# B CELL PRECURSOR GROWTH-PROMOTING ACTIVITY

## Purification and Characterization of a Growth Factor Active on Lymphocyte Precursors

By ANTHONY E. NAMEN, ANN E. SCHMIERER, CARL J. MARCH, ROBERT W. OVERELL, LINDA S. PARK, DAVID L. URDAL, AND DIANE Y. MOCHIZUKI

*From the Immunex Corporation, Seattle, Washington 98101*

Hemopoiesis in both mouse and man has been the focus of intense investigation in recent years. Dramatic progress has been achieved in resolving the various growth factors involved in many of the major hemopoietic lineages. The cDNAs encoding growth and differentiation factors active on multipotential cells (1–3) and on the granulocytic (4), eosinophilic (5), monocytic (6), and erythroid (7) lineages have now been cloned and expressed. In contrast, very little is known about the regulatory factors involved in the commitment and differentiation of precursor cells along the pathway of lymphogenesis. The pluripotent lymphoid stem cell has not been identified, nor have the factors or conditions required for commitment and expansion of the B cell lineage. The later stages of B cell growth and differentiation after the appearance of surface Ig and the emergence of the B cell from the bone marrow have been the most well studied and have revealed a number of factors that are active on mature peripheral B cells. These include IL-1 (8), IL-2 (9), IL-4 (10, 11), IL-5 (12), IFN-$\gamma$ (13), IFN-$\beta$2 (14), neuroleukin (15), and transforming growth factor $\beta$ (16).

Most of the available evidence on B cell generation has been limited to those B cells that are thought to be the immediate precursors of mature functional peripheral B cells. These pre-B cells have been defined as cells containing cytoplasmic $\mu$ chain but no cytoplasmic light chain and no surface Ig (17, 18). The information on these cells has primarily involved their phenotypic characterization and localization (19–23), Ig gene rearrangements (24–26), and mitogenic responses (27–29). It has also been demonstrated that pre-B cells can differentiate to antibody-secreting B cells in vitro (30, 31). In one report (32) IL-1 has been shown to induce the maturation of either pre-B cells from mixed cell cultures or the pre-B cell lymphoma 70 Z/3. In addition, it was reported that a humoral factor from the serum of NZB mice could enhance the maturation of B lineage precursor cells (33). Finally, Landreth et al. (34) have demonstrated the presence of a factor (or factors) that stimulated the formation of pre-B and B cells in cultures of human or mouse bone marrow.

Although there has been progress in understanding B cell genesis, the major impediment to the study of lymphocyte development has been the difficulties associated with obtaining highly enriched populations of viable B cell precursors.

 J. Exp. Med. © The Rockefeller University Press · 0022-1007/88/03/0988/15 $2.00
Volume 167 March 1988 988–1002

A long-term bone marrow culture system recently developed (35, 36) has proven to be a reliable source of highly enriched B cell precursors with which to characterize the factors required for the growth and maintenance of precursor B cells. The stromal cells isolated from our long-term cultures, as shown by others (37, 38), have proven to be the source of soluble factors that can support the in vitro growth of B precursor cells.

We have extended these observations and used factor-dependent B cell precursors obtained from long-term cultures as the basis for development of a rapid and quantitative proliferation assay to detect a soluble growth factor active on precursor B cells. In addition, we have derived from the stromal elements of a bone marrow culture, a clonal cell line (designated IxN/A6) that secretes this growth factor activity in serum-free medium. The development of both a rapid and specific bioassay as well as a clonal source of the growth factor has allowed us to use conventional biochemical methods to purify a factor active in the development of immature B cells which we have designated lymphopoetin 1 (LP-1).[1]

## Materials and Methods

*B Cell Precursor Population.* All cultures were initiated and maintained as described (36) in RPMI 1640 supplemented with 5% FCS (Irvine Scientific, Santa Clara, CA), 50 μM 2-ME, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine. Our cultures routinely yielded cells that were $B220^+$, surface $\mu^-$, $Ia^-$, Thy-$1.2^-$, and Mac-$1^-$ by cell surface analysis using an EPICS-c FACS. The cells exhibited a lymphocytic appearance with a large nucleus and a small rim of cytoplasm, which is consistent with a pre-B cell phenotype.

*LP-1 Biological Assay.* The nonadherent cells were removed from the adherent stromal layers by gentle pipetting, pelleted by centrifugation, and resuspended in 1–2 ml of Iscove's modified Dulbecco's Medium (IMDM) containing 5% FCS, 50 U/ml penicillin, 50 μg/ml streptomycin, and 2 mM L-glutamine (assay medium).

The cells were then applied to a small column of Sephadex G-10 to remove any contaminating adherent or stromal cells and were incubated at room temperature for 10 min (39). The precursor B cells were then eluted with assay medium, washed, and resuspended to $2.5 \times 10^5$ cells/ml in assay medium. The samples to be assayed were serially diluted in individual wells of a 96-well microtiter plate (No. 3596; Costar, Cambridge, MA) in a final volume of 50 μl. 50 μl of the cell suspension (12,500 cells/well) was added to each well and the plates were incubated for 48 h in a humidified $CO_2$ incubator containing 7.5% $CO_2$ and 5% $O_2$, balance $N_2$. The cultures were pulsed the final 4 h of incubation with 2 μCi/well of [$^3$H]TdR 60–80 Ci/mmol, New England Nuclear, Boston, MA) in a volume of 25 μl. Cultures were harvested with an automated harvester onto glass fiber filters and the radioactivity was determined by liquid scintillation counting. 1 U of LP-1 is defined as the amount of factor required to induce a half-maximal [$^3$H]TdR incorporation under the conditions of the LP-1 assay.

*Generation of the LP-1-producing Cell Line (IxN/A6) by Transfection.* Adherent bone marrow stromal cells were transfected with the plasmid pSV3*neo* containing the transforming sequences of SV40 (40) using the calcium phosphate procedure (41) as modified by Wigler et al. (42). The transfected adherent cells were removed with trypsin/EDTA and cloned by limiting dilution. The supernatants from the resultant clones were tested for growth factor activity and the best producer (IxN/A6) was expanded for further study. This cell line has been monitored monthly for the presence of mycoplasma by both mycoplasma culture and the Gen-Probe mycoplasma detection kit (San Diego, CA) and has been consistently found to be negative.

[1] *Abbreviations used in this paper:* G/M-CSF, granulocyte/macrophage colony-stimulating factor; IMDM, Iscove's modified Dulbecco's medium; LP-1, lymphopoetin 1; RP, reversed-phase.

*LP-1 Production.* Conditioned medium from IxN/A6 cells was generated in 1,750-$cm^2$ tissue culture roller bottles (No. 3029; Falcon Labware, Oxnard, CA) in the following manner. When the cells reached confluence the medium was replaced with 1 liter/roller bottle of serum-free RPMI supplemented with 2.3 gm/liter glucose and 1.54 gm/liter of sodium bicarbonate, containing as a stimulant 1 $\mu$g/ml LPS *Salmonella typhimurium*, (No. 3125-25; Difco Laboratories Inc., Detroit, MI). The bottles were gassed with 10% $CO_2$ in air and incubated for 6 d. The supernatant was harvested and then concentrated 20-fold in an Amicon Corp. (DC10; Danvers, MA) hollow fiber apparatus (10,000 mol wt cutoff). The crude concentrates were sterilized by filtration before assay and purification.

*Source of Other Factors.* The factors used and the corresponding assay systems include human rIL-1$\alpha$ (43–45), natural and recombinant human IL-1$\beta$ (43–45), human rIL-2 (46–48), murine IL-3 (48, 49), murine IL-4 (50, 51), murine IL-5 (52), murine granulocyte/macrophage colony-stimulating factor (GM-CSF) (53, 54), human G-CSF (55) and murine M-CSF (56), murine IFN-$\alpha$ and -$\beta$ (Lee Biomolecular, San Diego, CA), and human transforming growth factor $\beta$ (Calbiochem-Behring Corp., La Jolla, CA).

*DEAE-Sephacel Chromatography.* 1 liter of the crude concentrated conditioned medium was adjusted to pH 8 with 1 N NaOH and diluted with sterile distilled water to a conductivity of 1 m Siemen/cm (a conductivity of 1 m Siemen/cm corresponds to a sodium chloride concentration of 100 mM). The concentrate was then applied to a DEAE-Sephacel (Pharmacia Fine Chemicals, Piscataway, NJ) column (5.0 × 15 cm) previously equilibrated with 20 mM Tris-HCl (pH 8), containing 100 mM NaCl. The effluent was monitored by $OD_{280}$ and the protein-containing flow-through fraction was collected for further purification.

*SP-Trisacryl Chromatography.* The nonbinding fraction from the DEAE-Sephacel column was adjusted to pH 5 with 1 M citric acid (free acid). This was then applied directly to a column (3.2 × 12.5 cm) of SP-Trisacryl (LKB Instruments, Inc., Gaithersburg, MD) that had previously been equilibrated in 10 mM citrate (pH 5.0) containing 100 mM NaCl. The column was washed with five column volumes of equilibration buffer followed by washing with five column volumes of 20 mM Tris-HCl (pH 8). When the pH of the column effluent reached pH 8 and the $OD_{280}$ had returned to baseline values the column was eluted with a 1 liter linear gradient from 0.0 to 0.5 M sodium chloride in 20 mM Tris-HCl (pH 8). The elution was carried out at a flow rate of 50 ml/h and 10-ml fractions were collected and assayed for the presence of LP-1.

*Blue B Chromatography.* Pooled LP-1-containing fractions from two separate SP-Trisacryl elutions were pooled and applied directly to a column (2.5 × 40 cm) of Blue B agarose (Amicon Corp.) that had previously been equilibrated in 20 mM Tris-HCl (pH 8) containing 125 mM NaCl. The sample was applied at a flow rate of 15 ml/h. After washing with equilibration buffer until the $OD_{280}$ had returned to baseline values, the column was eluted with a 1 liter linear gradient from 0.125 to 2 M NaCl in 20 mM Tris-HCl (pH 8). 10-ml fractions were collected at a flow rate of 25 ml/h and assayed for LP-1 activity.

*Reversed-phase HPLC (RP-HPLC).* HPLC was performed with a Waters Associates (Milford, MA) liquid chromatograph equipped with two model 510 pumps, a model 720 system controller, and a model 441 absorbance detector monitoring at 214 nm essentially as previously described (48). Large sample volumes were pumped onto columns with a Milton Roy minipump (Laboratory Data Control, Rivera Beach, FL). Solvents were purchased from Burdick & Jackson Laboratories Inc. (Muskegon, MI).

Fractions containing LP-1 activity from the Sepharose Blue B column were applied at a flow rate of 5 ml/min to an 8 mm × 10 cm radial PAK cartridge (Waters Associates) containing 15–20 $\mu$m Vydac C-4 reversed-phase silica (The Separations Group, Hesperia, CA). Water containing 0.1% trifluoroacetic acid (TFA, solvent A) was flushed through the columns until the absorbance at 214 nm was down to baseline levels. At this time, a linear gradient was established that went from 0 to 100% solvent B (acetonitrile containing 0.1% TFA) at a rate of 1% solvent B/min and a flow rate of 1 ml/min.

Active fractions after a single HPLC phase were pooled, diluted with two volumes of 0.9 M acetic acid and 0.2 M pyridine (pH 4.0, buffer $A_2$), and were applied to the same

column that had been reequilibrated in the pyridine–acetate solvent system. A gradient of solvent $B_2$ (0.9 M acetic acid, 0.2 M pyridine, and 60% *N*-propanol) was established that went from 0 to 20% solvent $B_2$ in 10 min and from 20% $B_2$ to 84% $B_2$ in 100 min, at a flow rate of 1 ml/min.

The fractions containing activity from this second HPLC phase were pooled, diluted with two volumes of solvent $A_2$, and applied to a 3.9-mm by 30-cm radial PAK column packed with 10 μm Vydac C-18 reversed-phase silica that had been previously equilibrated in 20% solvent $B_2$. A gradient of solvent $B_2$ from 20 to 84% was used to elute protein off the column. Fractions containing activity from the third phase of HPLC were diluted with two volumes of solvent $A_1$ (0.1% TFA) and applied to the C-18 column previously equilibrated in 20% solvent $B_1$ (acetonitrile, 0.1% TFA) and a gradient of solvent $B_1$ established from 20 to 100% $B_1$ at a rate of change of 1% per minute and a flow rate of 1 ml/min was used to elute LP-1.

*Generation of the IxN/2b Cell Line.* The nonadherent precursor B cells from an 8-wk-old bone marrow culture were seeded into 96-well trays at 0.3 cells/well in assay medium containing 500 U/ml of partially purified LP-1. After 5 d of incubation, one-half of the medium was replaced with fresh assay medium containing LP-1. By the twelfth day after seeding, growth was visually obvious in 12 of the 96 wells. These clones were expanded and tested for their dependency upon exogenous LP-1. One of the clones selected, designated IxN/2b has been grown in the absence of any exogenous feeder or stromal cells for >10 mo. This cell line is absolutely dependent upon the presence of exogenous LP-1 for continued growth and viability.

*Radiolabeling of LP-1 and Binding to Intact Cells.* An aliquot (25 μl) of the fraction containing biological activity from the fourth HPLC column was radiolabeled with $^{125}I$ by the enzymobead procedure used for the radiolabeling of other lymphokines (57).

LP-1-dependent cells (IxN/2b) and LP-1 nonresponsive cells (CTLL, a murine IL-2-dependent T cell line [46]) were washed in RPMI 1640, suspended at a concentration of $2 \times 10^8$ cell/ml in RPMI 1640 containing 20 mM Hepes buffer, 0.2% sodium azide, and 2% BSA, pH 7.2, and an aliquot of radiolabeled LP-1 containing $2.0 \times 10^6$ cpm with or without 5 μl of the unlabeled LP-1 as a cold competitor was added. Tubes were incubated for 1 h at 37°C with rocking and then the cells washed three times in cold PBS by centrifugation. After the final wash the cell pellet was extracted in 50 μl of PBS containing 1% Triton X-100, PMSF (2 mM), pepstatin (10 μM), leupeptin (10 μM), *o*-phenanthroline (2 mM), and EGTA (2 mM). The suspension was centrifuged and aliquots of the supernatant were analyzed by autoradiography of the dried gel after polyacrylamide gel electrophoresis as previously described (57).

*SDS-PAGE.* SDS-PAGE was performed as described by Laemmli (58). Material purified through four phases of RP-HPLC was evaporated to dryness under vacuum in a SpeedVac apparatus (Savant Instruments, Inc., and redissolved in a small volume of SDS sample buffer without 2-ME. The sample was electrophoresed onto a 12% acrylamide gel, the gel in the appropriate lane was sliced into 1–2-mm sections, and each section was minced and eluted by diffusion overnight into 0.2% SDS and the biological activity in each fraction was then determined. The active fractions were pooled and an aliquot was analyzed after electrophoresis on a Phastgel PAGE System (Pharmacia Fine Chemicals) using a 10–15% gradient gel. Proteins were visualized using a silver-staining method recommended by Pharmacia Fine Chemicals.

*Protein Assay.* Protein determinations were carried out using a modified Bradford dye-binding assay (Bio-Rad Laboratories, Richmond, CA). BSA was used to establish a standard curve for each assay performed.

## Results

The development of an in vitro culture system for B cell precursors (35, 36) led to our initial interest in characterizing factors that might regulate their growth and differentiation. We speculated, based upon the apparent strict growth dependence of these precursors on their stromal feeder layers that these cells

produced a B cell precursor growth stimulatory activity. Characterization of this growth activity was considered feasible if we could both develop a quantitative assay and identify a good cellular source of the growth-promoting activity.

*Derivation of the IxN/A6 Cell Line.* Our initial attempts to develop a bioassay used cell-free supernatants from stromal bone marrow (feeder) layers as a source of factors. Generally the assay results were equivocal because a stimulation index of only two- to fourfold over background was observed (data not shown). This level of stimulation was consistent with results obtained by others (37, 38).

Initially, it was not clear if the low level of stimulation was due to a paucity of factor production, the presence of other suppressive or inhibitory materials in the conditioned medium, or an absolute requirement for direct cell contact. We considered that an immortalized cell line might circumvent some of these difficulties and allow us to establish a suitable cellular source of this growth factor. We therefore established stromal cell lines after the transfection of primary adherent stromal cells with the plasmid pSV3*neo* (40), which encodes the large and small T antigens of SV40. A number of immortal cell lines emerged from the transfected cultures but not from uninfected control cultures. One of the resultant clonal cell lines, designated IxN/A6 (Fig. 1 *B*), was found to produce a factor capable of supporting the growth of precursor B cells. Presumably, the immortalization of this cell line was due to the transfection by pSV3*neo*, as immunoprecipitation results confirmed that the cells expressed the SV40 large and small T antigens (data not shown). Both the IxN/A6 cells themselves and their culture supernatants could stimulate the proliferation of precursor B cells. Subsequently, it was found that in serum-free medium, stimulation of the IxN/A6 cells with 1 $\mu$g/ml LPS could increase the levels of factor produced fourfold.

*Biological Assay for LP-1.* The cellular basis of the LP-1 assay was a highly enriched population of precursor B cells obtained from our long-term bone marrow cultures. These cells exhibited a typical lymphocyte morphology as shown in Fig. 1 *A*. We found a further enrichment step, G-10 Sephadex passage, was necessary to obtain suitable biological assay results.

The results of a typical bioassay response to (*A*) LPS-stimulated crude IxN/A6 conditioned medium and (*B*) a 20-fold hollow fiber concentrate are graphically shown in Fig. 2. In this particular preparation the crude conditioned medium contained 19 U/ml and the 20× concentrate contained 642 U/ml of LP-1. The levels of LP-1 obtained in the large batches of crude conditioned medium ranged between 10 and 100 U/ml.

The LPS stimulant alone had no effect on the precursor B cells. The 20× hollow fiber concentration step often resulted in a recovery of >100% activity, presumably due to removal of inhibitory metabolic cellular products. The stimulation index of the LP-1 assay was routinely in the range of 15–20 times the level of background thymidine incorporation.

*Specificity of the LP-1 Assay.* Before initiating efforts to purify the LP-1 from the IxN/A6 cells, it was considered important to determine whether other known factors could stimulate pre-B cell proliferation and to assess the specificity of our established assay. We therefore evaluated the specificity of this assay system with other defined factors. None of the factors tested, including IL-1$\alpha$, IL-1$\beta$, IL-2, IL-3, IL-4, IL-5, GM-CSF, G-CSF, CSF-1, IFN-$\alpha$, $\beta$, and $\gamma$, neuroleukin, and

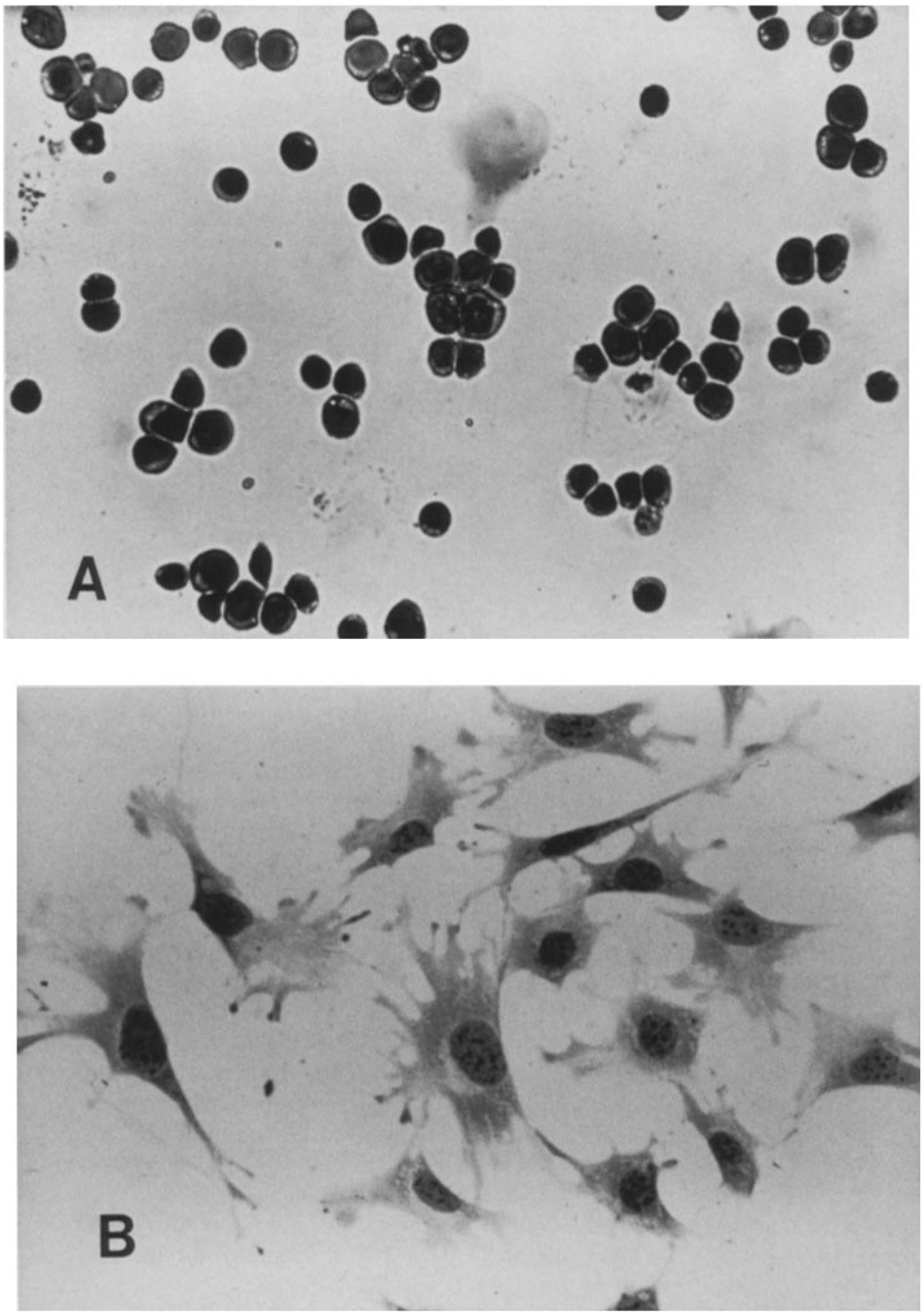


FIGURE 1. Typical morphology of precursor B cells derived from long-term bone marrow cultures and the IxN/A6. Wright-Giemsa–stained preparations of (*A*) nonadherent precursor B cells obtained from 8-wk-old bone marrow cultures; (*B*) the LP-1-secreting cell line IxN/A6 derived from the stromal elements of a bone marrow culture. × 400.

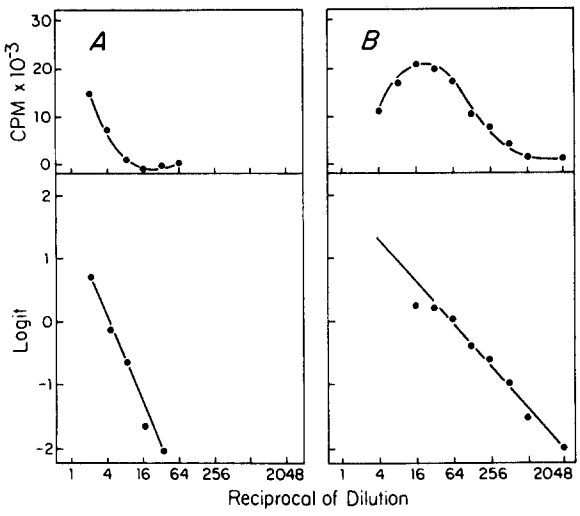


FIGURE 2. Proliferative response of long-term bone marrow culture-derived precursor B cells in the LP-1 quantitative microassay. (*A*) The response to crude conditioned medium from a 6-d stimulation of IxN/A6 cells, and (*B*) the response to a 20× concentrate of the same conditioned medium. The top portion of each panel depicts the raw data. Each cpm value is converted to a percentage of the maximum cpm. A logit transformation (63) is then applied to these percentages to obtain data that are a linear function of the $\log_2$ dilution. Values preceding the maximum of each dilution set are excluded from the linear regression analysis. Weighted linear regression is used to fit parallel lines to each sample and standard and units per milliliter can be computed in terms of the dilution that gives half-maximal activity.

TABLE I

*Purification of Lymphopoetin 1 (LP-1)*

| Step | SA | Purification | Yield |
|---|---|---|---|
| | *U/μg* | *-fold* | *%* |
| 1: Concentrate | 0.38 | 1 | —* |
| 2: DEAE-Sephacel (flow-through) | 4.63 | 12.2 | 100 |
| 3: SP-Trisacryl | 153 | 403 | 113 |
| 4: Blue B Agarose | 423 | 1113 | 80 |
| 5: RP-HPLC‡ | $8 \times 10^4$ | $2 \times 10^5$ | 50 |
| 6: SDS-PAGE‡ | $4 \times 10^6$ | $10 \times 10^6$ | 35 |

* The crude concentrated conditioned medium usually contains some level of toxic/inhibitory materials, presumably due to metabolic cellular products. Most of these inhibitory materials are removed by the DEAE-Sephacel step and the yields are based on the activity after DEAE-Sephacel chromatography.

‡ Specific activity based on protein values estimated from silver staining.

transforming growth factor β induced stimulation of thymidine incorporation over background levels throughout a range of factor concentrations (data not shown). In addition, purified LP-1 did not elicit any response in standard bioassays for IL-1, IL-2, IL-3, GM-CSF, IL-4, IL-5, G-CSF, CSF-1, and IFN. However, cell-free supernatants from the IxN/A6 cell line were found to stimulate the formation of murine macrophage-type colonies in soft agar. We subsequently determined that fractionation of the conditioned medium by DEAE-Sephacel chromatography completely resolved the colony-stimulating activity from the precursor B cell growth-promoting activity.

*Purification and Characterization of LP-1.* Table I summarizes the results of the procedures used to purify LP-1. At pH 8.0 and 100 mM NaCl, the LP-1 activity in the concentrated medium from IxN/A6 cells did not bind to a DEAE-Sephacel column, although 90% of the total protein present in the crude conditioned medium, including that responsible for macrophage colony-stimu-

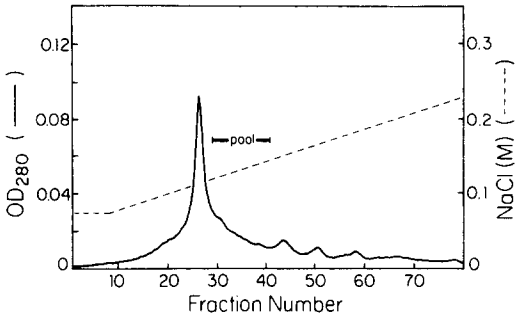


FIGURE 3. SP-Trisacryl chromatography of the nonbinding flow-through material after DEAE-Sephacel chromatography. A linear gradient ranging from 0.0 to 0.5 M NaCl in 10 mM Tris-HCl, pH 8, was used to elute the LP-1. The active fractions indicated (numbers 29–42) were pooled for subsequent purification.

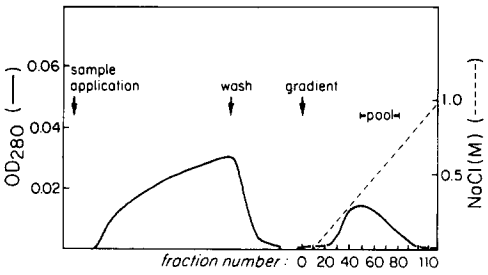


FIGURE 4. Chromatography on Blue B agarose. The active fractions from SP-Trisacryl chromatography were applied directly to the Blue B agarose. A steep gradient ranging from 0.0 to 2.0 M NaCl was then used to elute the LP-1. Those fractions containing biological activity were pooled as indicated for further purification by RP-HPLC.

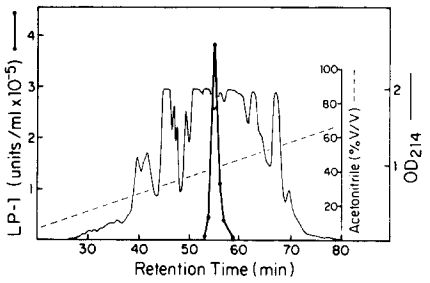


FIGURE 5. First-phase purification of LP-1 by RP-HPLC. The active fractions from a typical Blue B chromatography purification were applied to a $C_4$ column and eluted with increasing concentrations of acetonitrile in 0.1% TFA.

lating activity, did bind to the column. The chromatographs of subsequent steps, which included SP-Trisacryl, Blue B agarose, and four separate reversed-phase HPLC protocols, are shown in Figs. 3–6 and resulted in a 200,000-fold purification of LP-1 activity.

The processing of 800 liters of crude conditioned medium in this fashion resulted in four HPLC column fractions that contained a total of $5 \times 10^6$ U of LP-1 activity. Analysis of this material by SDS-PAGE and silver staining or after iodination, SDS-PAGE, and autoradiography (Fig. 7, lane *a*) revealed that a number of proteins were still present at this stage of purification. The major protein present in this material exhibited a molecular mass of 18 kD and faint bands above and below this band were detectable.

Two experiments were performed to identify the protein in this mixture that was responsible for LP-1 activity. In the first, LP-1 was applied to SDS-PAGE under nonreducing conditions. After the completion of electrophoresis the gel was cut into 1-mm bands and the protein in each band was eluted into medium and assayed for biological activity. The results are shown in Table II and indicated that the biological activity was associated with a protein of $M_r$ $25 \times 10^3$ and was

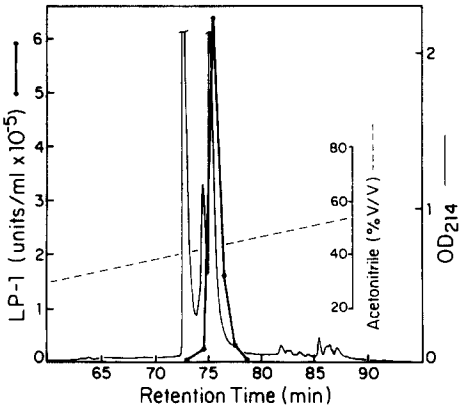


FIGURE 6. Fourth-phase purification of LP-1 by RP-HPLC. The active fractions after three phases of RP-HPLC were applied to a $C_{18}$ column and eluted with a linear gradient of increasing concentrations of acetonitrile in 0.1% TFA.

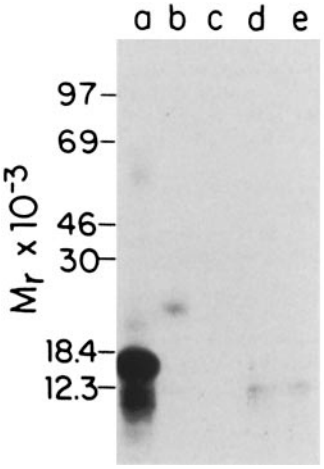


FIGURE 7. Binding of $^{125}$I–LP-1 to LP-1-dependent IxN/2b cells. (Lane *a*) Iodinated starting material. IxN/2b (lanes *b* and *c*) and CTLL (lanes *d* and *e*) cells ($2 \times 10^7$) were incubated with $^{125}$I–LP-1 ($2 \times 10^6$ cpm) both in the presence (lanes *c* and *e*) and absence (lanes *b* and *d*) of a 50-fold molar excess of unlabeled LP-1 for 1 h at 37°C. Cells were then harvested, washed, and extracted with PBS-1% Triton containing a mixture of protease inhibitors as described under Materials and Methods. Aliquots corresponding to $2 \times 10^6$ cells were boiled for 3 min in sample buffer containing 2% SDS and subjected to electrophoresis on a 10–20% linear gradient gel.

distinct from the region of the gel corresponding to that containing the $M_r$ 18 × $10^3$ protein band. No activity was isolated when electrophoresis was performed under reducing conditions.

In the second experiment, $^{125}$I-labeled LP-1 (Fig. 7, lane *a*) was absorbed to cells that responded to this factor (IxN/2b). After incubation with $^{125}$I–LP-1, the cells were washed and then extracted with PBS containing 1% Triton X-100. An aliquot of this material was then analyzed by SDS-PAGE and autoradiography. The results showed (Fig. 7, lane *b*) that of the $^{125}$I-labeled proteins present in the radiolabeled preparation, only a protein of $M_r$ 25,000 appeared to specifically bind to cells that responded to LP-1. Excess unlabeled LP-1 (Fig. 7, lane *c*) inhibited the binding of this protein and cells that do not respond to LP-1 failed to bind the $M_r$ 25 × $10^3$ protein (Fig. 7, lane *d*). The results of both experiments suggested that a protein of $M_r$ 25 × $10^3$ was responsible for B cell precursor growth-promoting activity.

The retention of PBGF activity after SDS-PAGE suggested that this procedure could be used as the final step in the purification of this protein. The results depicted in Fig. 8 showed that, as found in the pilot experiment, LP-1 activity

TABLE II
*Recovery of LP-1 After SDS-PAGE*

| Slice number* | Mol wt‡ | Amount recovered | Recovery |
|---|---|---|---|
| | $\times 10^3$ | *U/ml* | % |
| 1 | 43 | — | — |
| 2 | | — | — |
| 3 | | — | — |
| 4 | | 815 | — |
| 5 | 31 | 2,912 | 3.5 |
| 6 | | 11,374 | 13.6 |
| 7 | | 40,000 | 44.0 |
| 8 | | 19,011 | 22.8 |
| 9 | 21 | 1,951 | 2.3 |
| 10 | | 247 | — |
| 11 | | — | — |
| 12 | 14 | — | — |
| Total | | | 86.2 |

LP-1 that had been purified through four steps of HPLC was dried, reconstituted in nonreducing sample buffer, and applied to polyacrylamide gel as described in the Materials and Methods.

* After electrophoresis, the gel was sliced into 1-mm pieces and each piece was eluted and tested for biological activity in the LP-1 assay.

‡ Slice 1 corresponded to the position of the $M_r$ 43,000 marker; slice 5, with the $M_r$ 31,000 marker; slice 9 with the $M_r$ 21,000 marker; and slice 12, with the $M_r$ 14,000 marker.

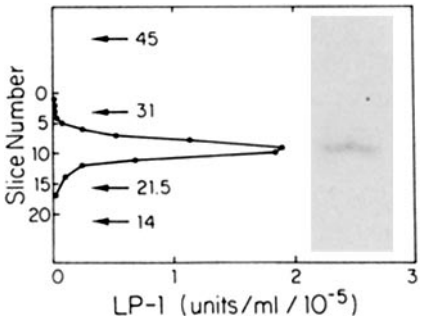


FIGURE 8. Purification of LP-1 by SDS-PAGE. The active fractions after four phases of RP-HPLC were evaporated to dryness, redissolved in nonreducing SDS-PAGE sample buffer, and electrophoresed on a 12% polyacrylamide gel. The gel was sliced, minced, and the activity was eluted in 0.2% SDS. An aliquot of each fraction was analyzed by Phastgel-PAGE and silver staining as recommended by Pharmacia Fine Chemicals.

eluted at a position coinciding with a protein of $M_r$ 25 × $10^3$. PAGE analysis of this bioactive fraction revealed a single protein detected by silver staining. The final specific activity was estimated to be 4 × $10^6$ U/μg protein, which would represent a purification of $10^7$-fold from the starting material. The purified LP-1 was active in the in vitro bioassay at a concentration of $10^{-13}$ M.

## Discussion

The later stages of B cell development have been well characterized at the cellular and molecular level. In contrast, very little is known about the events or factors involved in development of immature lymphocytes. Recently, a number of studies have appeared that use long-term lymphoid bone marrow cultures in

the study of B lymphocyte growth and development (59–61). Generally, the information derived from these studies has been restricted to phenotypic and morphological description of the culture system and the cells produced in these cultures. However, recent evidence that soluble factors from stromal cells could control precursor B cell growth has emerged (37, 38). In addition, it has been reported that precursor B and precursor T lymphocyte clones respond to IL-3 and IL-4 (62), cytokines previously associated with the proliferation and differentiation of a multipotential stem cell from the bone marrow and with more mature B and T cells, respectively.

To characterize the factors required for precursor B cell growth we exploited in vitro culture systems in two ways. First, precursor B cells cultured in vitro proliferate only when grown in the presence of stromal cells or when supplemented with medium conditioned by the growth of these cells. This property served as the basis for the development of a rapid and specific bioassay that could be used to detect this activity. A panel of purified natural or recombinant cytokines, including those that have been reported to stimulate various B cell responses, were tested in the LP-1 assay, and all failed to stimulate the proliferation of precursor B cells. In a similar fashion, the assays used to monitor the different activities associated with this panel all failed to respond to LP-1, indicating that the material responsible for the LP-1 activity was distinct from the well-characterized proteins detected by these assays. Secondly, we established a stable stromal cell line by transforming the primary stromal cell cultures with a plasmid vector encoding the transforming sequences of SV40. This procedure resulted in the isolation of a clonal cell line, IxN/A6, that secreted enhanced levels of LP-1 activity.

The purification strategy revealed that LP-1 activity was stable to a wide spectrum of conditions that included extremes of pH (2.1 to 8.0) and solvents containing acetonitrile or *n*-propanol. Resolution of the activity by PAGE gave conclusive proof that the LP-1 activity was associated with a protein of $M_r$ $25 \times 10^3$ and was stable in the presence of SDS and heat. In contrast, reduction of the protein by the addition of 2-ME completely destroyed its biological activity. Preparative SDS-PAGE resulted in the resolution of a single protein of $M_r$ $25 \times 10^3$ that coincided with the biological activity. Moreover, radiolabeled LP-1 bound specifically to cells that respond to this cytokine, suggesting that LP-1, like other polypeptide hormones, binds to specific receptors on the surface of cells. The purification protocol resulted in a $10^7$-fold purification with a final yield of 35%. The purified LP-1 exhibited a specific activity of $\sim 4 \times 10^6$ U/$\mu$g protein and was active at a concentration of $10^{-13}$ M.

The availability of this novel factor in pure form will allow us to elucidate its role within the developmental pathway of lymphogenesis and to define more precisely the repertoire of cells that have the capacity to respond to this cytokine.

## Summary

We have used a biological assay system we developed to biochemically purify a previously uncharacterized murine lymphopoetic growth factor designated lymphopoetin 1 (LP-1). This factor is capable of stimulating the proliferation and extended maintenance of precursor cells of the B lineage. A stromal cell line

producing LP-1 was established after transfection of primary stromal cultures with a plasmid encoding the transforming genes of SV40. This factor was purified to a single 25-kD species from the culture supernatant of an adherent stromal cell line. This material acts on immature lymphocytes, it binds to specific receptors on cells, and is distinct from previously described hematopoietic factors. LP-1 has been purified some $10^7$-fold with an overall recovery of 35%. The purified protein exhibits a specific activity of $\sim 4 \times 10^6$ U/$\mu$g of protein and is active at a half-maximal concentration of $10^{-13}$ M.

We wish to acknowledge the excellent technical assistance of Alan Alpert, June Eisenman, Ralph Klinke, Andrew Lewis, and Della Friend. We also thank Linda Troup for her assistance in the preparation of the manuscript. We also thank Robert J. Tushinski for performing the soft agar colony assays.

*Received for publication 19 October 1987 and in revised form 7 December 1987.*

## References

1. Fung, M. C., A. J. Happel, S. Ymer, D. R. Cohen, R. N. Johnson, H. D. Campbell, and I. G. Young. 1984. Molecular cloning of cDNA for mouse interleukin-3. *Nature (Lond.)*. 307:233.
2. Yang, Y. C., A. B. Ciarletta, P. A. Temple, M. P. Chung, S. Kovacic, J. S. Witek-Giannotti, A. C. Leary, R. Kriz, R. E. Donahue, G. G. Wong, and S. C. Clarke. 1986. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell.* 47:3.
3. Gough, N. M., J. Gough, D. Metcalf, A. Kelso, D. Grail, N. A. Nicola, A. N. Burgess, and A. R. Dunn. 1984. Molecular cloning of cDNA encoding a murine haematopoietic growth regulator, granulocyte-macrophage colony stimulating factor. *Nature (Lond.)*. 309:763.
4. Nagata, S., M. Tsuchiya, S. Aszno, Y. Kaziro, T. Yamazzki, D. Yamamoto, Y. Hiratz, N. Kabota, M. Oheda, H. Norura, and M. Ono. 1986. Molecular cloning and expression of a cDNA for human granulocyte colony stimulating factor. *Nature (Lond.)*. 319:415.
5. O'Garra, A. O., D. J. Warren, M. Holman, A. M. Popham, C. J. Sanderson, and G. G. B. Klaus. 1986. Interleukin 4 (B-cell growth factor II/eosinophil differentiation factor) is a mitogen and differentiation factor for preactivated murine B lymphocytes. *Proc. Natl. Acad. Sci. USA.* 83:5228.
6. Stanley, E. R., and P. M. Heard. 1977. Factors regulating macrophage production and growth: purification and some properties of the colony stimulating factor from medium conditioned by mouse L cells. *J. Biol. Chem.* 252:4305.
7. Graber, S. E., and S. B. Kranz. 1978. Erythropoetin and the control of red cell production. *Annu. Rev. Med.* 29:51.
8. Pike, B. L., and G. J. U. Nossal. 1985. Interleukin 1 can act as a B-cell growth and differentiation factor. *Proc. Natl. Acad. Sci. USA.* 82:8153.
9. Kehrl, J. H., J. H. Grove, P. K. Goldsmith, and A. S. Fauci. 1986. B cell growth and differentiation factors interact with receptors distinct from the interleukin 2 receptor. *Eur. J. Immunol.* 16:761.
10. Noma, Y., P. Sideras, T. Naito, S. Bergstedt-Lindquist, C. Asoma, E. Severinson, T. Tanabe, T. Kinashi, F. Matsuda, Y. Yzoita, and T. Hongjo. 1986. Cloning of cDNA encoding the murine IgG, induction factor by a novel strategy using SP6 promoter. *Nature (Lond.)*. 319:640.

11. Lee, F., T. Yokota, T. Otsuka, P. Meyerson, D. Villaret, R. Coffman, T. Mosmann, D. Rennick, N. Roehm, C. Smith, A. Zlotnik, and K. Aria. 1986. Isolation and characterization of a mouse interleukin cDNA clone that expresses B-cell stimulatory factor-1 activities and T-cell and mast cell stimulating activities. *Proc. Natl. Acad. Sci. USA.* 83:2061.
12. Kinishi, T., H. Harada, E. Severinson, T. Tanabe, P. Sideras, M. Konishi, C. Azuma, A. Tominaga, S. Bergstedt-Lindquist, M. Takahashi, F. Matsuda, Y. Yaoita, K. Takatsu, and T. Honjo. 1986. Cloning of complementary DNA encoding T-cell replacing factor and identity with B-cell growth factor II. *Nature* (*Lond.*). 354:70.
13. Leibson, H. J., M. Geffey, A. Zlotnik, P. Maurack, and J. W. Kappler. 1984. Role of gamma-interferon in antibody-producing responses. *Nature* (*Lond.*). 309:799.
14. Billiau, A. 1986. BSF-2 is not just a differentiation factor. *Nature* (*Lond.*). 324:415.
15. Gurney, M. E., B. R. Apatoff, G. T. Spear, M. J. Baumel, J. P. Antel, M. B. Bania, and A. T. Reder. 1987. Neuroleukin: a lymphokine product of lectin-stimulated T cells. *Science* (*Wash. DC*). 234:574.
16. Kehrl, J. H., A. B. Roberts, L. M. Wakefield, S. Jackowlew, M. B. Sporin, and A. S. Fauci. 1986. Transforming growth factor B is an important immunomodulatory protein for human B lymphocytes. *J. Immunol.* 137:3855.
17. Raff, M. C., M. Megson, J. J. T. Owen, and M. D. Cooper. 1976. Early production of intracellular IgM by B-lymphocyte precursors in mouse. *Nature* (*Lond.*). 259:224.
18. Levitt, D., and M. D. Cooper. 1980. Mouse pre-B cells synthesize and secrete $\mu$ heavy chains but not light chains. *Cell.* 19:617.
19. Kincade, P. W., G. Lee, T. Watanabe, L. Sun, and M. P. Scheid. 1981. Antigens displayed on murine B-lymphocyte precursors. *J. Immunol.* 127:2262.
20. McKearn, J. P., C. Bau, and J. M. Davie. 1984. Cell surface antigens expressed by subsets of pre-B cells and B cells. *J. Immunol.* 132:332.
21. Coffman, R. L., and I. L. Weissman. 1981. A monoclonal antibody which recognizes B cells and B cell precursors in mice. *J. Exp. Med.* 153:269.
22. Owen, J. J. T., M. D. Cooper, and M. C. Raff. 1974. *In vitro* generation of B lymphocytes in mouse fetal liver, a mammalian bursa equivalent. *Nature* (*Lond.*). 249:361.
23. Melchers, F. 1979. Murine embryonic B lymphocyte development in the placenta. *Nature* (*Lond.*). 277:219.
24. Honjo, T., S. Nakai, Y. Nishida, T. Katzoki, Y. Yamanakin-Karzoko, N. Takahaski, M. Obata, A. Shimizu, Y. Yaoita, T. Nikaido, and N. Ishida. 1981. Rearrangement of immunoglobulin genes during differentiation and evolution. *Immunol. Rev.* 59:33.
25. Denis, R. A., L. J. Treiman, J. I. St. Claire, and O. N. Witte. 1984. Long term cultures of murine fetal liver retain very early B lymphoid phenotype. *J. Exp. Med.* 160:1087.
26. Whitlock, C. A., S. F. Zeigler, L. J. Truman, J. I. Statford, and O. N. Witte. 1983. Differentiation of cloned populations of immature B cells after transformation with Abelson murine leukemia virus. *Cell.* 32:903.
27. Melchers, F. 1977. B lymphocyte development in fetal liver. I. Development of reactivity to B cell mitogens *in vivo* and *in vitro*. *Eur. J. Immunol.* 7:746.
28. Melchers, F., J. Andersson, and R. A. Phillips. 1975. B-lymphocyte subpopulations in the mouse organ distribution and ontogeny of immunoglobulin synthesizing and of mitogen sensitive cells. *Transplant. Rev.* 25:26.
29. Fairchild, S. S., and J. J. Cohen. 1978. B lymphocyte precursors. I. Induction of lipopolysaccharide responsiveness and surface immunoglobulin expression *in vitro*. *J. Immunol.* 121:1227.

30. Paige, C. J. 1983. Surface immunoglobulin-negative B-cell precursors detected by formation of antibody secreting colonies in agar. *Nature* (*Lond.*). 302:711.
31. Paige, C. J., R. H. Gisler, J. P. McKearn, and N. N. Iscove. 1984. Differentiation of murine B cell precursors in agar culture. Frequency, surface marker analysis and requirements for growth of clonable pre-B cells. *Eur. J. Immunol.* 14:979.
32. Giri, J. G., P. N. Kincade, and S. B. Mizel. 1984. Interleukin 1 mediated induction of K-light chain synthesis and surface immunoglobulin expression on pre-B cells. *J. Immunol.* 132:223.
33. Jyonouchi, H., M. B. Kimmel, G. Lee, P. N. Kincade, and R. A. Good. 1985. Humoral factors in very young NZB mice that enhance the maturation of normal B cell precursors. Partial purification and characterization. *J. Immunol.* 135:1891.
34. Landreth, K. S., D. Engelhard, M. H. Beare, P. N. Kincade, N. Kapoor, and R. A. Good. 1985. Regulation of human B lymphopoesis: effect of a urinary activity associated with cyclic neutropenia. *J. Immunol.* 134:2305.
35. Whitlock, C. A., and O. N. Witte. 1982. Long term culture of B lymphocytes and their precursors from murine bone marrow. *Proc. Natl. Acad. Sci. USA.* 79:3608.
36. Whitlock, C. A., D. Robertson, and O. N. Witte. 1984. Murine B cell lymphopoesis in long term culture. *J. Immunol. Methods.* 67:353.
37. Whitlock, C. A., G. F. Tidmarsh, C. Muller-Sieburg, and I. L. Weissman. 1987. Bone marrow stromal cell lines with lymphopoetic activity express high levels of a pre-B neoplasia-associated molecule. *Cell.* 48:1009.
38. Hunt, P., D. Robertson, D. Weiss, D. Rennick, F. Lee, and D. N. Witte. A single bone marrow-derived stromal cell type supports the *in vitro* growth of early lymphoid and myeloid cells. *Cell.* 48:997.
39. Ly, I. A., and R. I. Mishell. 1974. Separation of mouse spleen cells by passage through columns of Sephadex G-10. *J. Immunol. Methods.* 5:239.
40. Southern, P. J., and P. Berg. 1982. Transformation of mammalian cells to antibiotic resistance with a bacterial gene under control of the SV40 early region promoter. *J. Mol. Appl. Genet.* 1:327.
41. Graham, F. L., and A. J. van der Eb. 1973. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology.* 52:456.
42. Wigler, M., A. Pellicer, S. Silverstein, and R. Axel. 1978. Biochemical transfer of single-copy eucaryotic genes using total cellular DNA as donor. *Cell.* 14:725.
43. March, C. J., B. Mosley, A. Larsen, D. P. Cerretti, G. Braedt, V. Price, S. Gillis, C. S. Henney, S. R. Kronheim, K. Grabstein, P. J. Conlon, T. P. Hopp, and D. Cosman. 1985. Cloning, sequence, and expression of two distinct human interleukin-1 complementary DNAs. *Nature* (*Lond.*). 315:641.
44. Kronheim, S. R., C. J. March, S. K. Erb, P. J. Conlon, D. Y. Mochizuki, and T. P. Hopp. 1985. Human interleukin-1: purification to homogeneity. *J. Exp. Med.* 161:490.
45. Kronheim, S. R., M. A. Cantrell, M. C. Deeley, C. J. March, P. J. Glackin, D. M. Anderson, T. Hemenway, J. E. Merriam, D. Cosman, and T. P. Hopp. 1986. Purification and characterization of human interleukin-1 expressed in *Escherichia coli*. *Bio-Technology* (*NY*). 4:1078.
46. Gillis, S., M. M. Ferm, W. Ou, and K. A. Smith. 1978. T cell growth factor: parameters of production and a quantitative microassay for activity. *J. Immunol.* 120:2027.
47. Watson, J., S. Gillis, J. Marbrook, D. Mochizuki, and K. A. Smith. 1979. Biochemical and biological characterization of lymphocyte regulatory molecules. I. Purification of a class of murine lymphokines. *J. Exp. Med.* 150:849.
48. Urdal, D., D. Mochizuki, P. Conlon, C. J. March, J. Remerowski, J. Eisenman, C.

Ramthun, and S. Gillis. 1984. Lymphokine purification by reversed phase high-performance liquid chromatography. *J. Chromatogr.* 296:171.

49. Prestidge, R., J. D. Watson, D. L. Urdal, D. Mochizuki, P. Conlon, and S. Gillis. 1984. Biochemical comparison of murine colony stimulating factors secreted by a T cell lymphoma and a myelomonocytic leukemia. *J. Immunol.* 133:293.
50. Grabstein, K., J. Eisenman, D. Mochizuki, K. Shanebeck, P. Conlon, T. Hopp, C. March, and S. Gillis. 1986. Purification to homogeneity of B cell stimulating factor. *J. Exp. Med.* 163:1405.
51. Park, L. S., D. Friend, K. Grabstein, and D. Urdal. 1987. Characterization of the high affinity cell surface receptor for murine BSF-1. *Proc. Natl. Acad. Sci. USA.* 84:1669.
52. Swain, S. L. 1985. Role of IL-5 in the differentiation to antibody secretion of normal and tumor B cells. *J. Immunol.* 134:3934.
53. Cantrell, M., D. Anderson, D. P. Cerretti, V. Price, K. McKereghan, R. J. Tushinski, D. Y. Mochizuki, A. Larsen, K. Grabstein, S. Gillis, and D. Cosman. 1985. Cloning, sequence, and expression of a human granulocyte/macrophage colony-stimulating factor. *Proc. Natl. Acad. Sci. USA.* 82:6250.
54. Price, V., D. Y. Mochizuki, C. March, D. Cosman, M. Deeley, R. Klinke, S. Gillis, and D. Urdal. 1987. Expression, purification and characterization of recombinant murine GM-CSF and bovine IL-2. *Gene (Amst.).* 55:287.
55. Watson, J. D., P. S. Crosier, C. J. March, P. J. Conlon, D. Y. Mochizuki, S. Gillis, and D. L. Urdal. 1986. Purification to homogeneity of a human hematopoietic growth factor that stimulates the growth of a murine interleukin-3 dependent cell line. *J. Immunol.* 137:854.
56. Stanley, E. R., and L. J. Guilbert. 1981. Methods for the purification, assay, and characterization and target cell binding of a colony stimulating factor (CSF-1). *J. Immunol.* 42:253.
57. Park, L. S., D. Friend, S. Gillis, and D. L. Urdal. 1986. Characterization of the cell surface receptor for granulocyte-macrophage colony stimulating factor. *J. Biol. Chem.* 261:4177.
58. Laemmli, U. K. 1970. Cleavage of the structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* 227:680.
59. Kurland, J. I., S. F. Zeigler, and O. N. Witte. 1984. Long-term cultured B lymphocytes and their precursors reconstitute the B-lymphocyte lineage *in vivo*. *Proc. Natl. Acad. Sci. USA.* 81:7554.
60. Dorshkind, K., L. Schorest, and W. H. Fletcher. 1985. Morphologic analysis of long term bone marrow cultures that support B-lymphopoesis or myelopoesis. *Cell Tissue Res.* 239:375.
61. Muller-Sieburg, C. E., C. A. Whitlock, and I. L. Weissman. 1986. Isolation of two early B lymphocyte progenitors from mouse bone marrow: a committed pre-pre-B cell and a clonogenic Thy-$1^{10}$ hematopoietic stem cells. *Cell.* 44:653.
62. Sideras, P., and R. Palacios. 1987. Bone marrow pro-T and pro-B lymphocyte clones express functional receptors for interleukin IL-3 and IL-4/BSF-1 and non-functional receptors for IL-2. *Eur. J. Immunol.* 17:217.
63. Davis, B., M. Huffman, K. Knoblock, and P. Baker. 1983. Logit analysis of interleukin 2 (IL-2) activity using a BASIC program. *Comput. Appl. Laboratory.* 4:269.