

LONG-TERM CULTURE AND CLONING OF NONTRANSFORMED HUMAN B LYMPHOCYTES*

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The analysis of the cellular and molecular events that control activation, proliferation, differentiation, and tolerance induction in B lymphocytes is currently limited by the lack of availability of homogeneous populations of immunocompetent B lymphocytes that can be studied in several phases of their development. Separation of B lymphocytes from mixed cell populations is not a fully adequate means of obtaining such cells. Available long-term cell lines are also not suitable, since these represent transformed populations having only limited capacity to respond to immunologic stimuli (1, 2).

Long-term lines of cloned, nontransformed B lymphocytes would provide a powerful tool for the study of the critical events in B lymphocyte development and function. Several groups have succeeded in growing colonies of mouse and human B lymphocytes in soft agar using mitogens, 2-mercaptoethanol, or thymus factors (3-9). However, previous efforts to propagate and to clone the progeny of such colonies have not been successful. In this communication, we describe a technique for the extended culture and cloning of nontransformed human B lymphocytes derived from colonies established in soft agar. The requirements for establishing these lines and some of the characteristics of these propagated cells are presented.

Materials and Methods

Reagents. Noble agar and Bacto phytohemagglutinin-M (PHA-M; lot 657315)¹ were obtained from Difco Laboratories, Detroit, Mich. Purified PHA (lot K7508) was obtained from Burroughs Wellcome, Greenville, N. C. Pokeweed mitogen (PWM; lot A393903) was obtained from Grand Island Biological Co., Grand Island, N. Y. *Staphylococcus aureus* protein A (lot EM 15140) was obtained from Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.

Separation of Lymphocytes. Peripheral blood mononuclear cells were separated from heparin-

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¹Abbreviations used in this paper: EBNA, Epstein-Barr nuclear antigen; FACS, fluorescence-activated cell sorter; FCS, fetal calf serum; Fl, fluoresceinated; HBSS, Hanks' balanced salt solution; Ig, immunoglobulin; IL-2, interleukin 2; KLH, keyhole limpet hemocyanin; PFC, plaque-forming cell; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SRBC, sheep erythrocytes.

ized blood, obtained from normal volunteers (Blood Bank Department, Clinical Center, National Institutes of Health), by centrifugation on gradients of Ficoll-Hypaque (Pharmacia Fine Chemicals). Cells that banded at the interface were collected, washed in Hanks' balanced salt solution (HBSS), and resuspended at 1×10^6 cells/ml in culture medium. The culture medium used for these studies consisted of RPMI 1640 (Grand Island Biological Co.) supplemented with 50 U/ml penicillin, 0.6% L-glutamine, 100 $\mu\text{g}/\text{ml}$ gentamycin (Schering Corp., Kenilworth, N. J.), 5×10^{-5} M 2-mercaptoethanol, and 10% heat-inactivated fetal calf serum (FCS; Microbiological Associates, Walkersville, Md.). The mononuclear cells were then mixed with neuraminidase-treated sheep erythrocytes (SRBC) in a ratio of 1:40, in HBSS supplemented by 25% FCS absorbed with SRBC. Rosettes were formed by incubating this mixture at 37°C for 15 min, centrifugation at 200 *g* for 5 min, and incubation on ice for 1 h. The SRBC-lymphocyte suspension was then layered on Ficoll-Hypaque and centrifuged at 400 *g* for 20 min at 4°C. The rosetted lymphocytes in the pellet fraction and unrosetted lymphocytes at the interface were removed separately.

In some experiments, cell populations were further purified by a second round of rosette formation accomplished by the addition of a second aliquot of neuraminidase-treated SRBC, and subsequent centrifugation. Rosetted lymphocytes were suspended in 0.83% ice-cold ammonium chloride-0.17 M Tris buffer, pH 7.2, to lyse SRBC. The lymphocytes were then centrifuged, washed twice in HBSS, and resuspended in complete culture medium. We will refer to this cell population as T lymphocytes. Such populations contained <1% membrane immunoglobulin (mIg)-bearing cells and <1% monocytes, as judged by immunofluorescent and esterase staining, respectively.

The cells that failed to form rosettes were subsequently depleted of monocytes by incubation at 37°C for 24 h in 150 \times 15-mm plastic petri dishes (Falcon Labware, Oxnard, Calif.) in the presence of 20–25 ml complete culture medium. The nonadherent cells will be referred to as B lymphocyte-enriched cell populations. These populations contained 70–83% Ig-positive cells, 1–4% SRBC rosette-forming cells, and 10–17% monocytes.

Initial Suspension Culture. Suspensions of the B lymphocyte-enriched cell populations (1×10^6 cells/ml) were cultured in complete medium, alone or together with 0.5×10^6 autologous T cells that had received 2,500 rad from a cesium irradiator (Gammacell 40, Atomic Energy of Canada, Ltd., Commercial Products, Ottawa, Canada). PHA-M (12.5 $\mu\text{l}/\text{ml}$), protein A (10 $\mu\text{g}/\text{ml}$), or PWM (2.5 $\mu\text{g}/\text{ml}$) was added and cultures maintained for 72 h at 37°C in a water-saturated atmosphere of 5–7% CO₂ in air. The cultured cells were then washed, resuspended, and seeded in the upper layer of a two-layer agar system.

Colony Formation in Soft Agar. A two-layer soft agar system was prepared in 30 mm wells (3566; Costar, Cambridge, Mass.) as previously described (10–12). Briefly, the lower agar layer in each well consisted of a total volume of 2.5 ml composed of 1.25 ml of RPMI 1640, 0.375 ml of 2x RPMI 1640, 0.5 ml of heat-inactivated FCS, 0.375 ml of a stock 3.3% agar solution, and PHA-M (12.5 $\mu\text{l}/\text{ml}$), PWM (2.5 $\mu\text{g}/\text{ml}$), protein A (10 $\mu\text{g}/\text{ml}$), or supernatant factors from human peripheral blood cells stimulated with PHA or PWM. This layer, which had a final agar concentration of 0.5%, was prepared and allowed to equilibrate at 37°C in 5–7% CO₂ in air for 4–24 h before use. The upper agar layer consisted of a total volume of 0.85 ml composed of 0.3 ml of 2x RPMI 1640, 0.15 ml of distilled water, 0.15 ml of FCS, 0.15 ml of a 1.8% agar solution, and 0.1 ml of RPMI 1640 containing 1×10^6 cells from the initial suspension culture. The final agar concentration in the upper layer was 0.32%. The culture dishes were incubated at 37°C in a humidified atmosphere containing 5–7% CO₂. The development of colonies was observed through an inverted microscope; the number of colonies was determined at 4–6 d after seeding by counting colonies of ~50 or more cells from six culture plates for each experiment.

Preparation of Growth Factor-containing Culture Supernates. The production of growth factor was performed as described previously (13, 14) with slight modifications. Briefly, growth factor was prepared by culturing pooled normal human peripheral blood mononuclear cells at a density of 1.5×10^6 cells/ml in RPMI 1640 medium supplemented with 0.3% fresh glutamine, 1 mM nonessential amino acids (Microbiological Associates), 5×10^{-5} M 2-mercaptoethanol, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 10% heat-inactivated FCS. PHA-P at a final concentration of 2 $\mu\text{g}/\text{ml}$ or PWM at a final concentration of 2.5 $\mu\text{g}/\text{ml}$ was added. After 72 h

of incubation at 37°C the culture was harvested and the cells removed by centrifugation (2,000 rpm for 15 min at 4°C). This supernatant preparation, which was designated growth factor-containing culture supernate, was sterilized by filtration through 0.22- μ m filters (Steritil D-GS, Millipore Corp., Bedford, Mass.) and stored at -20°C until used.

Expansion of Cells from Colonies. Colonies were picked from soft agar, using a Pasteur pipette, under observation with a stereoscopic microscope. They were placed in individual wells of 96-well round-bottomed microtiter plates (Linbro Scientific, Inc., Hamden, Conn.), together with 1×10^4 autologous peripheral blood mononuclear cells, which had received 3,500 rad, or with 0.05% SRBC as filler cells. Cells were cultured in 100 μ l of RPMI 1640 medium supplemented with growth factor-containing culture supernate. The final concentration of such culture supernate was 40%. After 3-5 d, the cultures were fed with 100 μ l of fresh medium supplemented with culture supernate. The cultures were then fed every 3 d by removing 50-70 μ l of medium and replacing it with 100 μ l of medium supplemented with culture supernate. The cultures were split as necessary. On occasion, when the cells appeared to be in "crisis," SRBC (0.05%) or 10^4 - 10^5 irradiated human peripheral blood mononuclear cells were added to the cultures.

Detection of Cell-Surface Markers of Cells Harvested from Colonies. Colonies formed in soft agar were transferred with a Pasteur pipette to 96-well round-bottomed microtiter plates and expanded for 3-6 d. These cells were washed twice with RPMI 1640 to remove traces of agar. Cells grown from an individual colony were then tested either for their ability to form rosettes with SRBC or to ingest latex (15). Progeny of other colonies were tested for mIg by immunofluorescence. Fluoresceinated (Fl) rabbit anti-human Ig (specific for IgM, IgG, and IgA) and Fl-rabbit anti-human IgM (μ chain-specific) were purchased from Cappel Laboratories, Cochranville, Pa. Fl-rabbit antipeptide limpet hemocyanin (KLH) was used as a control reagent.

Limiting Dilution Cloning. Cells maintained in long-term culture were cloned by limiting dilution (16). Briefly, cells were suspended at densities of 0.3-3/0.2 ml of complete medium supplemented with growth factor-containing culture supernate, at a final concentration of 40%. Cell suspensions (0.2 ml) were placed in individual wells of flat-bottomed 96-well microtiter plates. Irradiated SRBC (0.05 or 0.1%) were added to some of these wells to improve cloning efficiency. 2 d later, the cultures were fed by adding ~100 μ l of medium supplemented with growth factor-containing culture supernate. The medium was changed every 3 d by removing most of the medium and replacing it with fresh medium supplemented with growth factor-containing culture supernates. Wells were inspected over a period of 10-12 d for cell growth. The log of percent of wells in which no growth occurred was plotted against the mean number of cells plated per well in order to determine whether likelihood of cell growth conformed to predictions of Poisson statistics. At 14 d, cells from selected wells with significant growth were transferred to larger wells for continued expansion.

Analysis of mIg Using the Fluorescence-activated Cell Sorter. Cell surface Ig was examined using the fluorescence-activated cell sorter (FACS II, B-D FACS Systems, Sunnyvale, Calif.). Aliquots of 1×10^5 - 5×10^5 cells in 0.2 ml RPMI 1640 containing 5% FCS and 1 mg/ml sodium azide were incubated on ice for 30 min with Fl-F(ab')₂ goat-anti-human Ig (IgA + IgG + IgM) (high F/P ratio, Cappel Laboratories) (1 μ l) or Fl-F(ab')₂ rabbit-anti-KLH, 4 mg/ml (a gift of Dr. Steven Kessler, Uniformed Services University of the Health Sciences, Bethesda, Md.) (0.5 μ l). All reagents were centrifuged for 30 min at 107,000 g before use. Staining for human κ or λ was done in a two-step incubation with either a rabbit anti- κ or rabbit anti- λ (a gift of Dr. Thomas Waldmann, National Cancer Institute), followed by Fl-guinea pig anti-rabbit IgG (a gift of Dr. Fred Finkelman, Uniformed Services University of the Health Sciences). Cells were washed twice before analysis in the FACS. Total scatter and total fluorescence windows were analyzed at appropriate gain settings.

Assay for Plaque-forming Cells (PFC). PFC were assayed by the micro-slide modification of the Jerne assay (17). PFC-secreting IgG were detected by using protein A-coupled SRBC as described by Gronowicz et al. (18). Cells were washed three times and suspended in HBSS immediately before assay. Cells (100 μ l) were mixed with 50 μ l of protein A-SRBC and 0.4 ml of 0.5% agarose (Indubiose, Accurate Chemical and Scientific Corp., Westbury, N. Y.) in Eagle's minimal essential medium and plated onto microscope slides. After 1 h incubation at 37°C, IgG PFC were developed for 2 h with a rabbit anti-human IgG anti-serum (1:200) followed by 1 h with guinea pig complement (1:40) in Dulbecco's phosphate-buffered saline

(Grand Island Biological Co.). A rabbit anti-human IgD antiserum was used as a control-developing reagent.

Chromosome Analysis. Chromosome number was determined essentially as described by Raveché et al. (19). Briefly, colchicine, at a final concentration of 0.05 $\mu\text{g}/\text{ml}$, was added to 1×10^6 cells. After either a 2-h or an overnight incubation, the cells were centrifuged for 5 min at 200 *g*. The pellet was resuspended in 0.075 M KCl for 30 min, centrifuged for 5 min at 200 *g*, and resuspended in 3:1 methanol:acetic acid fixative for 10 min. The cells were centrifuged again, resuspended in fixative, and slides prepared. They were stained for 15 min in a 4% Giemsa solution in Gurr's buffer, pH 6.8. 100 metaphases were analyzed. For analysis of cellular DNA content, cells were suspended in HBSS, centrifuged, and stained with propidium iodide (5 mg/ml Calbiochem-Behring Corp., San Diego, Calif.) in 0.1% sodium citrate according to the method of Krishan (20). The DNA content of at least 50,000 cells was measured in a Coulter TPS-1 cell sorter (Coulter Electronics, Inc., Hialeah, Fla.).

Results

B lymphocyte-enriched cell populations were cultured for 3 d with PHA (12.5 $\mu\text{l}/\text{ml}$), PWM (2.5 $\mu\text{g}/\text{ml}$), or protein A (10 $\mu\text{g}/\text{ml}$). They were then cultured in the upper layer of a two-layer soft agar culture system as described in Materials and Methods. Mitogen was included in the lower agar layer. Two types of colonies developed from these cells. The first, type I, were large colonies of 50–500 cells that developed within the upper layer 3–5 d after seeding. These colonies had a compact center and a diffuse periphery. Colonies of the second type, type II, appeared ~2 d later and formed on the surface of the upper agar layer. They were small and flat, comprised of 50–150 cells, and usually grew above the primary colonies. Both types of colonies began to degenerate on day 7–8 and had completely disintegrated by day 11.

The formation of type I colonies depended upon the presence of mitogen in both the initial suspension culture and in the subsequent soft agar culture. Indeed, if the initial suspension culture was omitted, or if the mitogenic agent was omitted from either culture step, the frequency of colonies was <5% of the number that appear when the stimulant was present in both cultures (Table I). Type II colonies occurred in higher frequency than type I colonies, although there was substantial variability among replicate cultures. Although formation of type II colonies had generally similar requirements to that for type I colonies, some type II colonies formed from cell populations not subjected to an initial suspension culture. All studies presented in this paper on the characteristics and stimulation requirements for colony formation will be limited to type I colonies. We do not know whether the cell types present in the two types of colonies are similar or different.

Identification of the Cells Found in Colonies. Colonies formed as a result of stimulation with each of the mitogens were picked from the soft agar on day 4 after seeding and transferred to microtiter wells. These cells were cultured in the presence of growth factor-containing culture supernate for 3–6 d.

Separate groups of 20 colonies were tested for individual characteristics (Table II). Surface Ig, as detected by fluorescence microscopy, was found on cells in 90–95% of colonies. More than 80% of cells in "positive" colonies were stained by F1 anti-Ig. Approximately 75% of colonies contained IgM-bearing cells. Only 5–10% of colonies contained SRBC rosette-forming cells and none contained cells that ingested latex. These results indicate that the great majority of colonies were mainly composed of B lymphocytes.

TABLE I
Induction of Type I Colonies from B Lymphocyte-enriched Cell Populations

Initial suspension culture*	Mitogen in soft agar	Number of colonies/10 ⁶ cells seeded (\pm SEM)			
		AT‡	BK	CD	RB
Omitted	None	2 (\pm 1)	1 (\pm 1)	3 (\pm 1)	2 (\pm 1)
Omitted	PHA	6 (\pm 2)	3 (\pm 1)	4 (\pm 2)	7 (\pm 2)
No mitogen	PHA	1 (\pm 1)	2 (\pm 1)	1 (\pm 1)	3 (\pm 2)
PHA	None	3 (\pm 1)	2 (\pm 1)	4 (\pm 2)	6 (\pm 3)
PHA	PHA	202 (\pm 18)	228 (\pm 19)	270 (\pm 21)	185 (\pm 15)
Omitted	None	3 (\pm 1)	5 (\pm 2)	3 (\pm 2)	5 (\pm 3)
Omitted	Protein A	18 (\pm 4)	10 (\pm 6)	7 (\pm 2)	14 (\pm 7)
No mitogen	Protein A	1 (\pm 1)	2 (\pm 1)	3 (\pm 2)	1 (\pm 1)
Protein A	None	9 (\pm 3)	6 (\pm 2)	11 (\pm 5)	5 (\pm 3)
Protein A	Protein A	401 (\pm 65)	361 (\pm 42)	245 (\pm 37)	370 (\pm 51)
Omitted	None	1 (\pm 1)	5 (\pm 4)	2 (\pm 1)	3 (\pm 1)
Omitted	PWM	2 (\pm 1)	4 (\pm 2)	2 (\pm 1)	3 (\pm 1)
No mitogen	PWM	1 (\pm 1)	1 (\pm 1)	3 (\pm 2)	2 (\pm 2)
PWM	None	1 (\pm 1)	2 (\pm 1)	3 (\pm 1)	3 (\pm 2)
PWM	PWM	301 (\pm 36)	281 (\pm 21)	322 (\pm 57)	265 (\pm 28)

* Cells were cultured for 3 d in the presence of no mitogen, PHA (12.5 μ l/ml), protein A (10 μ g/ml), or PWM (2.5 μ g/ml). In some instances the initial suspension culture was omitted as indicated in the table.

‡ Cells were obtained from four individuals designated AT, BK, CD, and RB.

TABLE II
Characteristics of Mitogen-stimulated Colonies

	Positive colonies/total*		
	PHA‡	Protein A	PWM
F1-anti-Ig	18/20	19/20	19/20
F1-anti-KLH	0/20	0/20	0/20
F1-anti-IgM	14/20	14/20	16/20
Rosette formation with SRBC	1/20	2/20	1/20
Latex ingestion	0/20	0/20	0/20

* Each reagent was tested on an independent group of 20 colonies picked on day 4 after seeding in soft agar.

‡ Mitogen used to initiate colony formation.

Factors Influencing Development of B Cell Colonies. In most respects our studies on conditions required for the development of B cell colonies yielded results very similar to those recently reported by Radnay et al. (6) and by Muraguchi et al. (8) and thus will only be summarized here. We observed that an initial suspension culture was required to endow B cells with colony-forming capacity and that mitogen (PHA, PWM, or protein A) had to be present during this suspension culture. Furthermore, the acquisition of colony-forming capacity was T cell dependent. Rigorous depletion of T cells from the B lymphocyte-enriched cell population ablated the ability of the B cells to develop colony-forming capacity. Irradiated T cells (10^5 cells irradiated at 2,500 rad) or growth factor-containing culture supernate produced by PHA stimulation allowed T cell-depleted B cell populations to acquire colony-forming capacity (Table III). The acquisition of colony-forming capacity during suspension culture was

TABLE III
Requirement for T Lymphocytes or Growth Factors in Initial Suspension Cultures

Mitogen	Rosetting of lymphocytes	Irradiated T cells*	Colonies/10 ⁶ seeded cells (± SE)
PHA	Once‡	—	261 (±52)
	Once	+	245 (±61)
	Twice§	—	6 (±2)
	Twice	+	279 (±65)
	Twice	PHA supernate	169 (±48)
PWM	Once	—	84 (±12)
	Once	+	281 (±76)
	Twice	—	3 (±1)
	Twice	+	320 (±59)
Protein A	Once	—	315 (±79)
	Once	+	323 (±92)
	Twice	—	20 (±4)
	Twice	+	285 (±65)

* T lymphocytes (5×10^5) that received 2,500 rad added to suspension culture.

‡ B lymphocyte-enriched cell population prepared by one round of rosetting contained 70% Ig⁺ cells and 4% SRBC rosette-forming cells.

§ B lymphocyte-enriched cell populations prepared by two rounds of rosetting contained 79% Ig⁺ cells and 1% SRBC rosette-forming cells.

|| Irradiated T cells omitted; PHA supernate added to culture medium at 1 vol/vol of medium.

time dependent; to obtain the maximum number of colonies required 72 h of initial suspension culture. Optimal mitogen doses for acquisition of colony-forming capacity were 12.5 μ l/ml for PHA, 2.5 μ g/ml for PWM, and 10 μ g/ml for protein A. There was a linear relationship between the number of cells seeded into soft agar and the number of colonies that appeared.

Mitogen Specificity of Colony Formation. B cell colony formation has been reported to display specificity for the mitogenic agent used (8). We observed that colonies appeared only when the same mitogenic agent was included in both the initial suspension culture and the subsequent soft agar culture (Table IV). Culture supernates of both PHA- and PWM-stimulated peripheral blood mononuclear cells could be substituted for mitogen in the soft agar culture. These supernates could support colony formation no matter which of the mitogens were used in the initial suspension culture.

A further demonstration of the mitogen specificity of colony formation was provided by experiments in which mixtures of two mitogens, each at optimal concentration, were used in both initial suspension cultures and subsequent soft agar culture. The numbers of colonies that formed was greater than that with either mitogen alone and, in most cases, was equal to the sum of the number of colonies obtained in response to each mitogen (Fig. 1). These data suggest that B cells differ in their capacity to acquire and express colony-forming ability under the influence of these mitogens. However, if the colony-forming unit consists of one (or more) B lymphocyte(s) together with a T lymphocyte, it is possible that the mitogen-specific cell is that T lymphocyte.

TABLE IV
Mitogen Specificity of B Cell Colony Formation

Stimulant in initial suspension culture*	Stimulant in agar					
	—	PHA	PWM	Protein A	PHA supernate‡	PWM supernate‡
Medium	0	2 (±1)§	1 (±1)	3 (±2)	4 (±2)	6 (±2)
PHA	6 (±2)	230 (±32)	4 (±2)	6 (±3)	250 (±39)	120 (±17)
PWM	4 (±1)	3 (±2)	301 (±29)	4 (±1)	175 (±48)	343 (±72)
Protein A	5 (±2)	4 (±1)	5 (±3)	352 (±27)	212 (±27)	205 (±31)

* T cells (5×10^5 /ml) that received 2,500 rad were added to B lymphocyte-enriched cells.

‡ Supernate from PHA- or PWM-stimulated peripheral blood mononuclear cells was incorporated in the lower agar layer at a final concentration of 25%.

§ Number (± SE) of colonies/ 10^6 cells seeded.

Development of Cell Lines from Colonies. Colonies were picked from soft agar at the 50–150 cell stage and transferred to microtiter wells for expansion. Growth in suspension culture was supported with growth factor-containing culture supernate, usually used at a final concentration of 40%. “Filler cells” in the form of 10^4 autologous irradiated peripheral blood mononuclear cells or 0.05% SRBC were used at the initiation of the suspension culture. The cultures were then fed every 3 d with growth factor-containing culture supernate for several weeks until the cells had increased in number to 1×10^5 – 2×10^5 . The lines were then transferred to 24-well Costar plates. They then entered a stationary phase, referred to as “crisis,” which resembles that observed during the expansion of T cell lines (21). If not further treated, such cultures died during the next month. To overcome this, we fed cultured lines that had entered crisis with irradiated peripheral blood mononuclear cells on a weekly basis. Depending on the number of cells per well, we added 10^4 – 10^5 irradiated cells to each well. During this period we continued to feed lines with growth factor-containing culture supernate every 3 d.

After ~2 wk of such treatment, some of the lines recommenced proliferation. We

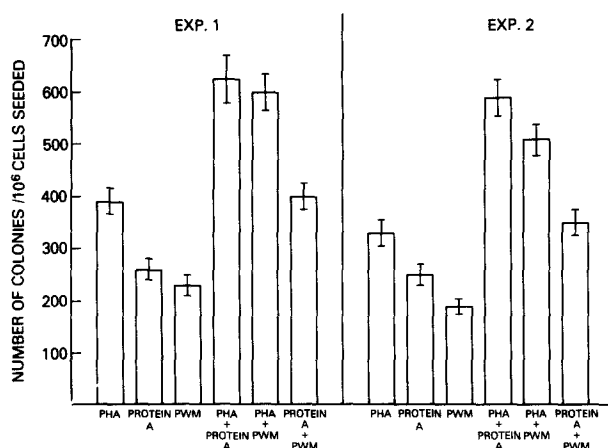


FIG. 1. Mitogen specificity of colony-forming units. PHA, protein A, and PWM were used alone or in mixtures in initial suspension culture and in subsequent soft agar culture. Concentrations of mitogens used were: PHA, 12.5 μ l/ml; protein A, 10 μ g/ml; PWM, 2.5 μ g/ml. B lymphocyte-enriched cell populations were prepared by one round of rosetting.

continued to feed with irradiated cells for 2–4 additional wk. The surviving lines were then capable of proliferation without further feeder cells although continued feeding with growth-factor containing culture supernate was essential.

An example of our success rate in establishing long-term B cell lines is provided by the following experiments. 25 colonies were picked from a soft agar plate containing PHA in the lower layer. Cells from each of these colonies proliferated in 96-well round and then flat-bottomed plates. One line was lost due to contamination. After 6 wk, the remaining 24 lines were transferred into 24-well Costar plates and all entered crisis within 1 wk. During the next 2 wk, 16 of these lines were lost. Of the remaining eight, one was lost to contamination and seven survived crisis and were established as proliferating lines in the absence of feeder cells. By fluorescence microscopy, six of the seven lines were made up of Ig⁺ cells and one line was Ig⁻. Thus, six B cell lines were established from an initial group of 25 colonies.

Lines that had survived crisis were examined for proliferation by measuring thymidine incorporation in the presence and absence of growth factor. As shown in Fig. 2, in the presence of growth factor-containing culture supernate, a linear relationship existed between the number of cells cultured and the amount of [³H]-thymidine incorporated during the last 18–24 h of a 2-d culture period. In the absence of growth factor, little, if any, thymidine incorporation was observed. Indeed, B cell lines cultured without growth factor-containing culture supernates die in ~48 h. The linear relationship between the number of B cells cultured and the magnitude of the response suggests that a single cell type was limiting.

Cell lines have been maintained in our laboratory for 1 yr or more by repetitive feeding and splitting of cultures as necessary. However, the limiting feature in the propagation of these cells is the growth factor. We have observed considerable variability among batches of culture supernates, in both toxicity for cells and capacity to support growth. In addition, only one of four lots of FCS tested was capable of supporting the generation of high levels of growth factor production by stimulated peripheral blood mononuclear cells. It is clear that the development of a reliable method to produce growth factor, either from normal cells or from tumor cells, would

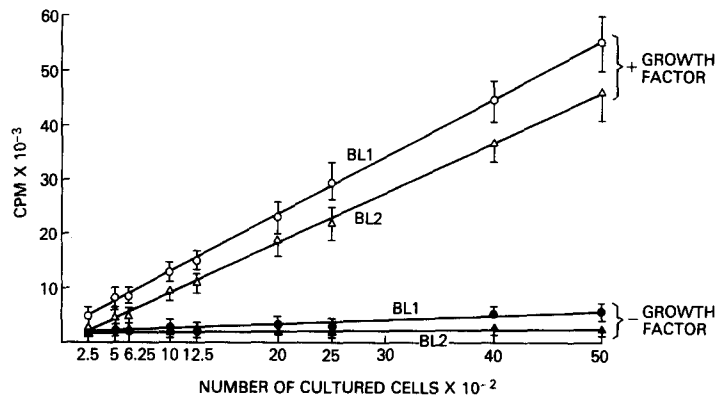


FIG. 2. Relation between number of cultured BL1 and BL2 cells and incorporation of tritiated thymidine. BL1 and BL2 are cell lines propagated from a PHA-stimulated colony and a protein A-stimulated colony, respectively. Cells were cultured for 48 h and tritiated thymidine (1 μ Ci) was present for the last 18 h of culture. Growth factor was prepared by stimulating human peripheral blood mononuclear cells with PHA and was added at a final concentration of 40%.

be a major step toward making propagation of cell lines a routine procedure. Our current procedure for the production of growth factor is to culture 1.5×10^6 peripheral blood mononuclear cells per milliliter from a pool of normal donors with $2 \mu\text{g/ml}$ PHA-P or $2.5 \mu\text{g/ml}$ of PWM. Supernates harvested from such cultures at 72, 96, or 120 h have substantial ability to support proliferation of B cell lines. Supernates harvested at 24 h or 48 h have much less activity (Fig. 3).

Cloning from Cell Lines. As discussed above, the colony-forming unit might potentially consist of more than a single cell. Consequently, long-term cell lines prepared from B lymphocyte colonies may not be the clonal progeny of a single precursor. We attempted to prepare cloned lines by limiting dilution culture from one cell line, BL1, derived from a PHA-stimulated colony. Initially, we tested the capacity of BL1 cells plated at 0.3, 1, and 3 cells/well to grow in the presence of growth factor. We found that very few wells yielded cell growth unless we added SRBC to them at a concentration of 0.1 or 0.05%. Both concentrations of filler cells yielded substantial numbers of wells with cell growth (Table V). We chose to carry out subsequent limiting dilution cultures using 0.05% SRBC as fillers because growing lymphocytes could be observed in such cultures without the need to lyse erythrocytes. BL1 cells were plated at densities of 0.3, 0.5, 1, 2, and 3/well. Their growth was evaluated over a period of 10–12 d. The log of percent of wells in which no cell growth occurred was plotted against the mean number of cells plated per well, yielding a straight line that intercepted the ordinate at 100% (Fig. 4). This is consistent with growth being dependent upon a single cell. Cloning efficiency was determined from the plating density which yielded 37% negative wells and was $\sim 36\%$.

BL1.1 and BL1.2 were lines propagated from these limiting dilution studies, in which BL1.1 was derived from a well that had received cells at a density of 0.3/well

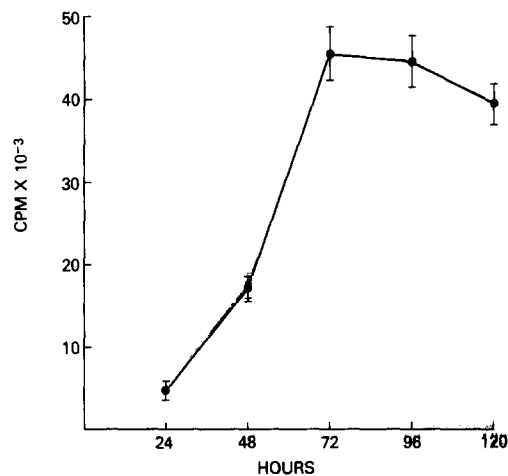


FIG. 3. Capacity of supernate from PHA-stimulated human peripheral blood mononuclear cells to support proliferation of BL1 cells. Human peripheral blood mononuclear cells pooled from several donors were cultured at $1.5 \times 10^6/\text{ml}$ in the presence of PHA-P ($2 \mu\text{g/ml}$). Culture supernates were collected at the end of cultures varying from 24 to 120 h. They were added to fresh complete medium at a final concentration of 40% and tested for their ability to support tritiated thymidine uptake by BL1 cells in 48-h cultures. Tritiated thymidine ($1 \mu\text{Ci}$) was added for the last 10 h of culture.

TABLE V
Growth of Cells Cultured at Limiting Dilution

Mean number of cells/well	Number of wells	Number of wells with cell growth*		
		No added "fillers"	0.1% SRBC	0.05% SRBC
0.3	56	0	7	7
1	45	0	14	15
3	39	2	31	29

* Wells scored for growth at 10-12 d after plating.

and BL1.2 was derived from a well that had received cells at a mean density of 1/well. These lines were maintained in the same manner as the parent line.

Characteristics of Long-term B Cell Lines. Cells from the BL1, BL1.1, and BL1.2 lines were examined for membrane Ig by staining with a F1-anti-Ig antibodies and analysis on the FACS-II. Approximately 90% of BL1 cells were fluorescent after staining with a F1-(Fab')₂-anti-Ig antibody; these cells were negative with an F1-(Fab')₂-anti-KLH antibody (Fig. 5). BL1.2 cells were stained with anti- κ , anti- λ , anti-KLH, or a mixture of anti- κ and anti- λ , and then counterstained with a F1 anti-rabbit Ig. The cells were negative after staining with anti-KLH. Anti- κ and anti- λ stained 69.5 and 56.6 of the cells, respectively (Fig. 6), and the mixture stained 96.1%. When BL1.1 was examined in the same way, 97.1% of the cells were positive with anti- λ and <1% positive with anti- κ (Fig. 6). These results indicate that the long-term lines express membrane Ig and that BL1 and BL1.2 are almost certainly not the progeny of single cells, since they contain both κ -bearing and λ -bearing cells. By contrast, BL1.1 appears to represent a cloned line, since all the cells are λ -positive and since, statistically, it is quite unlikely that more than one cell was seeded into the well from which BL1.1 grew out. All three lines failed to form rosettes with SRBC and failed to ingest latex particles.

Cells from line SH-1 were tested for Ig production at 5 mo after establishment by the protein A PFC method. The cells, when tested, had been maintained in several different preparations of growth factor. As shown in Table VI, SH-1 cells maintained in growth factor preparation 2 (GF-2) contained substantial numbers of IgG-secreting cells. In contrast, the same line maintained in GF-3 or GF-4 had very few IgG-

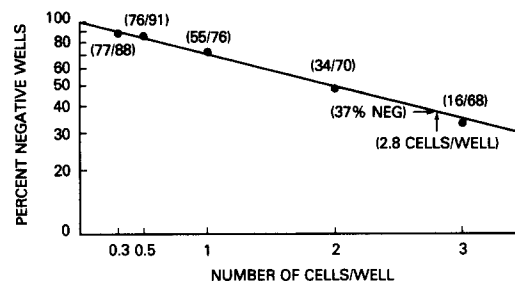


FIG. 4. Limiting dilution cloning of BL1 cells. BL1 cells were distributed in microtiter wells at mean densities varying from 0.3 to 3 cells/well, in the presence of 0.05% SRBC. The number of wells with cell growth was scored 10-12 d later. The ratio of number of wells with no cell growth to total number of wells is shown in parentheses next to each point. The arrows indicate the calculated mean number of cells plated per well to yield 37% negative wells.

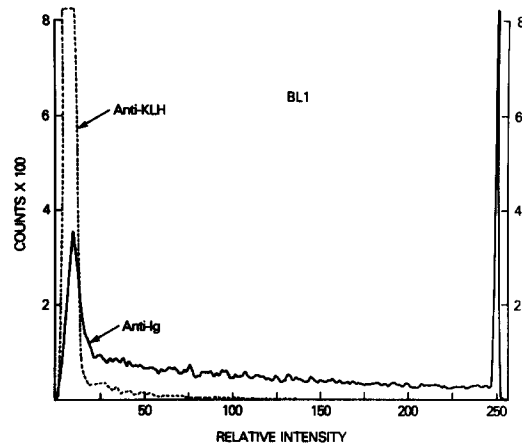


FIG. 5. FACS analysis of mIg-bearing cells from BL1. BL1 cells were stained with Fl-(Fab')₂-anti-Ig or Fl-(Fab')₂-anti-KLH and analyzed on the FACS II. Fluorescence intensity is indicated by channel numbers on the abscissa, and the number of cells with any given intensity on the ordinate.

producing cells. It is interesting that if a growth factor was removed 1 d before test, the number of IgG-secreting cells increased substantially, suggesting that as the cells cease proliferation, a fraction begin to secrete IgG. The significance of the development by anti- δ of 3,000–5,000 protein A PFC/ 10^6 cells in an SH-1 population maintained in GF-2 is uncertain. Further studies to clarify this are in progress.

A more definitive demonstration that IgG production by cells of a single line is dependent upon the growth factor preparation in which they are maintained is shown in Table VII. A group of SH-1 cells was grown in GF-3 for 1 wk, then transferred to GF-4 for another week. At this time, they were split and grown for 4 d in either GF-4 or GF-2. The cells grown in GF-4 contained very few IgG-secreting cells, whereas large numbers of IgG-secreting were found in the group grown in GF-2.

Several lines were examined for the Epstein-Barr nuclear antigen (EBNA) by immunofluorescence. These studies were carried out for us on different samples and at different times. We are grateful to Doctors Robert Gallo, Francis Ruscetti, Giovanna Tosata, Michael Blaese, and Ian Magrath, all of the National Cancer Institute, for performing these tests. All lines proved to be negative. The lines and the times at which they were tested are shown in Table VIII.

The DNA content of BL1 cells was determined by propidium iodide staining of lysed cells and analysis on a Coulter TPS-1 cell sorter. BL1 cells had a modal DNA content equivalent to that of normal diploid cells (Fig. 7). Chromosome number was determined on 100 metaphases prepared from BL1. In 93 cases, 46 chromosomes were observed. Of the remaining metaphases, 4 had 45 chromosomes, and metaphases containing 59, 65, and 92 chromosomes were observed once each. These results indicate that the great majority of BL1 cells had a normal number of chromosomes.

Discussion

The growth of long-term B lymphocyte cell lines and their cloning has the potential to provide a powerful tool with which to study the molecular and cellular regulation of B lymphocyte function. For this purpose, it would be desirable that the cells be nontransformed and that they resemble normal B lymphocytes as closely as possible.

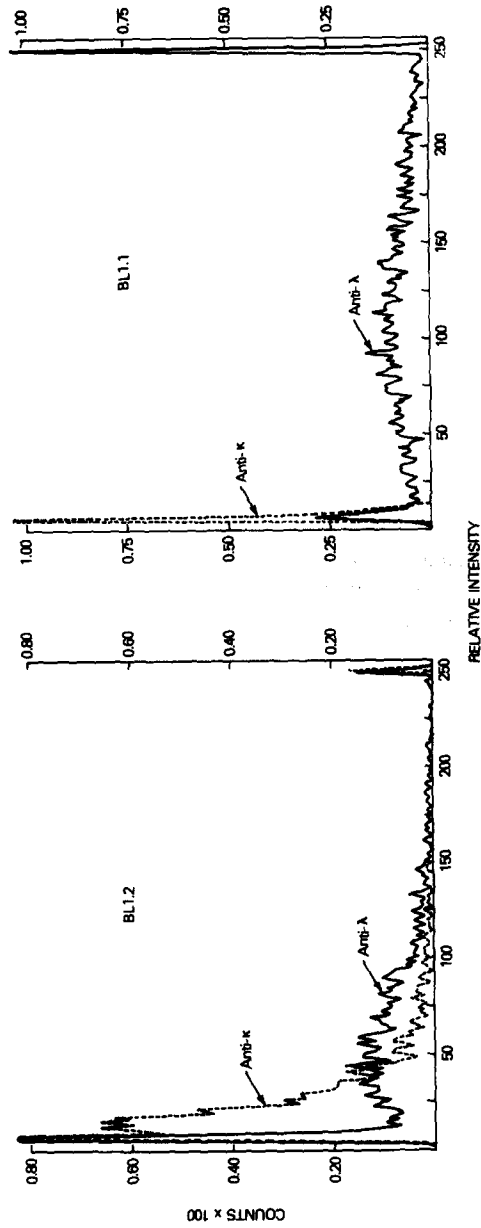


FIG. 6. FACS analysis of BL1.2 (left panel) and BL1.1 (right panel) stained with rabbit anti-k or rabbit anti-A, followed by FI-guinea pig anti-rabbit Ig.

TABLE VI
*IgG-secreting Cells in a Long-term B Cell Line**

Developing reagent	Protein A PFC (PFC/10 ⁶ cells)					
	GF2‡		GF3‡		GF4‡	
	+§	-	+	-	+	-
Anti-γ	22,700	40,710	562	1,803	40	1,300
Anti-δ	5,570	3,290	112	39	13	43

* Cells from line SH-1 were examined at ~5 mo after initiation. They were maintained in various growth-factor preparations.

‡ Growth-factor preparation.

§ + indicates that cells were maintained in growth factor until time of assay; - indicates that growth factor was removed 18 h before time of assay.

TABLE VII
Capacity of Different Growth Factors to Activate IgG-secreting Cells from SH-1

Developing reagent	Protein A PFC (PFC/10 ⁶ cells)	
	GF2*	GF4*
Anti-γ	14,420	120
Anti-δ	790	0

SH-1 cells were cultured for 1 wk in growth-factor preparation 3 and for an additional wk in growth-factor preparation 4. They were then split and one portion cultured for 4 d in preparation 2 and a second portion cultured in preparation 4. Protein A PFC were then measured.

* Growth-factor preparation.

TABLE VIII
Lack of EBNA in B Lymphocyte

Line	Time after establishment	EBNA
	<i>mo</i>	
BL1	4	Negative
BL1	10	Negative
BL1	11	Negative
BL1	12	Negative
BL1.1	7	Negative
BL1.1	8	Negative
BL2	4	Negative
BL2	10	Negative
BL2	11	Negative
BL2	12	Negative
BL3	4	Negative
BL3	10	Negative
BL3	11	Negative
BL3	12	Negative
SH1	6	Negative

BL1 and SH1 are B lymphocyte lines from PHA-stimulated colonies; BL1.1 is a line from a limiting dilution cloning (0.3 cells/well) of BL1; BL2 is a B lymphocyte line from a protein A-stimulated colony; BL3 is a B lymphocyte line from a PWM-stimulated colony.

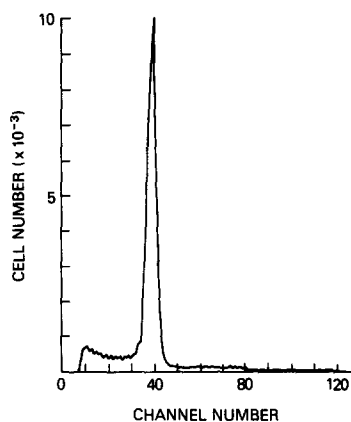


FIG. 7. Histogram of DNA content of BL1 cells that had been subjected to hypotonic lysis and stained with propidium iodide. The peak channel number for G_1 cells was the same as that obtained with normal human peripheral blood mononuclear cells stained at the same time.

In this paper, we have described an approach through which it has proved possible to propagate and clone nontransformed human peripheral blood B lymphocytes. At the same time these studies were carried out, a somewhat different approach to the propagation of long-term lines of mouse B lymphocytes by Howard et al. (22) in the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, has met with success and will be separately reported.

A key element of the procedure that we have used in the establishment of human B lymphocyte lines is the initial stimulation of B lymphocyte-enriched cell populations with mitogenic agents followed by colony formation in soft agar under continued mitogen stimulation or with the use of a growth factor-containing culture supernate. Our results here conform, in general, to those of Radnay et al. (6) and of Muraguchi et al. (8). The initial stimulation appears to be a T lymphocyte-dependent process; indeed, the mitogenic agents used in the suspension culture are generally considered to be T lymphocyte stimulants. T lymphocytes can be partially replaced with growth factor-containing supernates. Whether the mitogenic agent functions solely to stimulate T lymphocytes to produce growth factor *in situ* or, in addition, interacts with B lymphocytes in such a way as to make them sensitive to growth factor has not been determined. However, the failure of B lymphocytes to grow in soft agar without the initial suspension culture step indicates that some type of differentiation event is required to endow the B lymphocytes, or perhaps some postulated unit of a B lymphocyte with another cell type, with colony-forming ability.

Since T lymphocyte influences play a critical role in colony formation, it would be premature to consider that a colony represented the clonal progeny of a single B cell. Indeed, in one test of a long-term B cell line derived from a single colony, both κ -bearing and λ -bearing cells were found. This suggests that the original colony must have been mixed. Whether this represents a general characteristic of such colonies or is a fortuitous finding has not yet been established.

We should note that the B cell lines we have produced may be generated from a highly selected subset of B cells. First, the frequency of colonies that form in soft agar is generally of the order of 200–400/ 10^6 cells plated, indicating that only a small

proportion of the cells plated have the ability to form colonies or are appropriately stimulated to do so. Second, of the colonies transferred to suspension culture, only 25–35% survive the period of crisis and form stable long term lines, again providing a selective step. Thus, any general conclusions drawn from the study of these lines must be interpreted with care.

The growth of cells picked from colonies in soft agar is dependent upon the presence of a growth factor, or perhaps a collection of growth factors. We have mainly used culture supernates from PHA- or PWM-stimulated human peripheral blood mononuclear cells. Such supernates are known to contain interleukin 2 (IL-2) as well as several other lymphoid cell products (23). It has been suggested that IL-2, known to support the growth of certain types of T lymphocytes (24), is also critical to the proliferation of B lymphocytes. Although we have no decisive evidence concerning this, it should be noted that the time course of production of growth factor in our cultures differs from that of IL-2 production. The latter is found quite early in culture (25, 26), whereas the development of the human B cell growth factor we have used requires 72 h. Furthermore, if these growth factors are involved in the *in vivo* regulation of lymphocyte growth, it would seem likely that separate factors would regulate growth of IL-2-dependent T cells and of B cells, since immunization conditions optimal for one are not usually optimal for the other. Some preparations of the growth factor-containing medium also appear to contain the human analogue of T cell-replacing factor (27) as the capacity of the cells to differentiate into secreting cells depends on the growth factor preparation in which they are grown. A more detailed analysis of Ig secretion by these cells is now in progress.

The B lymphocytes found in the long-term lines and in the clones prepared from them do not express EBNA and have a normal number of chromosomes. However, a detailed karyotypic analysis has not yet been carried out. Because their growth is dependent upon growth factor, they display a retention of certain normal growth regulation properties. Indeed, their ability to secrete Ig is also under factor-dependent regulation. These lines promise to provide a very real resource for the study of the activation and regulation of B cell growth and differentiation.

Summary

B lymphocyte-enriched cell populations cultured with mitogens in initial suspension cultures formed colonies in soft agar when the same mitogenic agent was present in the lower layer of a two-layer soft agar system. Colony formation depended upon the presence of T cells in the initial culture, and was optimal after an initial 72-h culture with phytohemagglutinin (PHA; 12.5 μ l/ml), pokeweed mitogen (PWM; 2.5 μ g/ml), or protein A (10 μ g/ml). The colonies could be picked from the agar and propagated by feeding every 3 d with medium supplemented with a growth factor-containing tissue culture supernate. The growth factor-containing supernate was prepared by stimulating pools of human peripheral blood mononuclear cells for 72 h with PHA or PWM. The lines propagated in this manner were membrane Ig⁺, lacked sheep erythrocyte rosette-forming ability, and did not ingest latex. They lacked the Epstein-Barr nuclear antigen (EBNA) and had 46 chromosomes. Such lines have been propagated for over 1 yr. One line (BL1) was subjected to limiting dilution cloning and a line, BL1.1, was prepared that contained 96% λ -bearing cells and no κ -bearing

cells. This line was also EBNA negative. This procedure can thus be used to prepare and clone long-term lines of nontransformed human B lymphocytes.

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References

1. Fu, S. M., N. Chiorazzi, H. G. Kunkel, J. P. Halper, and S. R. Harris. 1978. Induction of *in vitro* differentiation and immunoglobulin synthesis of human leukemic B lymphocytes. *J. Exp. Med.* **148**:1570.
2. Lynch, R. G., J. W. Rohrer, B. Odermatt, H. M. Gebel, J. R. Autry, and R. G. Hoover. 1979. Immunoregulation of murine myeloma cell growth and differentiation: a monoclonal model of B cell differentiation. *Immunol. Rev.* **48**:45.
3. Sredni, B., L. A. Rozenszajn, I. Kalechman, and H. Michlin. 1975. Clonal proliferation of stimulated mouse B lymphocytes in soft agar culture. *Exp. Hematol.* **4**:45.
4. Metcalf, D., N. L. Warner, G. J. Nossal, J. F. A. P. Miller, K. Shortman, and E. Rabellino. 1975. Growth of B lymphocyte colonies *in vitro* from mouse lymphoid organs. *Nature (Lond.)*. **255**:630.
5. Kincade, P. W., P. Ralph, and M. A. S. Moore. 1976. Growth of B-lymphocyte clones in semisolid culture is mitogen dependent. *J. Exp. Med.* **143**:1265.
6. Radnay, J., I. Goldman, and L. A. Rozenszajn. 1979. Growth of human B-lymphocyte colonies *in vitro*. *Nature (Lond.)*. **278**:351.
7. Sredni, B., J. Gopas, and L. A. Rozenszajn. 1978. Induction of B lymphocyte colony growth *in vitro* by thymus-derived stimulating factor. *Eur. J. Immunol.* **8**:681.
8. Muraguchi, A., T. Kishimoto, T. Kuritani, T. Watanabe, and Y. Yamamura. 1980. *In vitro* immune response of human peripheral lymphocytes. V. PHA- and protein A-induced human B colony formation and analysis of the subpopulations of B cells. *J. Immunol.* **125**:564.
9. Rozenszajn, L. A., H. Michlin, I. Kalechman, and B. Sredni. 1977. Colony growth *in vitro* of mitogen-stimulated mouse B lymphocytes. *Immunology*. **32**:319.
10. Sredni, B., Y. Kalechman, H. Michlin, and L. A. Rozenszajn. 1976. Development of colonies *in vitro* of mitogen-stimulated mouse T lymphocytes. *Nature (Lond.)*. **259**:130.
11. Sredni, B., H. Y. Tse, and R. H. Schwartz. 1980. Direct cloning and extended culture of antigen-specific MHC-restricted, proliferating T lymphocyte. *Nature (Lond.)*. **283**:581.
12. Sredni, B., H. Y. Tse, C. Chen, and R. H. Schwartz. 1981. Antigen-specific clones of proliferating T lymphocyte. I. Methodology, specificity and MHC restriction. *J. Immunol.* **126**:341.
13. Morgan, D. A., F. W. Ruscetti, and R. C. Gallo. 1976. Selective *in vitro* growth of T lymphocytes from normal human bone marrow. *Science (Wash. D. C.)*. **193**:1007.
14. Sredni, B., D. Volkman, R. H. Schwartz, and A. S. Fauci. 1981. Antigen-specific human T cell clones. Development of human T cell clones which are antigen specific and which require HLA-DR compatible-presenting cells for stimulation in the presence of antigen. *Proc. Natl. Acad. Sci. U. S. A.* **78**:1858.
15. Cline, M. J., and R. I. Lehrer. 1968. Phagocytosis by human monocytes. *Blood*. **32**:423.
16. Lotze, M. T., J. L. Strausser, and S. A. Rosenberg. 1980. *In vitro* growth of cytotoxic human lymphocytes. II. Use of T cell growth factor (TCGF) to clone human T cells. *J. Immunol.* **124**:2972.

17. Golub, F. S., R. I. Mishell, W. O. Weigle, and R. W. Dutton. 1968. A modification of the hemolytic plaque assay for use with protein antigens. *J. Immunol.* **100**:133.
18. Gronowicz, E., P. Coutinho, and F. Melchers. 1976. A plaque assay for all cells secreting Ig of a given type or class. *Eur. J. Immunol.* **6**:588.
19. Raveché, E. S., J. Tjio, and A. D. Steinberg. 1979. Genetic studies in NZB mice. II. Hyperdiploidy in the spleen of NZB mice and their hybrids. *Cytogenet. Cell Genet.* **23**:182.
20. Krishan, A. 1975. Rapid flow cytofluorometric analysis of mammalian cell cycle by propidium iodide staining. *J. Cell Biol.* **66**:188.
21. Nabholz, M., A. Conzelmann, O. Acuto, M. North, W. Haas, H. Pollit, H. von Boehmer, H. Hengartner, J. P. Mach, H. E. Engers, and J. P. Johnson. 1980. Established murine cytolytic T-cell lines as tools for a somatic cell genetic analysis of T-cell function. *Immunol. Rev.* **51**:125.
22. Howard, M., S. Kessler, T. Chused, and W. E. Paul. 1981. Long-term culture of normal mouse B lymphocytes. *Proc. Natl. Acad. Sci. U. S. A.* In press.
23. Kurnich, J., K. Gronuic, A. K. Kimura, J. B. Lindblom, V. T. Skoog, O. Sooberg, and H. Wigzell. 1979. Long term growth in vitro of human T cell blast with maintenance of specificity and function. *J. Immunol.* **122**:1255.
24. Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* **150**:1510.
25. Alvarez, J. M., A. Silva, and M. O. de Landazuri. 1979. Human T cell growth factor. I. Optimal conditions for its production. *J. Immunol.* **123**:977.
26. Bonnard, G. D., K. Yasaka, and R. D. Maca. 1980. Continued growth of functional human T lymphocytes: production of human T cell growth factor. *Cell. Immunol.* **51**:390.
27. Schimpl, A., and E. Wecker. 1972. Replacement of T-cell function by a T-cell product. *Nat. New Biol.* **237**:15.