Clonal proliferation of murine lymphohemopoietic progenitors in culture

(hemopoietic stem cell/growth factor/B lymphocyte)

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Communicated by E. Donnall Thomas, March 30, 1992

ABSTRACT We have used a two-step clonal culture system to unequivocally demonstrate that individual primitive lymphohemopoietic progenitor cells have the capacity for differentiation along either the myeloid or the B-lymphoid lineage. Highly enriched murine marrow cells were plated individually in culture by micromanipulation in the presence of pokeweed mitogen-stimulated spleen cell conditioned medium, erythropoietin, steel factor (SF), and interleukin (IL) 7. Forty-five percent of the single cells formed primary colonies expressing multiple hemopoietic lineages. When aliquots from individual colonies were replated in secondary methyl cellulose culture containing SF and IL-7, 41% of the primary colonies gave rise to lymphocyte colonies. Cells of the lymphocyte colonies were blast-like and B220⁺, sIg⁺, Mac-1⁺, Gr-1⁺, Ly-1⁺, L3T4⁺, Ly-2⁺, and CD3⁺. Thirty to 70% of the cells were Thy-1⁺. μ -chain mRNA was detected in most of the cells by *in situ* hybridization with an antisense RNA probe. When lymphocyte colonies derived from a single cell were pooled and individually injected into *scid* mice, donor-type IgM was measurable in the serum of mice and spleens contained donor-type B cells. We then carried out initial screening of growth factors to identify growth factors that might replace pokeweed mitogen-stimulated spleen cell conditioned medium in the primary culture. Combinations of two factors that included SF plus IL-6, IL-11, or granulocyte colony-stimulating factor were all effective in the primary culture in the maintenance of the B-lymphoid potential. Interestingly, IL-3 could neither replace nor act synergistically with SF to support the lymphoid potential of the primary cultures. Our observations demonstrate that many primitive progenitors previously believed to be myeloid-committed also possess B-lymphoid potential. This culture system should prove valuable for elucidation of the mechanisms regulating early stages of lymphohemopoiesis.

The existence of pluripotent lymphohemopoietic stem cells was postulated long ago through observation of reconstitution of patients after bone marrow transplantation and through careful analysis of transplantation in experimental animals. More recently, retroviral labeling of individual hemopoietic progenitors has clearly demonstrated the existence of cellular precursors common to all of the blood cell lineages, both myeloid and lymphoid. However, despite all of this *in vivo* evidence, it has not yet been possible to detect and quantitate the existence of these pluripotent cells through *in vitro* culture. Clonal cell culture assays have been available for many years for analysis of committed myeloid progenitors. Long-term suspension culture systems have been devised that can be used to maintain exclusively myeloid or lymphoid development, depending on the conditions. Re-

cently described lymphocyte colony assay systems appear also to support the development of pre-B and B cells but not earlier precursors (1–3). Despite considerable investigation of the growth factor requirements of immature thymocytes, the early stages of T-cell ontogeny from lymphohemopoietic stem cells to CD3⁺, CD4⁺, CD8⁺ thymocytes are still also largely unknown. Therefore, the development of a clonal cell culture system capable of supporting the growth and development of lymphohemopoietic progenitors would provide a means for direct analysis of the early stages of development of primitive cells. Here we describe a two-step clonal culture system capable of supporting the clonal expansion of individual primitive lymphohemopoietic progenitors. That the individual primitive progenitors in the primary culture had the capacity to yield progeny committed to either the myeloid or B lymphoid lineages was demonstrated through harvesting individual colonies and replating aliquots of cells from each colony in parallel cultures optimized for supporting myeloid or B-lymphoid development.

MATERIALS AND METHODS

Growth Factors. The conditioned medium (CM) of COS cells transfected with an expression plasmid containing cDNA encoding mouse steel factor (SF) was provided by D. Donaldson (Genetics Institute, Cambridge, MA) and purified recombinant murine SF was provided by D. Williams (Immunex, Seattle). Serum-free CM from pokeweed mitogen-stimulated spleen cells (PWM-SCM) was prepared as described (4). Purified recombinant human interleukin (IL) 7 and murine IL-4 were provided by C. Flatynek (Sterling Drug, Malvern PA). The medium conditioned by Chinese hamster ovary cells that had been genetically engineered to produce murine IL-3 in a high titer ($\approx 30,000$ units/ml) was a gift from T. Sudo (Biomaterial Research Institute, Yokohama, Japan). Partially purified human urinary erythropoietin (Ep) (specific activity, 250 units/mg) was a gift from M. Kawakita (Kumamoto University, Kumamoto, Japan). Human recombinant IL-11 was provided by P. Schendel (Genetics Institute). Purified bacterially derived human granulocyte colony-stimulating factor (G-CSF) was provided by L. Sousa (Amgen Biologicals). Recombinant human IL-6 was a gift from M. Naruto (Toray Industries, Yokohama, Japan).

Primary Single-Cell Culture. Bone marrow cells were harvested from BDF1 mice 2 days after i.v. injection of 5-fluorouracil (5-FU) at 150 mg/kg (body weight), and cells with a density between 1.0631 and 1.0770 were collected with so-

Abbreviations: IL, interleukin; G-CSF, granulocyte colony-stimulating factor; Ep, erythropoietin; SF, steel factor; FITC, fluorescein isothiocyanate; PWM-SCM, conditioned medium from pokeweed mitogen-stimulated spleen cells; CM, conditioned medium; 5-FU, 5-fluorouracil.

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dium metrizamide gradients. The cells were further enriched for progenitors by negative immunomagnetic selection with a mixture of lineage-specific antibodies and the lineage-negative cells were sorted for Ly-6A/E-positive cells by using a FACStarplus cell sorter (Becton Dickinson). The final preparation of the marrow cells was ≈ 800 -fold enriched for cells producing multilineage colonies in the presence of IL-3, IL-6, and Ep (5). The enriched marrow cells were individually plated in methyl cellulose medium by micromanipulation (6) in 35-mm suspension culture dishes (Nunc). The culture mixture consisted of α modification of minimum essential medium (α medium), 1.2% (wt/vol) 1500 centipoise methyl cellulose (Fisher; $1 \text{ p} = 0.1 \text{ Pa-sec}$), 30% (vol/vol) fetal calf serum (HyClone), 1% deionized fraction V bovine serum albumin (Sigma), 0.1 mM 2-mercaptoethanol (Sigma), and a combination of growth factors consisting of 5% (vol/vol) PWM-SCM and 3% (vol/vol) COS-CM containing SF (6 units/ml), IL-7 (500 units/ml), and Ep (2 units/ml). Dishes were incubated at 37°C in a humidified atmosphere flushed with 5% CO₂/95% air for 11 days. In some experiments, COS-CM containing SF was replaced by purified recombinant murine SF (100 ng/ml).

Secondary Myeloid and Lymphoid Cultures. The primary colonies were individually lifted from the medium, washed, and divided into three aliquots. Cells in one aliquot were centrifuged onto a slide for cytological examination with May-Grunwald Giemsa. The other two aliquots were plated in secondary cultures for studies of myeloid or lymphoid potentials. The secondary myeloid culture was carried out in a 96-well microtiter plate in α medium containing 30% fetal calf serum, 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol, IL-3 (100 units/ml), 5% PWM-SCM, and Ep (2 units/ml). Small samples of cells were harvested every 2–3 days for cytological examination. Lymphocyte colony culture was carried out in 35-mm Nunc suspension culture dishes containing 1.2% methyl cellulose, 30% fetal calf serum, 1% bovine serum albumin, 0.1 mM 2-mercaptoethanol, and 3% COS-CM containing SF and IL-7 (500 units/ml) for 10–12 days. In some experiments COS-CM was replaced by purified recombinant murine SF (100 ng/ml).

Surface Phenotypes of Cells in the Lymphocyte Colonies. The lymphocyte colonies of single cell origin induced by SF and IL-7 were picked individually, pooled, and made into a single cell suspension. From 1 to 5×10^5 cells were then analyzed by flow cytometry using FACStar Plus flow cytometer. Antibodies used were (i) 14.8 [anti-B220, American Type Culture Collection (ATCC)], 53.7.3 (anti-Ly-1, Becton Dickinson), GK1.5 (anti-L3T4, ATCC), 53.6.7 (anti-Ly-2, Becton Dickinson), 30-H12 (anti-Thy1.2, Becton Dickinson), M1/70.15.11.5.HL (anti-Mac-1, ATCC), and RB6-8C5 (anti-Gr-1, a gift of A. L. Coffman of DNAX); (ii) fluorescein isothiocyanate (FITC) anti-mouse immunoglobulin (Becton Dickinson); and (iii) 145-2C11 (anti-CD3, Pharmingen, San Diego). Rat IgG (Zymed Laboratories), FITC-conjugated anti-rat immunoglobulin, and staining buffer were used as background controls for the three groups of antibodies above, respectively. Binding of the unlabeled primary antibodies in groups i and iii was detected with FITC-conjugated anti-rat immunoglobulin (Kirkegaard and Perry Laboratories, Gaithersburg, MD) and FITC-conjugated anti-hamster immunoglobulin (The Jackson Laboratory), respectively.

In Situ Hybridization. *In situ* hybridization was carried out essentially as described (7), with some adjustments for the pretreatment of cytological preparations. Cells from lymphocyte colonies were centrifuged onto poly(L-lysine)-coated microscope slides and fixed in 3% (wt/vol) paraformaldehyde for 15 min. Slides were then incubated in Pronase E (0.5 mg/ml; Sigma) for 5 min. Subsequent pretreatment, hybridization, and washing steps were done exactly as described

(7). As a template for the synthesis of RNA probes, a DNA segment spanning 317 base pairs of the C μ 3 exon of the mouse μ -chain gene (8) was generated by the polymerase chain reaction from cloned mouse genomic DNA (9) (a gift of K. Calame of Columbia University, New York) and cloned into the vector pBSKS (Stratagene) to give plasmid pIg μ 3. Asymmetric RNA probes were synthesized from linearized plasmids by using T7 (antisense RNA) or T3 (sense RNA) RNA polymerases and ³⁵S-labeled UTP ($\approx 1000 \text{ Ci/mmol}$; 1 Ci = 37 GBq; DuPont/NEN).

In Vivo Adoptive Transfer of Pre-B Colony Cells into *scid* Mice. All lymphocyte colonies derived from a single stem cell were pooled and placed in suspension culture for 1 week in the presence of 3% COS-CM containing SF and IL-7 (500 units/ml). One-half of each sample containing 7.5×10^6 cells was injected into the tail vein of 6-week-old C.B.-17 *scid* mice (Taconic Farms). Serum IgM and IgG levels were measured by ELISA at intervals of 1–2 weeks. Serum levels of non-b haplotype IgM were measured to distinguish donor-derived IgM from host-derived IgM by using anti-mouse non-b haplotype IgM, DS-1 (Pharmingen). One hundred and twelve days after injection, mice were sacrificed and 2×10^5 spleen cells were stained with 28.8.6s (anti-H-2K^bD^b, mouse IgG2a antibody) and FITC-conjugated goat anti-mouse γ -chain specific antibody (Cappel Laboratories) followed by biotin-conjugated goat anti-mouse μ -chain-specific antibody (Cappel Laboratories) and phycoerythrin-conjugated streptavidin (Becton Dickinson).

Analysis of Growth Factor Requirements in Primary Culture. For analysis of cytokine requirements by lymphohemopoietic progenitors, 50 enriched cells were cultured in the presence of Ep, IL-7, and various combinations of recombinant cytokines. PWM-SCM was omitted. Eight or 11 days later, 20 primary colonies were individually picked, pooled, and washed, and 5% of the pooled samples was plated in secondary methyl cellulose cultures containing SF and IL-7. Pre-B-cell colonies were counted 11 days after replating. Concentrations of cytokines in the primary culture were as follows: IL-7, 500 units/ml; Ep, 2 units/ml; SF, 3% COS-CM; IL-3, 200 units/ml; IL-4, 10 ng/ml; IL-6, 100 ng/ml; IL-11, 100 ng/ml; G-CSF, 100 units/ml.

RESULTS

Colony Formation from Single Progenitors in Primary Culture. To establish primary cultures that might support the growth of primitive cells while maintaining their multipotentiality, we used a combination of growth factors including SF (important for early hemopoietic cell development), IL-7 (to preserve B-lymphocyte phenotype), and PWM-SCM (an excellent source of general growth factors for hemopoietic cells). The enriched marrow cells were plated individually in methyl cellulose cultures by micromanipulation as described (6). As summarized in Table 1, of the total of 195 isolated progenitors in four experiments, 87 cells (45%) yielded primary colonies on day 11 of culture. A representative colony is shown in Fig. 1A. Upon staining, all colonies were found to contain blast cells from a few to $>90\%$ of the population. There were no obvious lymphocytes. Some of these colonies were neutrophil/macrophage colonies and others appeared to be multilineage in nature, revealing neutrophils, eosinophils, macrophages, erythrocytes, and/or megakaryocytes.

Development of Lymphocyte Colonies in Secondary Culture. Secondary lymphocyte cultures were established in the presence of SF and IL-7. Of the 87 cultures established with cells from the 87 primary colonies, 36 (41%) formed large compact unicentric colonies consisting of small round cells (Table 1). These colonies were similar to the pre-B-cell colonies that have been described (1–3). Because this culture system is not favorable for expression of the myeloid lineages, coexisting

Table 1. Lymphohemopoietic potentials of individual progenitors

Exp.	No. of single cells cultured	No. of single cells producing colonies in primary culture	No. of single cells producing lymphocyte colonies in secondary culture	No. of lymphocyte colonies derived from single cells
1	49	21	9	198 (26–540)
2	48	24	11	137 (4–420)
3	56	20	8	113 (12–308)
4	42	22	8	74 (16–168)
Total	195	87	36	133 (4–540)

In experiments 1–3, the source of SF was COS CM containing SF. In experiment 4, purified recombinant murine SF (100 ng/ml) was used. Data for the number of lymphocyte colonies derived from single cells are expressed as the mean; data in parentheses are the range.

macrophage colonies were very small. A photograph of a portion of the culture plate containing a putative lymphocyte colony and a small macrophage colony is shown in Fig. 1*B*. May–Grunwald Giemsa staining of the putative lymphocyte colonies revealed lymphoblast-like cells whose nuclei were sometimes clefted or lobulated.

We then used flow cytometry to analyze the cell surface markers of the cells pooled from all of the putative lymphocyte colonies derived from a single progenitor. All cells were B220⁺ and between 30 and 70% of the cells were Thy-1.2⁺. The cells were also sIg⁺, Ly-1⁺, L3T4⁺, Ly-2⁺, CD3⁺, Mac-1⁺, and Gr-1⁺. These observations indicated that a majority of the cells in the lymphocyte colonies apparently belonged to the B-lymphocyte lineage.

Detection of Immunoglobulin μ -Chain Gene Expression. The lymphocyte colonies were also analyzed for μ -chain gene expression by *in situ* hybridization. Upon hybridization with the antisense RNA probe, clusters of silver grains were detected over most but not all of the cells (Fig. 2*A*). Given the relatively low resolution of the autoradiographic hybridization signals with respect to cell density, we were unable to determine the exact percentage of cells expressing the μ -chain gene. No accumulation of silver grains above background was observed over any cells in control preparations that were hybridized with the sense RNA probe (Fig. 2*B*). Thus, hybridization signals were specific to the antisense probe, and the studies of the surface marker profiles and μ -chain mRNA expression strongly indicated that the majority of the cells in the lymphocyte colonies were pre-B cells.

In Vivo Transfer of Pre-B Colony Cells into *scid* Mice. Next we tested the pre-B colony cells for the ability to differentiate into functionally mature B cells *in vivo* by injecting cultured lymphocytes of single-cell origin individually into eight *scid*

mice. C.B.-17 *scid* mice are Igh-6^b, whereas BDF1 donor mice are Igh-6^{b/non-b}. Therefore, non-b haplotype IgM is donor-derived. Peak non-b IgM levels in the sera of all eight *scid* mice were >10 μ g/ml and of 2 control (noninjected) *scid* mice were always <1 μ g/ml. In some injected mice, the serum non-b IgM levels were as high as 90 μ g/ml at 7–8 weeks after cell injection and declined thereafter. In other mice, IgM levels continued to climb 3 months after cell injection. The total IgM levels were about twice as high as non-b IgM and the IgG levels were also similar to the levels of total IgM.

To further test for the presence of donor-derived B cells, on day 112 of observation, six of the *scid* mice expressing non-b haplotype IgM in the serum were sacrificed and their spleen cells were stained with anti-H-2K^bD^b and anti-IgM. C.B.-17 *scid* mice are H-2^d, whereas BDF1 mice are H-2^{b/d}. Therefore, H-2^b-positive cells are donor-derived. All mice had a significant number of H-2K^bD^b-positive cells and, in addition, almost all of the H-2K^bD^b-positive cells were surface IgM-positive B cells. In Fig. 3 we present a representative analysis. Two control (noninjected) *scid* mice revealed neither H-2K^bD^b-positive cells nor IgM-positive cells. There were correlations between the number of H-2K^bD^b-positive B cells and serum levels of non-b haplotype IgM (data not shown). These results clearly indicated that some, if not all, cells in the pre-B colonies matured to B cells and secreted immunoglobulin.

Analysis of Myeloid Potential of the Lymphohemopoietic Progenitors. In all of the experiments summarized in Table 1, we carried out detailed analysis of the myeloid capacity of the primary progenitors in secondary suspension culture. The analysis was based on cytological examination of the primary colonies and samples harvested from the secondary suspension cultures were optimized for expression of the myeloid

FIG. 1. Colonies identified in the primary and secondary cultures. (A) Typical primary colony derived from an isolated single progenitor. (B) A large lymphocyte colony and a small macrophage colony in the secondary culture containing SF and IL-7.

FIG. 2. Expression of immunoglobulin μ -chain mRNA. Cytospin preparations of the cells from lymphocyte colonies were hybridized with ³⁵S-labeled RNA probes corresponding to either the antisense (A) or the sense (B) strand of the C μ 3 exon at 0.3 mg per ml per kilobase of probe complexity at 50°C overnight. After washing and dehybridization, slides were coated with undiluted Kodak NTB2 photoemulsion and exposed at 4°C for 7 days.

FIG. 3. Identification of donor-derived B cells in spleen cells from *scid* mice. *scid* mice injected with pre-B colony cells were sacrificed 112 days after injection and spleen cells were analyzed for IgM and H-2K^bD^b. Representative analysis of control (A) and injected (B) mice are shown. Numbers in quadrants indicate the percentage of cells.

lineages. In one experiment, all 20 original primary colonies expressed two or more myeloid lineages. Of the 20, 8 colonies also expressed the B-lymphocyte lineage. The 8 colonies that coexpressed B-lymphoid potential included 2 neutrophil/macrophage colonies, 5 neutrophil/macrophage/mast cell/erythrocyte colonies, and 1 neutrophil/macrophage/mast cell/erythrocyte/megakaryocyte colony. The 12 colonies that did not produce pre-B cells also expressed a variety of types of multilineage myeloid cells including eosinophil lineage. There were no obvious differences in the myeloid potentials between colonies containing B-cell capability and colonies revealing no lymphoid potentials.

Growth Factor Requirements of the Lymphohemopoietic Progenitors. To begin to analyze the growth factor requirements of the lymphohemopoietic progenitors in culture, we tested various combinations of cytokines for the ability to yield primary colonies that upon replating yielded both myeloid and lymphoid cells. First, we observed that IL-7 and Ep are not necessary for maintenance of B-cell capability of the primary colonies (data not shown). Of known lymphohemopoietic cytokines, we have tested growth factors that regulate the early stages of hemopoiesis, including SF, IL-6, G-CSF, IL-11, IL-3, and IL-4. More specifically we focused on two-factor combinations that have been shown in our laboratory to work synergistically in support of multilineage

colony formation from marrow cells of 5-FU-treated mice (10–15). The results of the analysis are presented in Table 2. Of all two-factor combinations tested, the combinations containing SF plus IL-6, IL-11, or G-CSF were most effective in supporting the B-cell capability within the primary colonies. In contrast, none of the combinations containing IL-3 supported B-cell potential in the primary colonies even though the number and size of the primary colonies were comparable to the colonies detected in the SF-containing cultures. Synergistic interactions have been shown between IL-4 and IL-6 (12) and between IL-4 and IL-11 (14) in support of multilineage colony formation from marrow cells of 5-FU-treated mice. Colonies supported by these combinations also retained B-cell potential although less effectively than the colonies in SF-containing cultures.

DISCUSSION

We have used micromanipulation of single cells in a two-step culture system to demonstrate the development of both myeloid and lymphoid cells from single lymphohemopoietic progenitors *in vitro*. Other investigators have reported (16–19) the development of similar pluripotent progenitors in culture either through identification of T or B lymphocytes in multilineage myeloid colonies or through replating of myeloid

Table 2. Analysis of B-cell potentials in primary colonies supported by combinations of recombinant cytokines

Cytokines in culture						Replated on day 8			Replated on day 11	
						1° colonies, no.	Cells, no. in primary colony ($\times 10^{-4}$)	Pre-B-cell colonies, no.	Cells, no. in primary colony ($\times 10^{-4}$)	Pre-B-cell colonies, no.
SF	IL-3	IL-4	IL-6	IL-11	G-CSF					
+	+					20 $\pm$ 4	2.3	0	6.0	0
+		+				0			0.2*	0
+			+			19 $\pm$ 3	3.3	48 $\pm$ 8	9.4	38 $\pm$ 4
+				+		19 $\pm$ 3	1.9	25 $\pm$ 5	5.6	71 $\pm$ 9
+					+	18 $\pm$ 3	1.1	57 $\pm$ 1	6.4	54 $\pm$ 1
	+		+			16 $\pm$ 2	1.0	0	5.6	0
	+			+		19 $\pm$ 3	0.5	0	4.9	0
	+				+	15 $\pm$ 6	0.39	0	3.7	0
		+	+			15 $\pm$ 6	0.35	2 $\pm$ 1	2.8	5 $\pm$ 1
				+		18 $\pm$ 3	0.8	8 $\pm$ 1	2.4	9 $\pm$ 2
					+	0				

Fifty enriched marrow cells were cultured in the presence of Ep (2 units/ml), IL-7 (500 units/ml), and the designated cytokine combinations. Single factors failed to support primary colony formation except for IL-3 on day 11 of incubation. No pre-B-cell colonies were derived from these colonies. +, Cytokine present in culture. The number of pre-B-cell colonies was derived from 5% of 20 pooled colonies. Data are expressed as the mean or the mean $\pm$ SEM.

*On day 11 of incubation 2 $\pm$ 2 primary colonies were identified. Analysis of cell number is based on a total of six colonies.

colonies and observing expression of the lymphoid lineages in secondary cultures. The difficulty in the interpretation of these observations has been that in both cases it was not possible to exclude the possibility of contamination of the original myeloid colonies by committed T- or B-lymphocyte precursors. Indeed, experimental depletion and addition of T lymphocytes in comparable studies have indicated that insufficient T-cell depletion of the marrow could account for the T-lymphocyte-containing human myeloid colonies (20). Our results clearly establish the clonal derivation *in vitro* of myeloid and B-lymphoid progeny from the bone-marrow-derived lymphohemopoietic progenitors. The results also reveal that a significant number of the primitive progenitors present in marrow after 5-FU treatment originally thought to be restricted to myeloid differentiation also are capable of B-lymphoid development.

All of the lymphoid colonies that we obtained in secondary cultures consisted of B220⁺ cells that expressed the immunoglobulin μ -chain RNA but failed to express cell surface immunoglobulin. These cells, upon adoptive transfer into *scid* mice, reconstituted the production of serum immunoglobulin and spleen B cells in the immunodeficient animals. These results clearly demonstrate that the early stages of murine B-lymphocyte development can be achieved in culture from lymphohemopoietic progenitors. The cells in the lymphoid colonies did not express T-lymphocyte markers such as Ly-1, L3T4, Ly-2, or CD3. The expression of Thy-1.2 by 30–70% of the cells in these colonies remains a puzzle although other investigators have noted high levels of expression of this surface antigen by pre-B cells grown in the presence of IL-7 (2) and noted that Thy-1^{lo} B220⁺ bone marrow cells may represent the earliest stage of committed lymphocyte progenitors (21).

The two-step culture system for lymphoid and myeloid development was necessary for two major reasons. (i) PWM-SCM appears to be toxic for the proliferation of B-cell progenitors at later stages of development since replating of primary colonies into PWM-SCM-containing medium failed to support lymphocyte colony formation (data not shown). It is possible that inhibitory factors for late-stage B-cell development, such as IL-1 and IL-4, may be present in PWM-SCM, and our preliminary analysis of IL-1 α and IL-4 in secondary cultures indicates that both of these cytokines inhibit the development of pre-B colonies (data not shown). (ii) The overall cell proliferation from the original single lymphohemopoietic cell is so great that accurate quantitation of the development of the progenitors for lymphoid and myeloid cells in a single 35-mm culture dish is extremely difficult. However, it may now be possible to establish a one-step culture system since the assay may be carried out without PWM-SCM.

To gain insight into the contributing factors in the PWM-SCM, we carried out a brief survey of combinations of recombinant cytokines for their ability to support B-cell potential in primary colonies. Various two-factor combinations that had been shown to support multilineage colony formation from dormant progenitors were also shown to support B-cell potential in primary colonies. These studies showed that IL-6, IL-11, or G-CSF, cytokines likely to be present in PWM-SCM, could effectively replace the crude source of natural factors. Interestingly, IL-3 in combination with these same factors or in combination with SF proved incapable of maintaining the B-cell potential of the lymphohemopoietic progenitors in primary culture. IL-3 does not trigger cell cycling of dormant hemopoietic progenitors but rather functions as a permissive factor in support of proliferation and differentiation of the progenitors after they enter the active phase of the cell cycle (22). It is possible that IL-3 acts as a cell-cycle permissive factor only after stem cells are committed to the myeloid lineage and have lost the B-cell

potential. Again, a completely permissive one-step culture system would be necessary for testing this hypothesis.

As a first target, we have used our *in vitro* culture system to study the development of myeloid and B-lymphoid cells from individual lymphohemopoietic progenitors. The system readily yields these lineages in culture but, thus far, we have not identified any recognizable T lymphocytes or natural killer cells in the secondary culture. Adaptation of this system to human bone marrow could provide methods for quantitation of human lymphohemopoietic cells, which should assist in elucidation of the mechanisms regulating commitment and in monitoring the status of the hemopoietic system in myelosuppressed patients or in patients undergoing bone marrow transplantation.

We thank Dr. P. Kincade for kindly providing many cell lines and advice on immunostaining. We also are grateful for excellent technical assistance from Kathy Carr, Darlene Middleton, and Donna Jacobs. This work was supported by National Institutes of Health Grants DM32294 and GM4334-03 and National Science Foundation Grant 9106316, the Research Service of the Ralph H. Johnson Veterans Affairs Medical Center, and contributions from Westvaco and Kirin Brewery Co., Japan.

1. Lee, G., Namen, A. E., Gillis, S., Ellingsworth, L. R. & Kincade, P. W. (1989) *J. Immunol.* **142**, 3875–3883.
2. Suda, T., Okada, S., Suda, J., Miura, Y., Ito, M., Sudo, T., Hayashi, S.-I., Nishikawa, S.-I. & Nakauchi, H. (1989) *Blood* **74**, 1936–1941.
3. McNiece, I. K., Langley, K. E. & Zsebo, K. M. (1991) *J. Immunol.* **146**, 3785–3790.
4. Nakahata, T. & Ogawa, M. (1982) *Proc. Natl. Acad. Sci. USA* **79**, 3843–3847.
5. Shih, J. P., Zeng, H. Q. & Ogawa, M. (1992) *Leukemia* **6**, 193–198.
6. Suda, T., Suda, J. & Ogawa, M. (1983) *Proc. Natl. Acad. Sci. USA* **80**, 6689–6693.
7. Awgulewitsch, A. & Utset, M. F. (1991) in *Methods in Nucleic Acids Research*, eds. Karam, J. D., Chao, L. & Warr, G. (CRC, Boca Raton, FL), pp. 359–375.
8. Goldberg, G. I., Vanin, E. F., Zrolka, A. M. & Blattner, F. R. (1981) *Gene* **15**, 33–42.
9. Clarke, C., Berenson, J., Goverman, J., Boyer, P. D., Crews, S., Siu, G. & Calame, K. (1982) *Nucleic Acids Res.* **10**, 7731–7749.
10. Ikebuchi, K., Wong, G. G., Clark, S. C., Ihle, J. N., Hirai, Y. & Ogawa, M. (1987) *Proc. Natl. Acad. Sci. USA* **84**, 9035–9039.
11. Ikebuchi, K., Clark, S. C., Ihle, J. N., Souza, L. M. & Ogawa, M. (1988) *Proc. Natl. Acad. Sci. USA* **85**, 3445–3449.
12. Kishi, K., Ihle, J. N., Urdal, D. L. & Ogawa, M. (1989) *J. Cell. Physiol.* **139**, 463–488.
13. Musashi, M., Yang, Y.-C., Paul, S. R., Clark, S. C., Sudo, T. & Ogawa, M. (1991) *Proc. Natl. Acad. Sci. USA* **88**, 765–769.
14. Musashi, M., Clark, S. C., Sudo, T., Urdal, D. L. & Ogawa, M. (1991) *Blood* **78**, 1448–1451.
15. Tsuji, K., Zsebo, K. M. & Ogawa, M. (1991) *Blood* **78**, 1223–1229.
16. Messner, H. A., Izaquirre, C. A. & Jamal, N. (1981) *Blood* **58**, 402–405.
17. Fauser, A. A., Neumann, H. A., Bross, K. G., Kanz, L. & Lohr, G. W. (1982) *Blood* **60**, 1317–1320.
18. Minato, N., Hattori, M., Sudo, T., Kano, S., Miura, Y., Suda, J. & Suda, T. (1988) *J. Exp. Med.* **167**, 762–776.
19. Suda, T., Ohara, A., Suda, J., Okada, S., Tokuyama, N., Miura, Y., Sudo, T., Nishikawa, S. I. & Nakauchi, H. (1989) in *Experimental Hematology Today-1989*, eds. Gorin, N. C. & Douay, L. (Springer, New York), pp. 45–52.
20. Van der Maazen, R., De Witte, T., Preijers, F., Janssen, J., Blankenborg, G. & Wessels, H. (1987) *Exp. Hematol.* **15**, 1080–1085.
21. Tidmarsh, G. F., Heimfeld, S., Whitlock, C. A., Weissman, I. L. & Mueller-Sieburg, C. E. (1989) *Mol. Cell. Biol.* **9**, 2665–2671.
22. Suda, T., Suda, J., Ogawa, M. & Ihle, J. N. (1985) *J. Cell. Physiol.* **124**, 182–190.