

ROLE OF INTERLEUKIN 1 IN ANTI-IMMUNOGLOBULIN-INDUCED B CELL PROLIFERATION*

BY MAUREEN HOWARD, STEVEN B. MIZEL, LAWRENCE LACHMAN,
JOHN ANSEL, BARBARA JOHNSON, AND WILLIAM E. PAUL

From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20205, The Microbiology Program, Pennsylvania State University, University Park, Pennsylvania 16802, and Immunex Corporation, 51 University Building, Seattle, Washington 98101

The use of anti-Ig antibodies to stimulate polyclonal proliferation through Ig receptors expressed on the B cell membrane has been a popular model for antigen-driven B cell activation (1-7). Initially it was proposed that anti-Ig-induced proliferation proceeded independently of T cells and other accessory cells, as vigorous depletion of such cell types failed to prevent activation (3, 8). However, certain features of anti-Ig-induced B cell activation challenged the view that the response was a simple consequence of the interaction of anti-Ig and membrane Ig. In particular, the relationship between cell density and magnitude of the proliferative response was nonlinear and rapidly declined to background proliferation levels at cell numbers below 10^5 per microtiter well (3, 6). Furthermore, the optimum doses of anti-Ig used in such studies far exceeded the amount required to saturate membrane Ig receptors and induce capping of membrane components. Precise delineation of the stimuli required for anti-Ig-induced B cell activation requires a functional assay in which contaminating accessory cells potentially capable of endogenous factor production have been excluded. To this end, we have investigated conditions required for polyclonal activation of highly purified mouse B lymphocytes cultured at low cell density (e.g., 5×10^4 cells/well). Using such an assay, we have shown the role of a T cell-derived factor, designated B cell growth factor (BCGF),¹ in anti-IgM-induced B cell proliferation. (9). In this report we demonstrate that in the presence of optimum amounts of BCGF, anti-IgM-induced proliferation of B cells cultured at lower densities ($1-2 \times 10^4$ cells/well) is enhanced by the addition of a second soluble factor. Biochemical analyses identify this second cofactor as the previously described monokine interleukin 1 (IL-1).

Materials and Methods

Mice. BALB/cJ mice were obtained from The Jackson Laboratory, Bar Harbor, ME, and used at 8-12 wk of age. CH3/HeJ mice, also obtained from The Jackson Laboratory, were used at 5-8 wk of age.

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¹ *Abbreviations used in this paper:* BCGF, B cell growth factor; IEF, isoelectric focusing; IL-1, IL-2, interleukins 1 and 2; PAGE, polyacrylamide gel electrophoresis; PMA, phorbol myristate acetate; UV, ultraviolet.

Anti-IgM Antibodies. Affinity-purified goat anti-mouse Ig specific for μ heavy chains (anti-IgM) was prepared as described previously (6).

Culture Medium. The culture medium used throughout was RPMI 1640 (Gibco Laboratories, Grand Island, NY) supplemented with 10% fetal calf serum (Reheis, Kankakee, IL), penicillin (50 μ g/ml), streptomycin (50 μ g/ml), gentamycin (100 μ g/ml), L-glutamine (200 mM), and 2-mercaptoethanol (5×10^{-5} M) (2ME).

Growth Factor Preparations. EL4 supernatant was produced by stimulating a cloned subline of EL4 thymoma with 10 ng/ml phorbol myristate acetate (PMA) as described elsewhere (10). Cell-free supernatants were collected after 48 h and depleted of PMA by adsorption on activated charcoal (11). For some experiments, BCGF was partially purified from EL4 supernatants by phenylsepharose chromatography or isoelectricfocusing (IEF) (12).²

Macrophages were stimulated to produce growth factors by four different procedures.

(a) Stimulation of the cloned murine monocytic cell line P388D₁ with 1 μ g/ml PMA, as described by Mizel et al. (13). Cell-free supernatants were collected after 5 d and depleted of PMA by two successive adsorptions on activated charcoal. For some experiments, the supernatants were concentrated ~200-fold by Amicon filtration, then fractionated by gel filtration on an AcA54 column as described previously (10) to yield material of 10,000–20,000 mol wt.

(b) Treatment of P388D₁ cells with 2–4 min ultraviolet (UV) irradiation (200–400 mJ/cm²) using a bank of four Westinghouse FS20 bulbs followed by their culture for 24 h in serum-free RPMI as described (Ansel, J., T. Luger, and I. Green, manuscript submitted for publication).

(c) A superinduction protocol, designed to enhance IL-1 production by P388D₁ cells for factor purification purposes. This protocol involved co-culture of cells with protein and RNA synthesis inhibitors in addition to PMA for 5 h, removal of these agents by washing, then continued culture of the activated P388D₁ cells for a further 24 h (14).

(d) Stimulation of human acute monocytic leukemia cells with endotoxin and collection of cell-free supernatants 48 h later, as described by Lachman et al. (15).

B Cell Co-stimulator Assay. Full details of this assay are given elsewhere (9). Briefly, splenic B cells were purified by the procedure of Leibson et al. (16), then cultured at densities ranging from 10^3 to 5×10^5 /well in 200 μ l medium in flat-bottomed 96-well microtiter plates (0.32 cm² growth area). Some cultures contained affinity-purified goat anti-IgM antibody at 5–10 μ g/ml, and/or dilutions of the various growth factor preparations. Cultures were incubated at 37°C in a humidified atmosphere of air containing 7.5% CO₂ for 72 h. The proliferative response of these cultures was determined by adding [³H]thymidine (1 μ Ci, 6.7 Ci/mmol; New England Nuclear, Boston, MA) for the last 12–16 h of culture and measuring [³H]thymidine incorporation.

Purification of IL-1. Mouse and human IL-1 were purified according to previously published procedures (14, 17, 18). Mouse IL-1 in super-induced P388D₁ supernatants was purified to apparent homogeneity using the following protocol: gel filtration chromatography, IEF, sulphopropyl Sephadex cationic exchange chromatography, and tris glycinate polyacrylamide gel electrophoresis (PAGE). Human IL-1 was purified to high specific activity using the following procedures in sequence: hollow fiber diafiltration and ultrafiltration, IEF, and tris glycinate PAGE (18, 19). Criteria for purity of the preparations yielded by these protocols are outlined elsewhere (14, 17, 18).

IL-1 Assay. Mouse IL-1 was measured as a co-mitogen with phytohemagglutinin in a mouse thymocyte [³H]thymidine incorporation assay (19). Human IL-1 was measured as a direct mitogen for mouse thymocytes in a [³H]thymidine incorporation assay (18).

Results

BCGF Dependence of Anti-Ig-induced B Cell Proliferation. As has been shown previously (3, 6), a careful analysis of the relationship between B cell density and anti-IgM-induced proliferation revealed a sharp decline in response at cell numbers below 10^5 per microtiter well (Fig. 1A). This result strongly suggested that in addition to anti-IgM and the B lymphocytes, other elements in the culture were limiting. We have

² Farrar, J., M. Howard, J. Fuller-Farrar, and W. E. Paul. Biochemical characterization of a murine B cell growth factor distinct from interleukin 2. Manuscript submitted for publication.

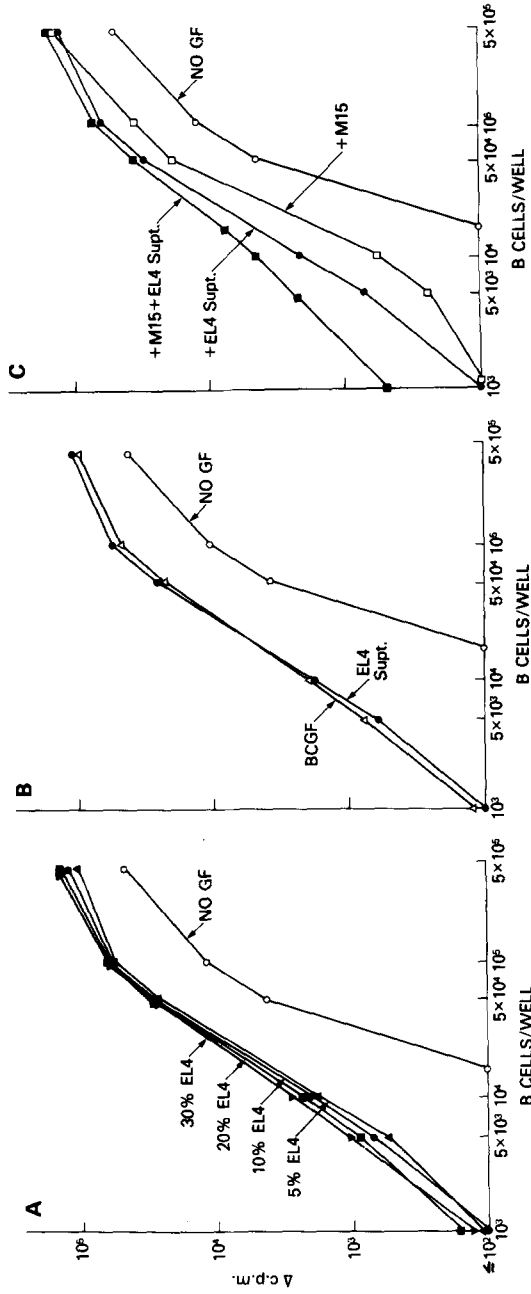


FIG. 1 Relationship between cell density and proliferative response of purified B cells cultured with anti-IgM (5 μ g/ml) alone (NO GF) or with various preparations of T cell or macrophage-derived factors. Cells were cultured for \sim 2.5 d, with the addition of [3 H]thymidine for the final 16 h. Results reflect the difference in [3 H] thymidine incorporation obtained in cultures containing anti-IgM versus those lacking anti-IgM. All results represent means of duplicate or triplicate cultures. (A) Cultures were supplemented with EL4 supernatant added at final concentrations ranging from 5 to 30%. (B) Cultures were supplemented either with 10% EL4 supernatant (\bullet), or with an IEF-purified preparation of BCGF (Δ) added to cultures at a 1:40 dilution, a concentration found to be saturating for co-stimulator activity at the B cell density of 5×10^4 /well. (C) Cultures were supplemented with 10% EL4 supernatant (\bullet), M15 added at final dilution of 1:16 (\square), or both EL4 supernatant and M15 (\blacksquare). M15 was purified by gel filtration, and corresponds to the hatched area shown in Fig. 2.

already shown (9) that induced supernatants from the mouse thymoma EL4 produce two striking effects in cultures of anti-IgM stimulated B cells: (a) a shift in the antibody concentration/response relation such that 5 $\mu\text{g/ml}$ anti-IgM plus EL4 supernatant induces levels of proliferation greater than those induced by 50 $\mu\text{g/ml}$ anti-IgM alone, and (b) a shift in the cell density/response relation such that a very substantial response to 5–50 $\mu\text{g/ml}$ anti-IgM can be obtained with as few as 5×10^4 B cells/well. Here we see that EL4 supernatant enhanced the B cell proliferative response at cell densities ranging from 5×10^3 /well to 5×10^5 /well, and that essentially identical results were obtained with concentrations of EL4 supernatant ranging between 5 and 30% indicating saturation conditions (Fig. 1 A). The B cell co-stimulating factor in EL4 supernatants has been examined in detail elsewhere (9, 12).² These studies show that the factor, designated BCGF, has an apparent molecular weight by gel filtration of 18,000 and is clearly distinct from interleukin 2 (IL-2) by gel filtration, phenylsepharose chromatography, sodium dodecyl sulfate-PAGE, and IEF, as well as by cellular absorption studies. BCGF partially purified by IEF caused the same maximal enhancement of response to anti-IgM as did unfractionated EL4 supernatant (Fig. 1 B). This strongly suggests that the enhancing activity of EL4 is attributable to its BCGF content.

A Monokine Enhances BCGF-dependent Anti-IgM-induced B Cell Proliferation. To explore the possibility of a macrophage-derived factor also being involved in anti-IgM-induced B cell proliferation, we selected B cell co-stimulator assay conditions that yielded low proliferative responses on a per cell basis to anti-IgM even in the presence of saturating amounts of EL4 supernatant, i.e., $1-2 \times 10^4$ B cells/microtiter well plus 10% EL4 supernatant (see Fig. 1 A), and examined the effects of supplementing these cultures with induced supernatants from the cloned murine macrophage cell line P388D₁. Others have shown that such supernatants are excellent sources of monokines such as IL-1 (13, 14, 20). Unfractionated PMA-induced P388D₁ supernatants were found to profoundly inhibit both BCGF-dependent anti-IgM-induced and unstimulated background levels of B cell proliferation (Table I). As unfractionated supernatants may contain both inhibitors and enhancing factors, we repeated these experiments using fractionated components of induced P388D₁ supernatants. Thus, P388D₁ supernatants were fractionated by gel filtration using a calibrated AcA54 column exactly as described elsewhere (10). Each column fraction was dialyzed against culture medium, and added at five serial dilutions to cultures containing 10^4 highly purified B cells, anti-IgM antibodies, and a saturating amount of the EL4 supernatant. The results obtained at a 1:16 dilution of column fractions revealed that proliferation of

TABLE I
Effect of Unfractionated PMA-induced Macrophage Supernatants on B Cell Proliferation

Macrophage factors	T cell factors	cpm/ 5×10^4 B cells	
		No anti-IgM	Plus Anti-IgM
—	—	1,713 \pm 267	2,133 \pm 301
P388D ₁ supernatant	—	10 \pm 41	189 \pm 34
—	EL4 supernatant	3,436 \pm 1,021	16,277 \pm 222
P388D ₁ supernatant	EL4 supernatant	358 \pm 27	482 \pm 103

25% P388D₁ supernatant; 10% EL4 supernatant; 5 $\mu\text{g/ml}$ anti-IgM.

such low numbers of B cells was markedly enhanced by the addition of macrophage-derived product(s) in the molecular weight range of 10–20,000 (Fig. 2). This material was not mitogenic for resting B cells, nor did it stimulate resting B cells in the presence of EL4 supernatant only. For convenience, the material was initially termed M15 to signify its origin (i.e., macrophage) and approximate molecular weight (i.e., 15,000). A pool was made of those fractions constituting the major peak of M15 activity (see hatched area of Fig. 2), and this pooled material was titrated under identical assay conditions. The results showed M15 to be (a) inhibitory at high concentrations and (b) active over a wide concentration range below the inhibitory doses (Fig. 3 A). These findings have been reproduced using several different batches of M15 prepared from separate PMA-induced P388D₁ supernatants. Similar B cell co-stimulator activity was found in supernatants obtained from UV-irradiated P388D₁ cells (Fig. 3 B), thereby allaying concern regarding the potential involvement of PMA in the co-stimulatory activity of P388D₁ supernatants.

The effect of M15 on the cell density/response relationship of anti-IgM-induced B cell proliferation was tested using a 1:16 dilution of M15, i.e., the optimal concentration of this material (see Fig. 3 A). B cell proliferation was enhanced at all cell densities tested (Fig. 1 C). When the same experiment was performed in the presence of a saturating amount of the T cell-derived lymphokine BCGF, a synergistic effect of the two factors was observed, particularly at low densities (Fig. 1 C). Identical results were obtained when a PMA-free source of M15 (i.e., supernatant of UV-irradiated P388D₁ cells) was used (Fig. 4 A). The results shown in Fig. 1 have been corrected for proliferation obtained in the absence of anti-IgM antibodies, and thus represent anti-IgM-dependent activation. We emphasize that both M15 (see Figs. 2 and 3) and

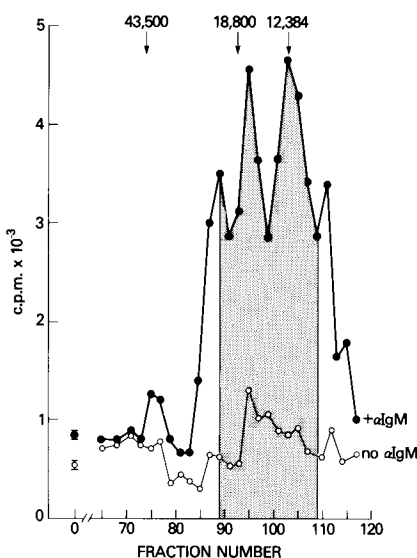


FIG. 2. Gel filtration analysis of B cell co-stimulator activity in PMA-induced P388D₁ supernatant. Fractions collected from an AcA54 column were dialysed against culture medium, then added at a final concentration of 1:16 to cultures containing 10⁴ purified B cells per well and 10% EL4 supernatant with or without anti-IgM antibodies (5 μg/ml). Proliferation was assessed at day 2.5 by [³H]thymidine uptake. The calibrating molecular weight markers were ovalbumin (43,500), myoglobin (18,800), and cytochrome *c* (12,384). All results represent duplicate cultures.

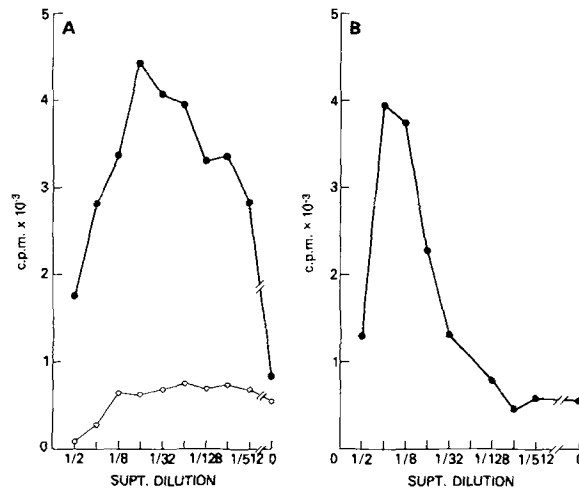


FIG. 3. B cell co-stimulator activity in (A) a 10,000–20,000-mol wt pool made from PMA-induced P388D₁ supernatant (equivalent to hatched area of Fig. 2), or (B) supernatant collected from UV-irradiated P388D₁ cells (refer to Materials and Methods). Supernatants were added at final dilutions of from 1/2 to 1/512 to cultures containing 10^4 purified B cells and 10% EL4 supernatant, with (●) or without (○) anti-IgM antibodies (5 μ g/ml). Proliferation was assessed at day 2.5 by [³H]-thymidine uptake. Results represent duplicate cultures.

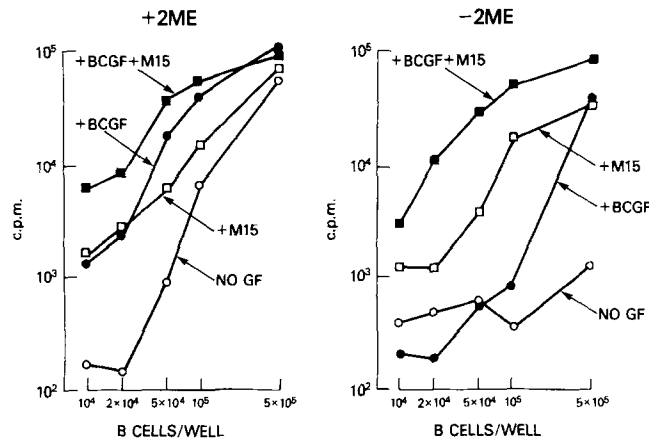


FIG. 4. Proliferative response of purified B cells cultured with anti-IgM (5 μ g/ml) together with various co-factors either in culture medium containing 2×10^{-5} M 2ME or in 2ME-free medium. Co-factor supplements were: (a) phenyl Sepharose-purified BCGF, prepared as in reference 12 (also footnote 2) and added at a 1:50 final dilution found to be saturating for co-stimulator activity at the B cell density of 5×10^4 /well; (b) supernatant collected from UV-irradiated P388D₁ cells and added at a final dilution of 1:4 (designated M15); (c) medium only (NO GF); (d) a mixture of (a) and (b). Other assay conditions were as in Fig. 1.

BCGF (9) produce little proliferation in the absence of anti-IgM antibodies. In summary, these data strongly imply roles for both BCGF and M15 in anti-IgM-induced B cell proliferation.

Absolute Need for M15 in 2ME-free Cultures. M15-mediated enhancement of anti-Ig induced BCGF-dependent B cell proliferation was highly reproducible from experiment to experiment. However, the magnitude of its effect was often relatively small (two- to fourfold), making it difficult to clearly determine whether BCGF and M15

acted synergistically or additively. This problem was resolved by taking advantage of the observation that B cell proliferation experiments performed in culture medium lacking 2ME show an exaggerated need for exogenously added M15. Fig. 4 shows B cell proliferative responses to anti-IgM plus cofactors in medium containing or lacking 2ME. While the maximum proliferation levels obtained in the presence of anti-IgM, BCGF, and M15 were the same in both media, a significant response to anti-IgM alone, i.e., in the absence of exogenously added BCGF and M15, was only obtained when B cells were cultured in 2ME-containing medium; in the absence of 2ME a response to anti-IgM and BCGF was only observed at 5×10^5 cells/well, the highest cell density tested (Fig. 4B). In cultures lacking 2ME, M15 has a marked costimulatory effect at all densities. These data prompt speculation regarding the possibility of endogenous 2ME-mediated M15 production by residual macrophages and/or B cells in our standard BCGF co-stimulator assay. The striking M15-dependent enhancement of B cell proliferation observed in 2ME-free cultures clearly establishes the synergy between BCGF and M15, suggesting that both co-factors operate on the same population of B cells.

Identification of M15 as IL-1. In terms of approximate molecular weight and cellular origin, M15 resembles the previously described monokine IL-1. To investigate the possible identity of these two factors, we tested murine and human IL-1, purified according to previously established procedures, for M15 activity, i.e., ability to enhance the proliferation of low numbers of purified B cells cultured with anti-IgM and saturating doses of EL4 supernatant.

Murine IL-1 can be obtained in large quantities by a superinduction procedure involving pre-culture of P388D₁ cells with PMA in the presence of protein and RNA inhibitors (14). Mizel and Mizel (14) have purified the IL-1 in this crude supernatant to apparent homogeneity by a modification of a previously published fractionation scheme involving gel filtration, IEF, sulpho-propyl Sephadex cationic exchange, and tris glycinate PAGE. As expected, the 15,000-mol wt material obtained by gel filtration of supernatant from superinduced P388D₁ cells showed excellent M15 activity when added to cultures of highly purified B cells, anti-IgM, and saturating amounts of EL4 supernatant (data not shown). The 15,000-mol wt material was then further purified by IEF, sulpho-propyl Sephadex cationic exchange, and tris glycinate PAGE. Fractions from the tris glycinate gel were dialyzed, then assayed for M15 activity using the B cell co-stimulating assay described above and for IL-1 activity using the conventional mouse thymocyte proliferation assay. The two activity profiles were indistinguishable (Fig. 5), indicating either that M15 is IL-1 or that there is a great biochemical similarity between them.

Human and murine IL-1 are functionally interchangeable in a variety of T cell assays (S. B. Mizel, unpublished observations). Thus, to further assess the relationship between M15 and IL-1, we tested purified human IL-1 for M15 activity. Human IL-1 can be obtained in large quantities by incubating freshly collected acute monocytic leukemia cells with endotoxin (15). Lachman et al. (17) have purified the IL-1 in this crude supernatant to high specific activity by sequential fractionation involving hollow fiber filtration, IEF, and tris glycinate PAGE (17, 18). This combination of procedures generally yields a preparation that is free of endotoxin, as assessed by the Limulus assay (L. Lachman, unpublished data). The 10–50,000-mol wt material obtained by hollow fiber filtration was subjected to IEF, and dialyzed fractions were

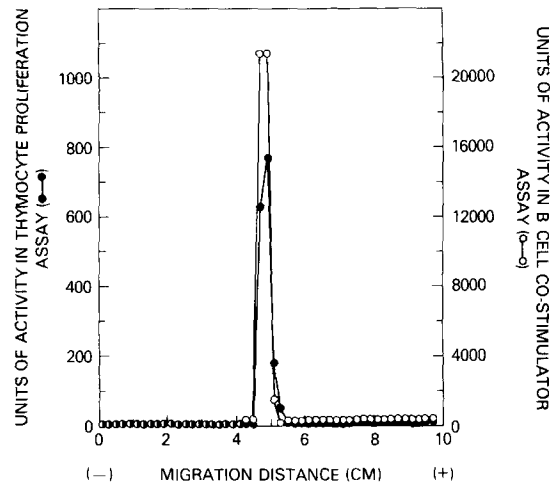


FIG. 5. B cell co-stimulating activity of highly purified murine IL-1. Murine IL-1 from superinduced P388D₁ supernatants was purified to apparent homogeneity as described in text. Dialyzed fractions obtained from the final tris glycinate gel electrophoresis were added at several dilutions ranging from 1/8 to 1/2048 to cultures of 2×10^4 B cells, 10% EL4 supernatant, and anti-IgM (5 $\mu\text{g}/\text{ml}$) (○), or to cultures of 1.5×10^6 mouse thymocytes and phytohemagglutinin (●). In both cases, proliferation was assessed at day 2.5 by [³H]thymidine uptake. For each fraction, relative units of activity represent the inverse of the dilution that produced 50% of the maximum proliferation obtained in that assay. All results represent means of duplicate cultures.

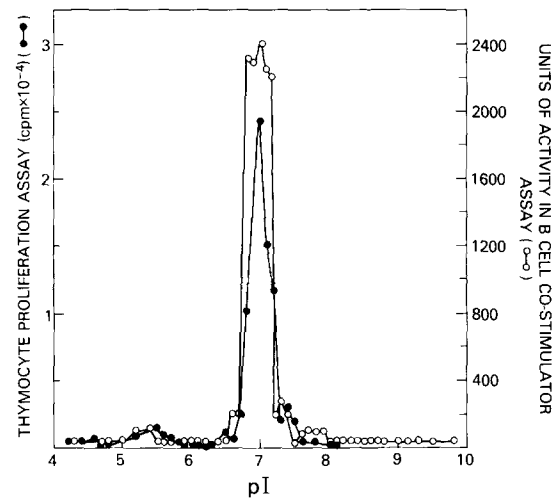


FIG. 6. B cell co-stimulating activity of partially purified human IL-1. Supernatants from endotoxin-stimulated human monocytic leukemia cells were fractionated by hollow fiber filtration, and the 10-50,000-mol wt component further purified by IEF. Dialyzed fractions were then added at several dilutions ranging from 1/50 to 1/3,200 to cultures of 2×10^4 B cells, 10% EL4 supernatant, and anti-IgM (5 $\mu\text{g}/\text{ml}$) (○), or at 1:100 (final dilution) to cultures of 1.5×10^6 mouse thymocytes (●). In both cases, proliferation was assessed at day 2.5 by [³H]thymidine uptake. Units of B cell co-stimulator activity were calculated as outlined in Fig. 5. All results represent duplicate cultures.

assayed for M15 and IL-1 activity. The two activities had a concordant distribution with a peak in activity in the pI 7.0 fractions (Fig. 6). The pI 6.8-7.2 fractions from IEF that contained both M15 and IL-1 activity were pooled and electrophoresed on

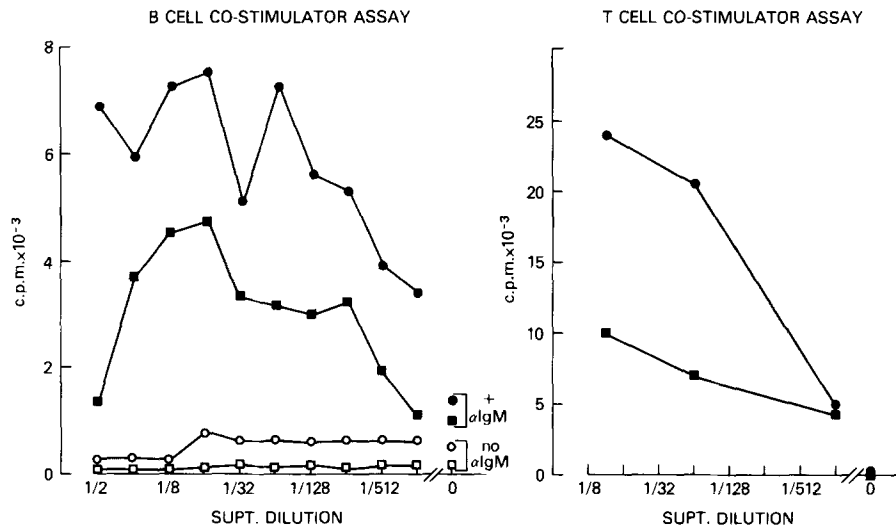


FIG. 7. B cell co-stimulating activity of purified human IL-1. Two separate preparations (●, ■) of human IL-1 from endotoxin-stimulated monocytic leukemia cells were purified to high specific activity as described in text. Each preparation was added at the various dilutions indicated to cultures of 2×10^4 B cells, 10% EL4 supernatant with or without anti-IgM at $5 \mu\text{g}/\text{ml}$ (B cell co-stimulator assay), or mouse thymocytes (T cell co-stimulator assay). Proliferation was assessed at day 2.5 by [^3H]thymidine uptake. Results represent means of duplicate cultures.

a tris glycinate polyacrylamide gel. In two separate experiments, the fraction that contained IL-1 activity was shown to be also rich in M15 activity (Fig. 7).

The biochemical results correlating B cell and thymocyte co-stimulating activities from two separate sources of IL-1 provide strong evidence that M15 is the monokine IL-1 and thus that IL-1, together with BCGF, plays a key role in the stimulation of anti-IgM treated B cells to enter the S phase of the cell cycle.

Discussion

We report here conditions for polyclonal activation of small numbers of highly purified mouse B lymphocytes. Three stimuli are required for induction of DNA synthesis by the particular subset of small B lymphocytes investigated: one delivered by antibodies specific for the IgM receptor expressed on the B cell membrane; one delivered by the T cell-derived factor BCGF; and one delivered by the 15,000-mol wt macrophage-derived factor IL-1. BCGF is a newly described lymphokine discussed in detail elsewhere (9, 12).² Identification of the monokine involved in this process as IL-1 is based on correlation of B cell and T cell co-stimulating activities following an extensive series of biochemical purification procedures. Indeed, the purest preparations of murine and human IL-1 currently available show excellent B cell co-stimulating activity in our assay systems. We do not believe that the B cell co-stimulatory activity contained in IL-1-containing supernatants can be attributed to the PMA generally used for IL-1 induction for several reasons: (a) PMA-free IL-1-containing supernatants obtained from UV-irradiated P388D₁ cells contain the B cell-costimulant; (b) The IL-1-rich supernatant obtained by the superinduction protocol is produced by exposing P388D₁ cells to PMA for 5 h, extensively washing the cells, culturing them for 24 additional h and collecting that supernatant. No PMA can be detected in the purified

preparation, as assessed by the inclusion of isotopically labeled PMA in test batches of supernatant (Mizel, unpublished data); (c) The human IL-1-containing supernatant is induced by endotoxin rather than by PMA; and (d) The B cell co-stimulant resided in IL-1 purified to apparent homogeneity. We wish to emphasize that BCGF and IL-1 do not appear to be mitogenic for resting B cells. Furthermore, BCGF derived from an H-2^b line (EL4) functions on BALB/c (H-2^d) B cells and IL-1 derived from an H-2^d line (P388D₁) functions on CBA (H-2^k) B cells (unpublished data). Thus, these factors act on anti-IgM-activated B cells in a non-antigen-specific, non-H-2-restricted manner. The fact that BCGF and IL-1 co-stimulatory effects are synergistic rather than additive suggests the two factors operate on the same B cell subset. We show elsewhere that BCGF does not lead to the appearance of antibody-forming cells, but is nevertheless an essential component in both an antigen-induced antibody-forming cell response (9) and in the induction of cells with cytoplasmic Ig in response to anti-IgM.³ Preliminary experiments suggest a similar role for IL-1 (unpublished observations). The involvement of accessory cells in anti-IgM-induced B cell proliferation has previously been a matter of controversy; however, involvement of either macrophages or T cells has been reported by some laboratories (28–30). We suggest that failure to observe the need for one or both cell types reflects the use of assay conditions in which small numbers of one or both accessory cells are present with consequent endogenous factor production. Our own experiments show a need for exogenous BCGF and IL-1 only at low cell densities, despite the rigorous B cell purification procedure we have used. Thus it would appear that very small numbers of accessory cells can provide B cell stimulatory co-factors.

Three major models to explain antigen-specific B cell activation have previously been proposed: (a) activation occurs as a result of the binding of antigen to membrane Ig (8, 31); (b) activation occurs via an unrelated nonspecific receptor, with membrane Ig serving to specifically focus molecules capable of binding to this receptor (32); and (c) activation requires both a signal resulting from the binding of the Ig receptor and a signal delivered by an interaction at another antigen-nonspecific receptor on the B cell membrane (33). In this study, we arrive at the novel conclusion that IgM-specific induction of B cell proliferation requires three stimuli: one specific for the antigen receptor, and two antigen-nonspecific stimuli. Preliminary kinetic analyses investigating the relative roles of BCGF and IL-1 in the B cell cycle suggest the two factors function independently, with BCGF operating on G₀ or early G₁ cells and IL-1 acting at some later point of G₁ (34; M. Howard and E. Rabin, unpublished data). It seems likely that the time of action of the co-stimulant correlates with that time that receptors for them are expressed on the B cell surface, just as T cell sensitivity to IL-2 appears to correlate with the expression of receptors for IL-2 on T cells that have been stimulated with lectins or antigens (35–37). However, no direct evidence for receptors for BCGF or IL-1 on activated mouse B cells has yet been obtained.

This report establishes a role for IL-1 in stimulation of B cells. While others have previously proposed roles for IL-1 in B cell responses (38–44), such experiments were conducted using less pure B cell populations cultured at high cell density and thus failed to distinguish a direct action on B cells vs. secondary actions via other cell

³ Nakanishi, K., M. Howard, A. Muraguchi, J. Farrar, K. Takatsu, T. Hamaoka, and W. E. Paul. 1983. Soluble factors involved in B cell differentiation: identification of two distinct T cell replacing factors (TRF). *J. Immunol.* In press.

types. A particular problem in this respect is that IL-1 appears responsible for the generation of at least some T cell-derived lymphokines, e.g., IL-2 (22–25) and colony-stimulating factor (26). With the recent identification of several distinct B cell-specific lymphokines³ (9, 16, 27, 30, 45–48), one could certainly envisage IL-1-mediated factor cascades in many biological assays for B cell function. While such objections may be leveled at our experiments, we offer the following considerations: (a) We have used very highly purified B cells cultured at low cell densities, thus greatly reducing the possibility of accessory cell contamination. (b) We show elsewhere (34) that IL-1 may be added quite late in the B cell cycle, thus providing little opportunity for a factor cascade to develop. (c) Other T cell-derived B cell-specific lymphokines, e.g., BCGF, and the T cell-replacing factors from the EL4 (EL-TRF) and the B15K12 (B15-TRF) T cell lines³ fail to replace the need for IL-1 (unpublished data). Nevertheless, ultimate proof of IL-1 acting directly on B cells will await reagents capable of demonstrating its specific binding to cellular receptors. The observation that IL-1 dependency of B cell proliferation is heightened by omitting 2ME from the culture medium is reminiscent of the earlier studies of Mongini et al. (28), who demonstrated an accessory cell requirement for anti-IgM induced B cell proliferation under 2ME-free conditions. 2ME has been used extensively as an additive to culture medium in a variety of tissue culture systems to either augment the magnitude or facilitate observation of the particular phenomenon under study. While 2ME has been directly implicated as a lymphocyte mitogen and polyclonal activator (49, 50), our experiments suggest an alternative mode of action, namely as a stimulant that induces IL-1 production by residual macrophages and/or B cells.

We wish to emphasize that the conclusions reached from these studies reflect the activation requirements of the subset of B cells that can proliferate in response to anti-IgM antibodies. This subset has recently been quantitated as comprising ~50% of normal splenic B cells (21). Such cells are absent from *xid* mice and appear to be members of the Lyb-5⁺ population of normal B cells. Thus, our data do not exclude the existence of a separate subset of B cells which interact with T cells in an H-2-restricted manner, as demonstrated by others (51–53). Finally, it is difficult to relate the findings of this investigation to those recently obtained in other laboratories using elegant single-cell assays for B cell growth (54–56). As such studies have not used anti-IgM antibodies as the primary activant, different B cell subsets may be under study. Furthermore, the increased sensitivity of single-cell assays over those described in this report may allow detection of trace amounts of growth factors in cell supernatants or indeed in the fetal calf serum contained in culture medium itself, producing results in apparent conflict with ours. Attempts to resolve some of these issues are in progress.

Summary

In this report we describe conditions for polyclonal activation of small numbers of highly purified mouse B lymphocytes. Three signals are required for induction of DNA synthesis by the particular subset of small B lymphocytes investigated: a signal delivered by antibodies specific for the IgM receptor expressed on the B cell membrane; a signal delivered by a T cell-derived factor (B cell growth factor [BCGF]); and a signal delivered by the macrophage-derived factor interleukin 1 (IL-1). The conclusion that IL-1 has B cell co-stimulator activity is based on the findings that highly purified preparations of mouse and human IL-1 have the capacity to cause

proliferation in B cells treated with anti-IgM and BCGF. Such cultures show an absolute dependence on exogenously added IL-1 when 2-mercaptoethanol is omitted from the medium. BCGF and IL-1 each act in a non-antigen-specific, non-H-2-restricted, synergistic manner. Their requirement is not observed when B cells are cultured at high density, presumably reflecting accessory cell contamination and endogenous factor production under these conditions. The B cell activation induced by these three signals is restricted to proliferation without the production of antibody-forming cells.

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