cells. Because *BCR/ABL* stimulates the growth of the progenitor cells, these cells

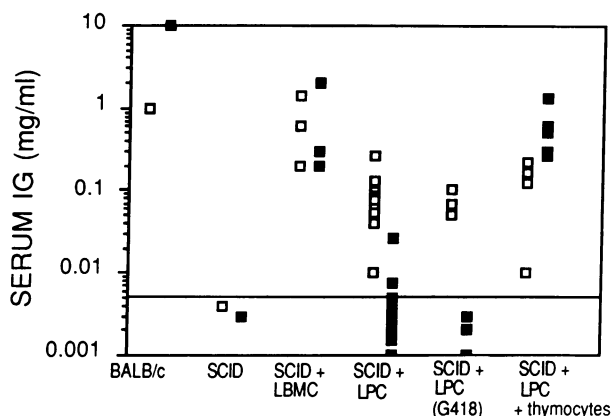


FIG. 4. Serum immunoglobulin levels in SCID mice reconstituted with *BCR/ABL*-transformed LPCs. SCID mice were reconstituted with $1-5 \times 10^6$ cells from LBMCs, P210-expressing LPCs, P210-expressing LPCs that had been passaged in G418 (250 μ g/ml) for 2 weeks before transfer [LPC (G418)], or P210-expressing LPCs together with nylon wool-passed thymocytes (LPC + thymocytes), as described. Serum IgM (\square) or IgG (\blacksquare) levels were analyzed by ELISA at 4 weeks after reconstitution. The assay was developed with peroxidase-conjugated anti-class-specific antibodies. Positive wells were detected with tetramethylbenzidine. Absorbance was read at 405 nm on a Bio-Rad enzyme-linked immunosorbent assay reader (model 2550).

can be expanded to numbers useful for biochemical analyses. This procedure should enable one to begin to identify the genes involved in B-cell development as well as signaling mechanisms that permit a cell to progress through the B lineage.

Although their growth is stimulated by *BCR/ABL*, the progenitor cells retain their ability to give rise to mature B cells that appear to function normally in terms of their response to T-cell help and upon antigenic challenge. The diversity of the B cells in LPC-reconstituted SCID mice has not been formally examined. Several findings suggest that the progenitor cell is not restricted: (i) diverse D-J rearrangements are detected *in vitro*, (ii) B cells of all immunoglobulin subclasses are generated, (iii) antibodies are produced upon immunization with antigens such as TNP and sheep erythrocytes (Table 2 and unpublished data), and (iv) isoelectric focusing analysis shows the serum immunoglobulin to be as heterogeneous as in normal BALB/c mice (P.A.S. and O.N.W., unpublished work). Because B cells in the LPC-reconstituted mice are clonally derived from a common progenitor, this system may be valuable for studying the generation of antibody diversity and the evolution of antibody structure during an immune response.

Nontransformed, interleukin-3-dependent pro-B-cell clones that are Lyl⁺ and can differentiate to IgM⁺ B cells *in vitro* and *in vivo* have been reported (35, 36). The LPCs described here are not Lyl⁺ and they are not interleukin 3-dependent (P.A.S. and O.N.W., unpublished work). We cannot exclude the possibility that *BCR/ABL* abrogates an interleukin 3 requirement. *BCR/ABL*, however, does not abrogate all cytokine requirements because the cells remain dependent on the S17 stromal layer for growth. IL-7 also does not appear to be the primary factor required for progenitor cell growth. The LPCs cannot be maintained in IL-7-supplemented medium, and the S17 stroma produce minimal, if any, levels of IL-7. Others have indicated that IL-7-dependent lymphoid cells can induce IL-7 production in stromal cells that do not constitutively secrete that cytokine (32). The observation that the IL-7-dependent clone 4 pre-B cell line does not grow well on S17 stroma would argue against that process as being a generalized phenomenon and lends further support to the hypothesis that the S17 stroma supports B lymphopoiesis without producing IL-7. The absence of IL-7 may be critical in allowing the outgrowth of an earlier cell type than the pre-B cell, which predominates in long-term bone-marrow cultures established on mixed stroma.

The ability of *BCR/ABL* to stimulate growth without arresting differentiation has precedent in other oncogene systems (37–39). A well-characterized system is avian erythro-leukemia virus, in which transformation of avian erythroblasts by the oncogene *erbB* results in growth and differentiation, whereas transformation by *erbB* plus *erba* results in growth only (39). These findings suggest that complete transformation and differentiation arrest require oncogene cooperativity. The progression of chronic myeloid leukemia lends additional support to this concept. In the chronic phase, there is proliferation and differentiation of predominantly myeloid

elements. In the transition to blast crisis, a second event is thought to occur, causing an arrest of differentiation and an accumulation of immature myeloid or lymphoid cells. In the experimental system described in this paper, the LPCs are not grossly leukemic, at least in the first 60 days after transfer (P.A.S. and O.N.W., unpublished work). This system provides a model in which to study the effects of additional oncogenes on leukemogenesis as well as the signals that regulate growth versus differentiation.

We are grateful to A. Berk, J. Braun, A. Muller, and J. Young for critical review of the manuscript; to Carol Crookshank for assistance in preparation of the manuscript; and to Mike Cohan for photography. This work was supported, in part, by grants to O.N.W. and K.D. from the National Institutes of Health. P.A.S. is an Associate of the Howard Hughes Medical Institute. O.N.W. is an Investigator of the Howard Hughes Medical Institute. K.D. is a recipient of a Research Career Development Award (AI00843).

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