DIFFERENTIAL SENSITIVITY OF HUMAN B CELL SUBSETS TO ACTIVATION SIGNALS DELIVERED BY ANTI-μ ANTIBODY AND PROLIFERATIVE SIGNALS DELIVERED BY A MONOCLONAL B CELL GROWTH FACTOR

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It has been demonstrated from several laboratories that there exists a number of antigen-nonspecific soluble factors that can transmit signals for growth and/or differentiation among various types of leukocytes and that thus play a potentially major role in the regulation of immune responses (1, 2). Certain studies have demonstrated that monocyte-derived interleukin 1 (IL-1)[1] induces certain thymus-derived T cell subsets to secrete IL-2, which in turn can maintain the continuous in vitro proliferation of other T cell subsets (3, 4). In this regard, by using IL-2, it has recently become possible to maintain the long-term culture of antigen-specific mouse or human T cell clones (5–10).

Compared with the use of IL-1 and IL-2 in T cell systems, the modulation of bone marrow-derived B cell activation, proliferation, and differentiation by soluble factors is poorly understood. Recent studies of the long-term culture of normal mouse and human B cells have indicated the existence of T cell-derived B cell growth factors (BCGF) that deliver signals to activated B cells to maintain a proliferative state (11–13). Indeed, we and others have reported that there are separate signals required for B cell proliferation vs. differentiation in both the mouse and human models (14–17).

With regard to the sources of human BCGF, we have recently demonstrated that substantial BCGF activity was contained in culture supernatants of phytohemagglutinin (PHA)-stimulated human mononuclear cells (MNC) (18) and that BCGF and IL-2 elaborated by mitogen-stimulated peripheral blood T cells are separate molecules (19). Finally, we have most recently developed a human T-T hybridoma that produces BCGF in the absence of other demonstrable T cell factor activity (20). In the present study, using this monoclonal BCGF, we have examined the various activation signals required by purified human peripheral blood or tonsillar B cells for proliferative responses to BCGF. The data strongly suggest that there are at least two subsets of human B cells with regard to their relative susceptibility to proliferative signals delivered by BCGF, based on their state of in vivo activation.

[1] *Abbreviations used in this paper:* AET, S-(2-aminoethyl)-isothiuronium bromide hydrobromide; BCE, B cell-enriched; BCDF, B cell differentiation factor; BCGF, B cell growth factor; ELISA, enzyme-linked immunosorbent assay; FCS, fetal calf serum; IL, interleukin; MNC, mononuclear cells; PBMC, peripheral blood mononuclear cells; PHA, phytohemagglutinin; PWM, pokeweed mitogen; SAC, *Staphylococcus aureus* Cowan I; sIg, surface immunoglobulin.

Materials and Methods

Reagents. Purified PHA was obtained from Wellcome Research Laboratories, Beckenham, England. Pokeweed mitogen (PWM) was purchased from Gibco Laboratories, Grand Island Biological Co., Grand Island, NY. *Staphylococcus aureus* Cowan I (SAC) was prepared in our laboratory as previously described in detail (17). $F(ab')_2$ fragment goat anti-μ-specific antibody, $F(ab')_2$ fragment nonimmunized goat IgG, fluorescein-conjugated $F(ab')_2$ fragment goat anti-μ human immunoglobulin (Ig) (μ, γ, α, heavy, and light chains), fluorescein-conjugated $F(ab')_2$ fragment goat anti-mouse IgG, chromatographically purified human IgG, and human IgM, and $F(ab')_2$ fragment rabbit anti-human Ig were purchased from N. L. Cappel Laboratories Inc., Cochranville, PA. A monoclonal antibody (4F2) was prepared in our laboratory as previously described (21, 22).

Cell Preparations. Peripheral blood mononuclear cells (PBMC) from healthy donors were separated by the standard Hypaque-Ficoll gradient method (18). B cell-enriched (BCE) populations from PBMC were obtained by depletion of T cells and monocytes (18). Briefly, T cells were depleted by twice rosetting with *S*-(2-aminoethyl)-isothiuronium bromide hydrobromide (AET)-treated sheep erythrocytes. Monocytes were depleted by adhering 50×10^6 T cell-depleted cells to plastic flasks containing RPMI 1640 with 1% fetal calf serum (FCS) for 45 min at 37°C. Within this peripheral BCE population, there were 60–80% surface Ig (sIg)-positive cells as determined by staining with a fluorescein-conjugated $F(ab')_2$ fragment goat anti-human Ig, and 8–20% monocytes as determined by nonspecific esterase staining (23). Thus, sIg-positive cells were purified thoroughly by rosetting with ox erythrocytes coated with rabbit anti-human Ig (24). Because this procedure reduced the monocytes to <1% and increased sIg-positive cells up to 80–90% in the BCE population, these sIg-positive cells were used as purified peripheral blood B cells. Human tonsils were obtained at tonsillectomy from 12- to 22-yr-old patients with chronic tonsillitis and were dispersed into single-cell suspensions (25). BCE populations from tonsillar MNC were obtained by the methods described above. After two depletions of AET rosette-forming cells followed by depletion of plastic-adherent cells, there were 83–92% of sIg-positive cells and <0.1% of esterase-positive cells. Thus, this BCE population was used as purified tonsillar B cells. In certain experiments where indicated, tonsillar BCE suspensions were separated into fractions according to cell volume by using counterflow centrifugation-elutriation (model J-6B, Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) by the method described previously in detail (26) with certain modifications (T. L. Gerrard and A. S. Fauci, unpublished observations). Briefly, 500×10^6 cells in 3 ml of elutriator buffer (phosphate-buffered saline added with 1.25% of human albumin and 50 μg/ml of gentamycin) were injected into the elutriator chamber and spun with the J-6B elutriator at a constant speed of 2,020 ± 10 rpm, and the countercurrent flow rate was started at 7.5 ml/min and increased every 200 ml by 0.5-ml/min increments. Each 50-ml aliquot was collected from the exit part of the elutriator centrifuge. Cellular volume was measured with a Coulter Channelyzer (Coulter Electronics Inc., Hialeah, FL). Cells that were positive for 4F2 antigen were enumerated by treating cells with mouse monoclonal 4F2 antibody followed by incubating with fluorescein-conjugated $F(ab')_2$ goat anti-mouse IgG (27). Sheep erythrocyte receptor-positive cells were enumerated by rosetting the cells with AET-treated sheep erythrocytes (18).

BCGF Preparations. Conventional BCGF was prepared as previously described from the supernatants of co-cultures of PBMC from two allogeneic donors in the presence of PHA (18). Monoclonal BCGF was obtained from a human T-T cell hybridoma cell line, $2B_{11}$, which had been established in our laboratory (20). Cell-free supernatants were collected from unstimulated $2B_{11}$ cells or a subclone of $2B_{11}$, which showed substantial BCGF activity measured by the method described previously (18). In this monoclonal BCGF preparation, there was no IL-2 activity as determined by measurement of [^{3}H]thymidine incorporation by an IL-2-dependent cell line as described by Farrar et al. (28), and there was no T cell-replacing factor activity as determined by measurement of the induction of plaque-forming cells from SAC-stimulated normal B cells as described by Falkoff et al. (17), or from the Epstein-Barr virus-transformed B blastoid cell line, CESS, described previously (29).

B Cell Costimulatory Assay. A human B cell costimulatory assay was performed by the methods described for the murine model by Howard et al. (30), with slight modifications. Briefly, purified B cells from peripheral blood or tonsillar MNC were cultured in 200 μl of

RPMI 1640 containing 10% FCS (Dutchland Laboratories, Inc., Denver, PA), 5×10^{-5} M 2-mercaptoethanol, penicillin (100 μg/ml), and streptomycin (100 μg/ml) in flat-bottomed 96-well microtiter plates (Costar, Data Packaging, Cambridge, MA) at densities as indicated in each experiment. Some cultures contained $F(ab')_2$ fragment goat anti-human μ antibody (1–75 μg antibody/ml), $F(ab')_2$ fragment nonimmunized goat IgG (15–150 μg/ml), soluble human IgM or IgG (4–1,000 μg/ml), and/or various dilutions of the BCGF preparation as indicated. Cultures were incubated in 100% humidity in 5% CO_2 in air at 37°C. In the standard proliferation assays, the cultures were pulsed with 1 μCi of [^{3}H]thymidine (6.7 Ci/mM; New England Nuclear, Boston, MA) over the last 16–20 h of a 3-d culture. Incorporation of [^{3}H]thymidine was measured by standard liquid scintillation counting techniques after harvesting by a Titertek cell harvester (Flow Laboratories, Inc., Rockville, MD).

Measurement of the Amount of Ig in Culture Supernatants. Total IgG and IgM in culture supernatants were measured by using enzyme-linked immunosorbent assays (ELISA) as has been previously reported (31).

Results

Effect of BCGF on Co-culture of Peripheral Blood B Cells with Soluble Anti-μ Antibody. To determine the conditions for the co-culture system, 1×10^5 purified B cells from the peripheral blood of normal donors (enriched by the protocol described in Materials and Methods) were cultured with 15 μg/ml of $F(ab')_2$ fraction of goat anti-μ antibody in the presence of BCGF (50 or 5% vol/vol). Substantial proliferation was observed only when both goat anti-μ antibody and BCGF were added to the culture and not when either was added alone (Fig. 1, groups E–H). This costimulatory effect of BCGF with goat anti-μ antibody on B cell proliferation was found in the culture with not only monoclonal BCGF but also with conventional BCGF (PHA-conditioned medium), which was prepared by culturing allogeneic human MNC from two donors together with PHA for 96 h as previously described (18). Purified B cells manifested substantial responses to the T cell-independent B cell mitogen, SAC, in the absence

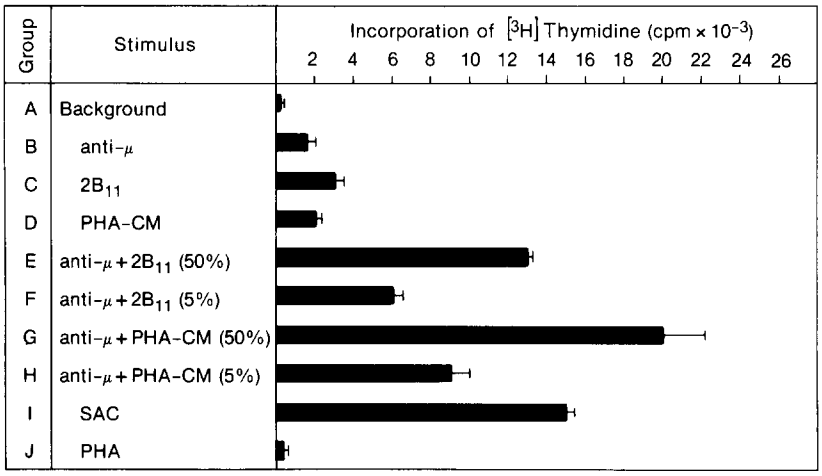

FIG. 1. Incorporation of [^{3}H]thymidine by purified B cells in response to goat anti-μ antibody with or without BCGF. 1×10^5 purified B cells from the peripheral blood of normal donors were cultured with 15 μg/ml $F(ab')_2$ fragment of goat anti-μ antibody in the presence or absence of $2B_{11}$ (BCGF derived from a human T-T cell hybridoma) or PHA-conditioned medium (BCGF prepared by the conventional method described in Materials and Methods) at a final concentration of 50 or 5% vol/vol. Cultures were incubated for 3 d; [^{3}H]thymidine was added for the last 16 h. Data represent the mean ± SEM of triplicate experiments.

of added BCGF of a magnitude comparable to that of co-culture of anti-μ antibody and BCGF. No response was observed to the T cell mitogen, PHA, in the presence or absence of added anti-μ antibody.

Specific Binding of Anti-μ Antibody to the IgM Receptor on B Cells. To demonstrate the specific interaction between $F(ab')_2$ goat anti-IgM antibody and membrane IgM, 1×10^5 purified peripheral blood B cells were co-cultured with $F(ab')_2$ fragment goat anti-μ antibody or with nonspecific $F(ab')_2$ fragment goat Ig in the presence of BCGF (25% vol/vol). Although substantial B cell proliferative responses were observed by the co-culture with $F(ab')_2$ fragment anti-μ antibody, no proliferation was observed with $F(ab')_2$ fragment nonspecific goat Ig (Table I, groups E–G).

In further attempts to define the specific binding of anti-μ antibody to membrane IgM, we added soluble human IgM and IgG at various concentrations to cultures in which 1×10^5 freshly separated peripheral blood B cells were co-cultured with anti-μ antibody (15 μg/ml) and BCGF (25% vol/vol). Data in Fig. 2 demonstrate that, although 20 μg/ml of soluble IgM enhanced the incorporation of [^{3}H]thymidine of B cells that were stimulated with anti-μ antibody and BCGF, the incorporation was reduced to background levels with 100 μg/ml or more of soluble IgM. In contrast to IgM, the same concentration of IgG did not show any effect on anti-μ-stimulated B cell proliferation. Furthermore, concentrations of 4–500 μg/ml of IgM had no effect on SAC-induced B cell proliferation, ruling out the possibility of nonspecific suppression of responses or of toxicity to B cells by these concentrations of IgM (data not shown).

Optimal Conditions for Synergistic Effect of Anti-μ Antibody and BCGF on Peripheral Blood or Tonsillar B Cell Proliferation. To study the relationship between the level of proliferative responses of B cells and the concentration of BCGF in culture, 1×10^5 purified peripheral blood B cells were cultured with serial dilutions of the monoclonal BCGF in the presence of anti-μ antibody (15 μg/ml), and proliferation of B cells was measured after 3 d of culture. As the concentration of BCGF in culture increased, the incorporation of [^{3}H]thymidine by B cells increased to a concentration of 25% vol/vol

TABLE I

Induction of B Cell Proliferation by Costimulation with Goat Anti-μ Antibody and Monoclonal BCGF

Group	Co-culture*		Incorporation of [^{3}H]thymidine‡
	Antibody	BCGF§	
	μg/ml		*cpm*
A	—	—	259 ± 21
B	—	+	2,342 ± 652
C	$F(ab')_2$ goat anti-μ, 15	—	2,627 ± 219
D	$F(ab')_2$ goat anti-μ, 15	+	22,560 ± 558
E	$F(ab')_2$ goat IgG, 15	—	385 ± 127
F	$F(ab')_2$ goat IgG, 15	+	936 ± 142
G	$F(ab')_2$ goat IgG, 150	+	958 ± 122

* 10^5 purified B cells were co-cultured with antibodies in the presence or absence of BCGF.

‡ Incorporation of [^{3}H]thymidine was measured on day 3.

§ BCGF was added to cultures at a concentration of 25% vol/vol.

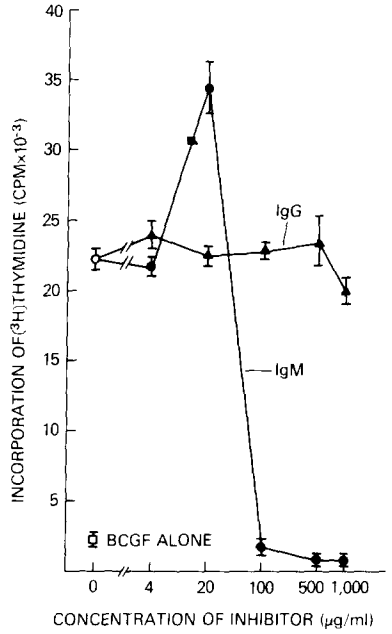

FIG. 2. Inhibition of anti-μ-induced B cell proliferation by soluble IgM. 1×10^5 purified B cells from the peripheral blood of normal donors were cultured with 15 μg/ml of anti-μ antibody alone (□), anti-μ antibody and 25% vol/vol of BCGF (○), anti-μ antibody, BCGF, and various doses of inhibitors: soluble IgM (●), soluble IgG (▲). Cultures were incubated for 3 d; [^{3}H]thymidine was added for the last 16 h. Data represent the mean ± SEM of triplicate experiments.

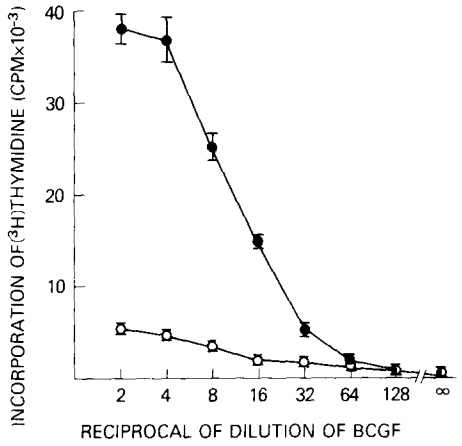

FIG. 3. The relationship between the level of proliferative responses of B cells and the concentration of BCGF. 1×10^5 purified peripheral blood B cells were cultured with serial dilutions of BCGF in the presence (●) or absence (○) of anti-μ antibody (15 μg/ml). Cultures were incubated for 3 d; [^{3}H]thymidine was added for the last 16 h. Data represent the mean ± SEM of triplicate experiments.

of BCGF in the presence of anti-μ antibody (Fig. 3). Thus, we used the monoclonal BCGF at a concentration of 25% vol/vol for the subsequent costimulatory assays.

To study the relationship between B cell density in the microwell cultures used and the magnitude of proliferative responses of B cells, various numbers of purified B cells

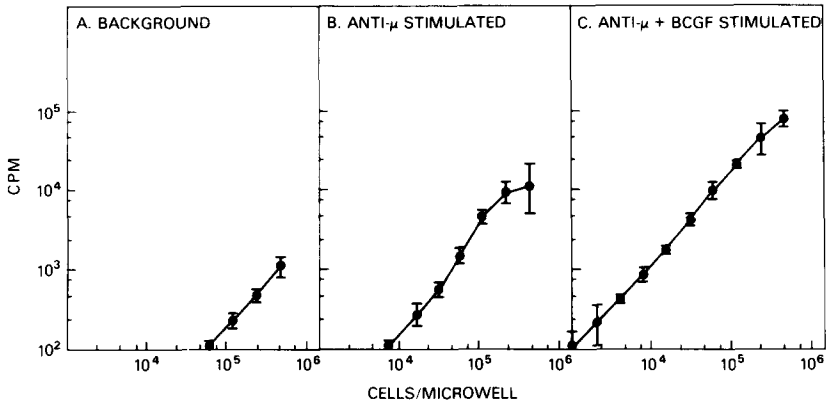

FIG. 4. The relationship between B cell density in microwell cultures and the magnitude of proliferative responses of B cells. Various numbers of purified peripheral blood B cells ranging from 10^3 to 5×10^5 cells/well were cultured in medium alone (A) or with 15 μg/ml of anti-μ antibody (B) or with 15 μg/ml of anti-μ antibody and 25% vol/vol of BCGF (C). Cultures were incubated for 3 d; [^{3}H]thymidine was added for the last 16 h. Data represent the mean ± SEM of triplicate experiments.

ranging from 10^3 to 5×10^5/well were cultured with anti-μ antibody (15 μg/ml) in the presence or absence of BCGF (25% vol/vol), and proliferation of B cells was measured after 3 d of culture. When B cells were cultured with anti-μ antibody in the absence of BCGF at a high cell density, usually as many as 6.25×10^4 cells/well, significant proliferation (>10^3 cpm of [^{3}H]thymidine incorporation) was observed (Fig. 4B). However, when B cells were cultured with anti-μ antibody and BCGF, substantial responses to anti-μ antibody were found with as little as 7.8×10^3 cells/well (Fig. 4C). This relationship between cell density and the requirement for exogenous BCGF in the proliferative response of B cells to anti-μ stimulation suggests that at high cell densities, anti-μ-stimulated B cells receive the required second signal for proliferation from contaminating accessory cells. At low cell densities in which accessory cells are limited, exogenous BCGF is required to deliver this second signal to the anti-μ-stimulated B cells. These data confirm those of Howard et al. (30) in the murine model.

The effect of BCGF on peripheral blood B cells co-cultured with various concentrations of anti-μ antibody is shown in Fig. 5. Purified 1×10^5 sIg+ cells were cultured with anti-μ antibody at a final concentration ranging from 1 to 75 μg/ml in the presence or absence of BCGF (25% vol/vol). In three separate experiments, in the presence of BCGF, substantial B cell proliferation was observed in an antibody dose-dependent fashion, with the maximal response noted at 15 μg/ml of antibody. Although no proliferation was induced at low concentrations of anti-μ antibody, significant proliferation was observed at relatively high concentrations of anti-μ antibody in the absence of BCGF. Freshly separated tonsillar B cells also showed substantial proliferation in the co-culture system of anti-μ antibody and BCGF (Fig. 6).

Costimulation with Anti-μ Antibody and BCGF Results in Proliferation of B Cells without Maturation to Ig-secreting Cells. To more precisely characterize the response of B cells to costimulation by anti-μ antibody and BCGF, 1×10^5 BCE cells from peripheral

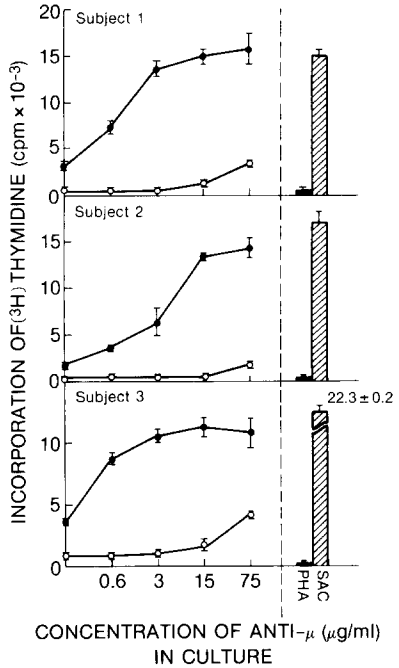

FIG. 5. Synergistic effect of low dose of anti-μ antibody and BCGF on peripheral blood B cells. 1 $\times$ 10^5 purified B cells from three individual normal donors were cultured with various concentrations of anti-μ antibody in the presence (●) or absence (○) of BCGF (25% vol/vol). Cells were also cultured with PHA (2 μg/ml) in the presence of anti-μ antibody (15 μ/ml) or SAC (1:10^4 dilution). Cultures were incubated for 3 d; [^{3}H]thymidine was added for the last 16 h. Data represent the mean ± SEM of triplicate experiments.

blood or tonsillar MNC were cultured with anti-μ antibody (15 μg/ml) and BCGF (25% vol/vol) and assayed on day 3 for proliferation by measuring the incorporation of [^{3}H]thymidine and on day 7 for maturation to Ig-secreting cells by measuring Ig (total IgM and IgG) in the culture supernatants by ELISA. As shown in Table II, tonsillar B cells cultured with PWM and irradiated T cells showed the expected polyclonal proliferative response concomitant with polyclonal Ig production, whereas B cells cultured with anti-μ antibody and BCGF showed substantial proliferative responses without the induction of Ig production. This observation was also made in peripheral blood B cells stimulated with anti-μ antibody in the presence of BCGF. These data indicate that although costimulation with anti-μ antibody and monoclonal BCGF can induce blast transformation and DNA synthesis, it cannot induce maturation to Ig synthesis and secretion.

Relationship of Cell Size and State of In Vivo Activation to Responsiveness to BCGF. In virtually all the costimulatory assays performed with anti-μ antibody and BCGF, relatively high background proliferative responses of B cells to BCGF in the absence of anti-μ antibody were consistently observed (2–8 $\times$ 10^3 cpm). This observation prompted us to investigate the possibility that a B cell subpopulation in peripheral blood or tonsillar lymphocytes had been preactivated in vivo sufficiently to respond to BCGF alone in the absence of an in vitro activation signal such as anti-μ antibody. To study the potential relationship between B cell subsets and their responsiveness to

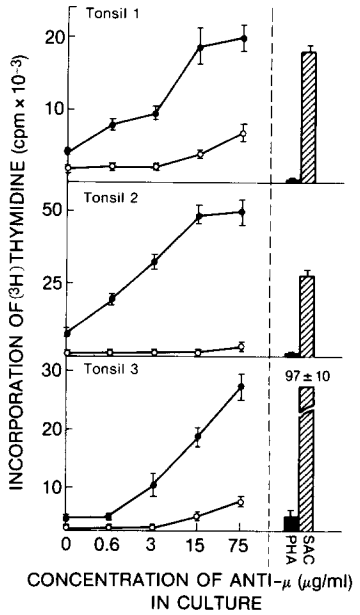

FIG. 6. Synergistic effect of low dose of anti-μ antibody and BCGF on tonsillar B cells. 1×10^5 purified B cells from tonsils of three subjects were cultured with various concentrations of anti-μ antibody in the presence (●) or absence (○) of BCGF (25% vol/vol). Cells were also cultured with PHA (2 μg/ml) in the presence of anti-μ antibody (15 μg/ml) or SAC (1:10^4 dilution). Cultures were incubated for 3 d; [^{3}H]thymidine was added for the last 16 h. Data represent the mean ± SEM of triplicate experiments.

TABLE II

Proliferation vs. Differentiation and Ig Secretion of Human Tonsillar B Lymphocytes

Stimulant*	[^{3}H]thymidine uptake‡	Total Ig in culture supernatant§
	$\times 10^{-3}$ *cpm*	*ng/ml*
	1.1 ± 0.2	9
Anti-μ	0.3 ± 0.1	7
BCGF	2.4 ± 0.6	14
Anti-μ plus BCGF	21.7 ± 2.3	33
PWM plus irrT‖	16.8 ± 4.1	>1000

* 1×10^5 tonsillar B cells were cultured with anti-μ (15 μg/ml), BCGF (25% vol/vol), PWM (10 μg/ml), or 1×10^5 2,000-rad-irradiated autologous T cells as indicated.

‡ Uptake of [^{3}H]thymidine was measured on day 3.

§ Total Ig (IgM and IgG) in each culture supernatant was assayed by ELISA on day 7.

‖ Irradiated T cells.

BCGF based on the size of B cells, we used countercurrent centrifugation-elutriation (26) to obtain B cell populations of different sizes. The cell-size profile of two B cell subsets obtained by elutriation is demonstrated in Fig. 7. Characterization of the surface markers of these unfractionated or fractionated purified tonsillar B cells is summarized in Table III. Of note is the fact that almost all of the cells in the fraction

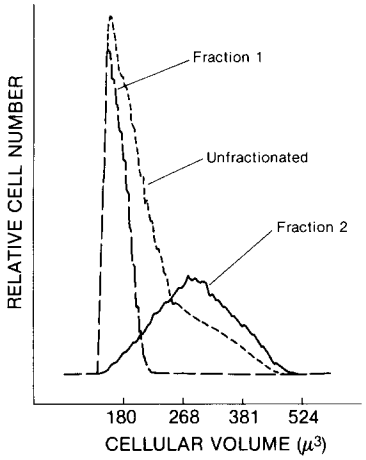

FIG. 7. Cell-size profile of two B cell subsets. Tonsillar BCE suspensions were separated into two fractions according to cell volume by using counterflow centrifugation-elutriation as described in Materials and Methods. Cellular volume was quantitated with a Coulter Channelyzer.

TABLE III

Characterization of Tonsillar B Cells Fractionated by Counterflow Centrifugation

Fraction	Mean volume	Surface marker				Esterase staining positive
		sIg+	Ia+	4F2+	ER+	
	μ^3	%				
Unseparated B cells	215	83	88	22	<0.1	<0.1
Fraction 1	185	92	ND*	<0.1	<0.1	<0.1
Fraction 2	325	72	ND	85	<0.1	<0.1

* Not determined.

of larger B cells (fraction 2) expressed not only sIg but also the 4F2 antigen, which is a non-HLA, non-Ia cell-surface marker present on human monocytes and a subset of activated but not resting lymphocytes (21, 22), whereas cells in the fraction of smaller B cells (fraction 1) did not express 4F2 antigen on their surface. These separate fractions were cultured with or without anti-μ antibody (0.6–15 μg/ml) in the presence or absence of BCGF (25% vol/vol), and incorporation of [^{3}H]thymidine was measured on day 3. Smaller cells (fraction 1) manifested substantial proliferative responses to costimulation with anti-μ antibody and BCGF, but very little proliferation was observed with anti-μ antibody or BCGF alone (Fig. 8). Larger B cells (fraction 2) manifested substantial proliferative responses to BCGF without anti-μ antibody, and the addition of anti-μ antibody did not affect the response to BCGF (Fig. 8). This observation indicates that small (presumably resting) B cells need at least two signals (anti-μ antibody and BCGF) for initiation of proliferation, whereas large (activated) B cells need only one signal (BCGF) for initiation of proliferation or more likely for amplification of the low level of proliferation that had already been introduced in vivo.

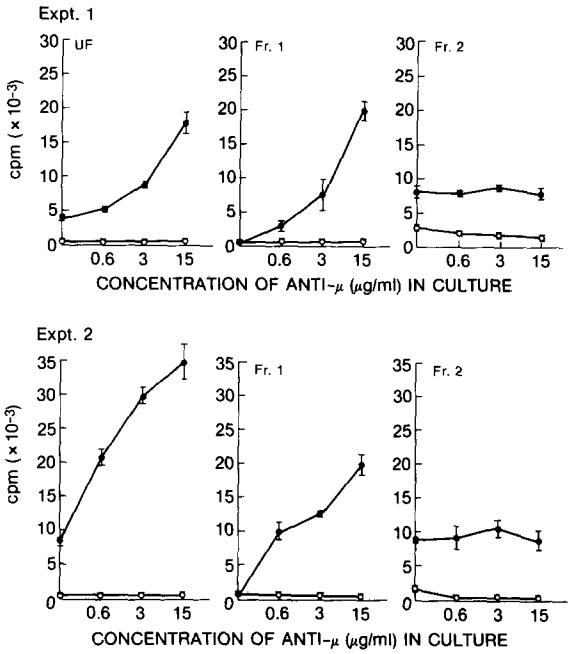

FIG. 8. Effect of anti-μ antibody and BCGF on B cells of different sizes. 1×10^5 unfractionated B cells, smaller B cells (fraction 1 in Fig. 7), and larger B cells (fraction 2 in Fig. 7) obtained from tonsils were cultured with or without anti-μ antibody (0.6–15 μg/ml) in the presence (●) or absence (○) of BCGF (25% vol/vol). Cultures were incubated for 3 d; [^{3}H]thymidine was added for the last 16 h. Data represent the mean ± SEM of triplicate experiments.

Discussion

The present study demonstrates a synergistic relationship between activation signals delivered to human B cells in the form of anti-μ antibody and proliferative signals delivered by BCGF for the ultimate proliferation of B cells. The BCGF used was a monoclonal protein produced by a human T-T cell hybridoma we had previously described (20). This particular growth factor sustains proliferation in B cells that are already proliferating and induces proliferation in B cells that have been activated but are not yet proliferating (20). Furthermore, by using size separation techniques, we were able to separate at least two fractions of tonsillar lymphocytes that differed in their requirement for those activation signals that rendered them sensitive to the proliferative signals of the monoclonal BCGF. Such observations have clear-cut implications for our attempts to more precisely delineate the minimal, optimal, and synergistic signals involved in the activation, proliferation, and differentiation of human B cells.

With regard to the use of anti-μ antibody to activate human B cells, it has been proposed in several studies that the binding of specific antigen to sIg on B cells triggers activation of the antigen-specific B cells and initiates a complex series of events whereby B cells evolve into Ig-producing cells (32). This being the case, cross-linking of the sIg on B cells by anti-Ig antibody directed against the appropriate sIg could activate B cells in a polyclonal manner, resulting in subsequent proliferation and differentiation if combined with other appropriate signals. In this regard, Sell and

Gell (33) initially described the stimulation of blastogenesis of rabbit B cells by anti-allotype sera. It has subsequently been demonstrated by Kishimoto et al. (16, 34) in the rabbit system and by Parker et al. (15) in the murine system, that anti-Ig antibody in the presence of T cell-derived factors induces Ig production in B cells. Since that time, anti-Ig antibody has been extensively used to selectively induce the proliferation of B cells, particularly in the murine system (35, 36). However, this approach has been less successful in the human system and although there have been several reports of human B cell activation by anti-Ig, results have been controversial, probably because of the various types of anti-Ig antibodies used and the differing cellular requirements for activation (37–40).

In the present study, we used purified B cells from peripheral blood or tonsillar lymphocytes and the $F(ab')_2$ fraction of goat anti-human IgM antibody. With low concentrations of goat anti-μ antibody, the whole population of purified B cells manifested very little proliferation. However, a marked synergistic effect on B cell proliferation was noted with anti-μ antibody in the presence of BCGF. Regarding the question of specific binding of anti-μ antibody to the IgM receptor on B cells, it was demonstrated that the synergistic effect of anti-μ antibody and BCGF on B cell proliferative responses was completely inhibited by the addition of soluble human IgM, but not by human IgG (Fig. 2). It is not clear why the addition of 20 μg/ml of soluble IgM-enhanced anti-μ induced B cell proliferation, but it is quite conceivable that the immune complexes made up of IgM and anti-μ antibody enhance the lymphocyte proliferative responses, as previously described (41, 42).

It has previously been reported that high concentrations of heterologous $F(ab')_2$ fragment rabbit anti-μ antibody induced B cell proliferation without a requirement for BCGF (40). In that study, Chiorazzi et al. clearly demonstrated that in the absence of added BCGF, purified rabbit anti-human IgM or IgG antibodies induced proliferation of human tonsillar or peripheral blood B cells. The optimal concentration of antibody was 100–400 μg/ml. This requirement for a relatively high concentration of antibody to induce BCGF-independent B cell proliferation has also been reported in the murine system (35). Furthermore, cross-linking of sIg by large molecules such as the particulate SAC also resulted in BCGF-independent B cell proliferation (17). In contrast, in the present study, low concentrations of anti-μ antibody induced barely detectable levels of proliferation in tonsillar or peripheral blood B cells. In the presence of BCGF, however, these same low concentrations of anti-μ antibody induced substantial proliferation (Figs. 5 and 6). This observation of synergy with low concentration anti-μ antibody and BCGF is the same as that made in the murine model by Howard et al. (30). Of note is the fact that higher concentrations of anti-μ antibody in our system did indeed induce a certain degree of B cell proliferation in the absence of BCGF similar to the BCGF-independent proliferation reported by us (17, 18) with SAC and by Chiorazzi et al. (40) with high concentrations of rabbit anti-Ig. These observations fit quite well with a more precise delineation of the model of human B cell function proposed by us and others (18, 43). It is likely that SAC or high concentrations of anti-Ig can provide a powerful enough signal for the B cell to not only be activated but also to undergo a degree of self-limited proliferation in the absence of BCGF. On the other hand, a weaker triggering signal such as low concentration anti-μ antibody only provides an activation signal, without driving the cell to express the early rounds of proliferation. This signal can, however, induce the

B cell to express receptors for BCGF (18), which then allow it to respond synergistically to exogenous BCGF by substantial proliferation. Thus it appears that the strength of the initial activating signal in vitro determines whether the B cell will go on to proliferate (in the case of a strong stimulus) as well as whether there will be synergy with exogenous factors such as BCGF (as with weak stimuli). Furthermore, it was shown in the present study by using a low concentration of anti-μ stimulation that (*a*) as cell density in cultures decreases, anti-μ-induced B cell proliferation is rapidly reduced to background levels (Fig. 4 B); and (*b*) the synergistic effect of anti-μ antibody and BCGF was observed in cell densities as low as 7.8×10^3/well, whereas no proliferation was observed with anti-μ antibody alone (Fig. 4 C). This observation again strongly suggests the requirement of BCGF for a B cell proliferative response to a weaker triggering signal.

In the present study, costimulation of B cells with anti-μ antibody and BCGF resulted in proliferation without differentiation into Ig-secreting cells (Table II). In this regard, it has recently been reported that there are separate signals for B cell proliferation vs. differentiation in mouse (14–16) and in man (17, 18, 43). We are currently studying differentiation factors derived from either mitogen-stimulated MNC cultures (29, 44) or human T-T cell hybridomas (20) which can induce differentiation in B cells stimulated with anti-μ antibody and BCGF.

An observation of particular interest that emerged from the present study was that B cells were differentially sensitive on the basis of size to either the activation signal delivered by anti-μ antibody or the proliferative signal delivered by BCGF. BCGF could directly stimulate larger B cells to proliferate even in the absence of the activation signal delivered by anti-μ antibody. In fact, anti-μ antibody did not at all increase the proliferative response of these larger B cells to BCGF, which strongly suggests that the activation signal was superfluous. Thus, it is highly likely that these larger tonsillar B cells were already activated in vivo; they certainly already expressed receptors for BCGF, since they proliferated quite well to BCGF alone. In fact, these larger B cells were shown to be already activated in that they expressed the 4F2 antigen (Table III) found on activated but not resting lymphocytes (J. H. Kerl and A. S. Fauci, manuscript in preparation) (21, 22). In contrast, the smaller tonsillar lymphocytes did not respond to BCGF alone and hence did not express functional receptors for BCGF. Furthermore, they did not express the 4F2 activation antigen and they required activation by anti-μ antibody in order to respond by proliferation to BCGF. These data are consistent with those reported in the murine model by DeFranco et al. (45, 46). These authors demonstrated that virtually all resting B cells were stimulated by low concentrations of anti-μ antibody to become larger (i.e., to proceed from G_0 phase to G_1 phase of the cell cycle), and that a subpopulation of these cells entered S phase in response to high concentrations of anti-μ antibody or lipopolysaccharide. These latter reagents apparently acted directly on the B cells or stimulated other cells to produce BCGF, which may be the actual stimulus to G_1 phase B cells to enter S phase (46). Anderson and Melchers (47) have also demonstrated a different pattern of the responsiveness of resting and activated (large) B cells to nonantigen-specific T cell factors, with only the activated B cells being able to proliferate and differentiate in response to such factors in the absence of antigen. Taken together, these results extend and expand the model of B cell activation with

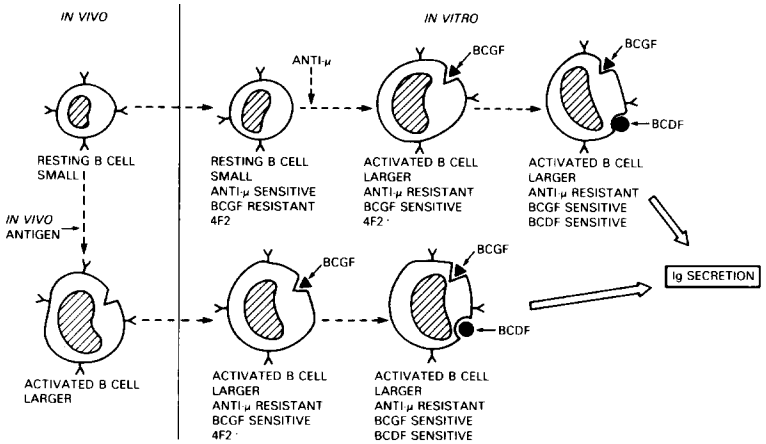

FIG. 9. Proposed scheme of human B cell activation and proliferation. In the in vivo situation, certain resting B cells may be activated by exposure to specific antigen. Upon activation, the cells become larger and express receptors for growth factors. Thus when B cells are removed from the host, they are in various states of activation depending on prior exposure to these various activation signals in vivo. In the examination of the B cell repertoire in vitro, certain cells will have already been activated in vivo and thus will be larger and will express activation antigens such as 4F2. These activated cells are already sensitive to the proliferative signals delivered by BCGF and will in fact proliferate to BCGF without needing an in vitro activation signal such as stimulation with anti-μ antibody. Once these cells are exposed to BCGF, they will proliferate and go on to express receptors for a B cell differentiation factor termed BCDF. If exposed to BCDF, they will differentiate and secrete Ig. In contrast, the smaller resting B cell that has not received an activation signal in vivo is resistant to direct stimulation by BCGF and does not express the 4F2 activation antigen. Upon triggering with anti-μ antibody, the resting B cell becomes larger, expresses the 4F2 antigen, and becomes sensitive to the proliferative signals to BCGF. It thus assumes the same profile of the B cell that had been activated in vivo. When this in vitro-activated B cell is exposed to BCGF, it proliferates, expresses receptors for BCDF, and will differentiate upon exposure to differentiation signals.

regard to the relationship between activation and proliferation (Fig. 9). B cells require activation in order to express receptors for BCGF that are required for sustained proliferation. This activation signal can be delivered by either anti-μ, which is the minimal activation signal that results in virtually no proliferation in the absence of exogenous BCGF, or by high concentration anti-μ antibody or SAC, which activates cells and induces a degree of proliferation without exogenous BCGF. These larger cells can still respond to exogenous BCGF and thus still express receptors for BCGF. B cells may also be activated in vivo. Complete activation that drives a cell through proliferation as well as differentiation clearly can occur in vivo since spontaneous Ig-secreting cells are routinely demonstrated in vitro within the circulating B cell repertoire (48). Lesser degrees of in vivo activation can trigger a cell to express BCGF receptors, such that when the cells are studied in vitro they respond directly to BCGF and do not require the activation signal of anti-μ antibody.

Such studies of the combination of in vivo and in vitro activation of human B cells using purified growth and differentiation factors produced from human T-T cell hybrids should provide new and potentially important avenues of approach to the delineation of the precise mechanisms of activation, proliferation, and differentiation of human B lymphocytes.

Summary

The present study demonstrates the minimal, optimal, and synergistic signals involved in the activation of normal human peripheral blood and tonsillar B cells to proliferation. Initial activation signals were delivered to B cells by low concentrations of anti-μ antibody which did not induce proliferation by themselves. However, marked synergy was seen when anti-μ antibody was added to cultures in the presence of monoclonal B cell growth factor (BCGF) obtained from a human T-T cell hybrid such that the B cells underwent substantial proliferation. This latter proliferation was seen without maturation into Ig-secreting cells, which indicates that the BCGF is not a differentiation signal but a signal that drives the cell up to but not beyond the proliferative phase. Of note was the fact that B cells reflected differential sensitivity on the basis of size to either the activation signal delivered by anti-μ antibody or the proliferative signal delivered by BCGF. BCGF directly stimulated the larger B cells in the normal tonsillar B cell repertoire to proliferate without the requirement for an in vitro activation signal, which indicates that the cells had already received some form of activation signal in vivo. Indeed, these cells expressed the 4F2 antigen found on activated but not resting lymphocytes. In contrast, the smaller tonsillar B lymphocytes did not express the 4F2 activation antigen and required activation by anti-μ antibody, which did not of itself induce proliferation, but which acted in synergy with BCGF for substantial proliferation of the B cells. These studies thus provide a useful model of human B cell activation, proliferation, and differentiation and allow a more precise delineation of each phase in this cascade.

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References

1. Möller, G., editor. 1982. Interleukins and lymphocyte activation. *Immunol. Rev.* **63:**1.
2. Oppenheim, J. J., B. M. Stadler, R. P. Siraganian, M. Mage, and B. Mathieson. 1982. Lymphokins: their role in lymphocyte responses. *Fed. Proc.* **41:**257.
3. Farrar, J., J. Fuller-Farrar, P. Simon, M. Hilfiker, B. Stadler, and W. Farrar. 1980. Thymoma production of T cell growth factor (interleukin 2). *J. Immunol.* **125:**2555.
4. Gillis, S., and S. Mizel. 1981. T cell lymphoma model for the analysis of interleukin 1-mediated T cell activation. *Proc. Natl. Acad. Sci. USA.* **78:**1133.
5. Gillis, S., and K. A. Smith. 1977. Long-term culture of tumor-specific cytotoxic T cells. *Nature (Lond.).* **268:**154.
6. Baker, P. E., S. Gillis, and K. A. Smith. 1979. Monoclonal cytolytic T-cell lines. *J. Exp. Med.* **149:**273.
7. Watson, J. 1979. Continuous proliferation of murine antigen-specific helper T lymphocytes in culture. *J. Exp. Med.* **150:**1510.
8. Schreier, R. P., B. J. Skidmore, J. T. Kurnick, S. N. Goldstein, and J. M. Chiller. 1979. Propagation of antigen-specific T cell helper function *in vitro*. *J. Immunol.* **123:**2525.
9. Sredni, B., H. Y. Tse, and R. H. Schwartz. 1980. Direct cloning and extended culture of antigen-specific MHC-restricted, proliferating T lymphocytes. *Nature (Lond.).* **283:**581.
10. Sredni, B., D. Volkman, R. H. Schwartz, and A. S. Fauci. 1981. Antigen-specific human T

cell clones: development of clones requiring HLA-DR-compatible presenting cells for stimulation in presence of antigen. *Proc. Natl. Acad. Sci. USA.* **78:**1858.

11. Howard, M., S. Kessler, T. Chused, and W. E. Paul. 1981. Long-term culture of normal mouse B lymphocytes. *Proc. Natl. Acad. Sci. USA.* **78:**5788.
12. Sredni, B., D. G. Sieckmann, S. Kumagai, S. House, I. Green, and W. E. Paul. 1981. Long-term culture and cloning of nontransformed human B lymphocytes. *J. Exp. Med.* **154:**1500.
13. 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.
14. Dutton, R. W. 1975. Separate signals for the initiation of proliferation and differentiation in the B cell response to antigen. *Transplant. Rev.* **23:**66.
15. Parker, D. C., J. F. Fothergill, and D. C. Wadsworth. 1979. B lymphocyte activation by insoluble anti-immunoglobulin: induction of immunoglobulin secretion by a T cell-dependent soluble factor. *J. Immunol.* **123:**931.
16. Kishimoto, T., T. Miyake, Y. Nishizawa, T. Watanabe, and Y. Yamamura. 1975. Triggering mechanism of B lymphocytes. I. Effect of anti-immunoglobulin and enhancing soluble factor on differentiation and proliferation of B cells. *J. Immunol.* **115:**1179.
17. Falkoff, R. J. M., L. P. Zhu, and A. S. Fauci. 1982. Separate signals for human B cell proliferation and differentiation in response to *Staphylococcus aureus.* Evidence for a two-signal model of B cell activation. *J. Immunol.* **129:**97.
18. Muraguchi, A., and A. S. Fauci. 1982. Proliferative responses of normal human B lymphocytes. Development of an assay system for human B cell growth factor (BCGF). *J. Immunol.* **129:**1104.
19. Muraguchi, A., T. Kasahara, J. J. Oppenheim, and A. S. Fauci. 1982. B cell growth factor and T cell growth factor produced by mitogen-stimulated normal human peripheral blood T cells are distinct molecules. *J. Immunol.* **129:**2486.
20. Butler, J. L., A. Muraguchi, H. C. Lane, and A. S. Fauci. 1983. Development of a human T-T cell hybridoma secreting B cell growth factor. *J. Exp. Med.* **157:**60.
21. Eisenbarth, G. S., B. F. Haynes, J. A. Schroer, and A. S. Fauci. 1980. Production of monoclonal antibodies reacting with peripheral blood mononuclear cell surface differentiation antigens. *J. Immunol.* **124:**1237.
22. Haynes, B. F., M. E. Hemler, D. L. Mann, G. S. Eisenbarth, J. Shelhamer, H. S. Mostowski, C. A. Thomas, and A. S. Fauci. 1981. Characterization of a monoclonal antibody (4F2) that binds to human monocytes and to a subset of activated lymphocytes. *J. Immunol.* **126:**1409.
23. Gerrard, T. L., and A. S. Fauci. 1982. Activation and immunoregulation of antigen-specific human B lymphocyte responses. Multifaceted role of the monocyte. *J. Immunol.* **128:**2367.
24. Romagnani, S., M. G. Giudizi, F. Almerigogna, and M. Ricci. 1980. Interaction of Staphylococcal protein A with membrane components of IgM- and/or IgD-bearing lymphocytes from human tonsil. *J. Immunol.* **124:**1620.
25. Watanabe, T., K. Yoshizaki, T. Yagura, and Y. Yamamura. 1974. *In vitro* antibody formation by human tonsil lymphocytes. *J. Immunol.* **113:**608.
26. Contreras, T. F., J. F. Jemionek, H. C. Stevenson, V. M. Hartwig, and A. S. Fauci. 1980. An improved technique for the negative selection of large numbers of human lymphocytes and monocytes by counter centrifugation-elutriation. *Cell. Immunol.* **54:**215.
27. 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.
28. Farrer, J., W. Benjamin, M. Hilfiker, M. Howard, W. Farrar, and J. Fuller-Farrar. 1982.

The biochemistry, biology, and role of IL-2 in the induction of cytotoxic T cells and antibody-forming B cell responses. *Immunol. Rev.* **63:**129.

29. Muraguchi, A., T. Kishimoto, Y. Miki, T. Kuritani, T. Kaieda, K. Yoshizaki, and Y. Yamamura. 1981. T cell-replacing factor (TRF)-induced IgG secretion in a human B blastoid cell line and demonstration of acceptors for TRF. *J. Immunol.* **127:**412.
30. Howard, M., J. Farrar, M. Hilfiker, B. Johnson, K. Takatsu, T. Hamaoka, and W. E. Paul. 1982. Identification of a T cell-derived B cell growth factor distinct from interleukin 2. *J. Exp. Med.* **155:**914.
31. Lane, H. C., D. F. Volkman, G. Whalen, and A. S. Fauci. 1981. In vitro antigen-induced, antigen-specific antibody production in man. Specific and polyclonal components, kinetics, and cellular requirements. *J. Exp. Med.* **154:**1043.
32. Möller, G., editor. 1975. Concept of B lymphocyte activation. *Transplant. Rev.* **23:**1.
33. Sell, S., and P. G. H. Gell. 1965. Studies on rabbit lymphocytes *in vitro*. I. Stimulation of blast transformation with an antiallotype serum. *J. Exp. Med.* **122:**423.
34. Kishimoto, T., and K. Ishizaka. 1975. Regulation of antibody response *in vitro*. IX. Induction of secondary anti-hapten IgG antibody response by anti-immunoglobulin and enhancing soluble factor. *J. Immunol.* **114:**585.
35. Sieckmann, D. G., R. Asofsky, D. E. Mosier, I. M. Zitron, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. I. Parameters of the proliferative response. *J. Exp. Med.* **147:**814.
36. Sieckmann, D. G., J. Scher, R. Asofsky, D. E. Mosier, and W. E. Paul. 1978. Activation of mouse lymphocytes by anti-immunoglobulin. II. A thymus-independent response by a mature subset of B lymphocytes. *J. Exp. Med.* **148:**1628.
37. Adinolfi, M., B. Gardner, F. Gianelli, and M. McGuire. 1967. Studies on human lymphocytes stimulated *in vitro* with anti-gamma and anti-mu antibodies. *Experientia (Basel).* **23:**271.
38. Oppenheim, J. J., G. N. Rogentine, and W. D. Terry. 1969. The transformation of human lymphocytes by monkey antisera to human immunoglobulins. *Immunology.* **16:**123.
39. Gausset, P., G. Delespesse, C. Hubert, B. Kennes, and A. Govaeris. 1976. *In vitro* response of subpopulations of human lymphocytes. II. DNA synthesis induced by anti-immunoglobulin antibodies. *J. Immunol.* **116:**446.
40. Chiorazzi, N., S. M. Fu, and H. G. Kunkel. 1980. Stimulation of human B lymphocytes by antibodies to IgM and IgG: functional evidence for the expression of IgG on B lymphocyte surface membranes. *Clin. Immunol. Immunopathol.* **15:**301.
41. Block-Shtacher, N., K. Hirschhorn, and J. W. Uhr. 1968. The response of lymphocytes from non-immunized humans to antigen-antibody complexes. *Clin. Exp. Immunol.* **3:**889.
42. Möller, G. 1969. Induction of DNA synthesis in normal human lymphocyte cultures by antigen-antibody complexes. *Clin. Exp. Immunol.* **4:**65.
43. Yoshizaki, K., T. Nakagawa, T. Kaieda, A. Muraguchi, Y. Yamamura, and T. Kishimoto. 1982. Induction of proliferation and Ig production in human B leukemic cells by antiimmunoglobulin and T cell factors. *J. Immunol.* **128:**1296.
44. Teranishi, T., T. Hirano, N. Arima, and K. Onoue. 1982. Human helper T cell factor(s). II. Induction of IgG production in B lymphoblastoid cell line and identification of T cell-replacing factor-like factor(s). *J. Immunol.* **128:**1903.
45. DeFranco, A. L., E. S. Raveche, R. Asofsky, and W. E. Paul. 1982. Frequency of B lymphocytes responsive to anti-immunoglobulin. *J. Exp. Med.* **155:**1523.
46. DeFranco, A. L., J. T. Kung, and W. E. Paul. 1982. Regulation of growth and proliferation in B cell subpopulations. *Immunol. Rev.* **64:**161.
47. Anderson, J., and F. Melchers. 1981. T cell dependent activation of resting B cells:

requirement for both nonspecific unrestricted and antigen-specific Ia-restricted soluble factors. *Proc. Natl. Acad. Sci. USA.* **78:**2497.

48. Fauci, A. S., G. Whalen, and C. Burch. 1980. Activation of human B lymphocytes. XVI. Cellular requirements, interactions, and immunoregulation of pokeweed mitogen-induced total immunoglobulin producing plaque-forming cells in peripheral blood. *Cell. Immunol.* **54:**230.