

# REGULATION OF B-LYMPHOCYTE CLONAL PROLIFERATION BY STIMULATORY AND INHIBITORY MACROPHAGE-DERIVED FACTORS\*

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The macrophage has been implicated in the elaboration of a spectrum of molecules which alter or modulate the functions of hemopoietic and lymphoid cells. These include colony-stimulating factor (CSF),<sup>1</sup> required for the renewal and clonal proliferation of granulocyte-macrophage stem cells in semisolid culture (1), lymphocyte-activating factor which potentiates the mitogenic response of T lymphocytes to lectin and histocompatibility antigen (2-4), as well as factors which increase the helper function of T lymphocytes (5, 6) and the promotion of soluble mediator production by lymphocytes (7-9). The role of the macrophage in the regulation of humoral immune responses is however, less well understood. In contrast to the reported potentiation by cell-free macrophage supernates of B-cell differentiation into antibody-secreting cells in response to sheep erythrocytes (SRBC) and hapten-protein conjugates (10, 11), mitogen-induced B-lymphocyte proliferative responses are suppressed in the presence of macrophages (12-15).

We have elected to investigate the immunoregulatory role of the macrophage in B-cell activation by utilizing a two-layer semisolid culture system which prevents macrophage-lymphocyte, as well as lymphocyte-lymphocyte contact, and in which only diffusible interactions may occur. In this system, the capacity of a B lymphocyte to undergo focal proliferation and generate a clone or colony is taken as the immunological end point of B-cell activation, a process dependent upon 2-mercaptoethanol (16) and mitogens native to laboratory-grade agar (17). As previously reported, this functional population of clonable B lymphocytes, the majority of which bear Fc receptors, IgM, and Ia antigens on their surface (18, 19), are heterogeneous with respect to size and density and are widely distributed in lymphoid tissues (20, 21).

We report here that B-cell activation under these conditions is dependent upon diffusible factor(s) elaborated by macrophages. In addition, the ability of

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<sup>1</sup> Abbreviations used in this paper: AM, agar-derived mitogen; CSF, colony-stimulating factor; Con A, concanavalin A; DS, dextran sulfate; FCS, fetal calf serum; [<sup>125</sup>I]IUDR, [<sup>125</sup>I]iododeoxyuridine; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; PEC, peritoneal exudate cells; PGE, E-series prostaglandins; SRBC, sheep erythrocytes.

indomethacin to prevent suppression of B-cell activation as a result of an accumulation of macrophage-derived factor(s), indicates a role for prostaglandin, and suggests that the dual regulatory influence that macrophages exert on B-cell activation operates via the elaboration of individual molecules. Preliminary evidence is presented that immunomodulating agents such as lipopolysaccharide may alter the production of these B-lymphocyte regulatory factors by macrophages.

### Materials and Methods

*Animals.* Female B6D2F<sub>1</sub> mice (Cumberland View Farms, Clinton, Tenn.) were used at 2-3 mo of age and provided spleen and lymph nodes, as well as peritoneal macrophages. Macrophages for some experiments were also obtained from C57BL/6 mice (Jackson Laboratories, Bar Harbor, Maine) and CBA/Cum mice (Cumberland View Farms) of either sex. Neonatal spleens were harvested from two litters of CBA/Cum mice less than 24 h after birth.

*Preparation of Cell Suspensions.* Spleen and axillary, mesenteric and subcutaneous abdominal lymph nodes were sterilely excised, sliced into 1-mm fragments and teased through a stainless steel mesh. Large fragments of connective tissue and fat were removed and the cells washed three times, counted, appropriately diluted, and kept on ice until needed. Stimulated peritoneal exudates were induced by intraperitoneal injections of 1.5 ml thioglycollate medium (Difco Laboratories, Detroit, Mich.). 4 days later, the mice were killed by cervical dislocation their abdominal skin reflected, 8 ml of ice-cold serum-free McCoy's 5A Modified Medium containing 5 U of heparin/ml injected i.p., and the peritoneal contents were gently agitated and withdrawn into a syringe fitted with an 18-gauge needle. The peritoneal exudate cells (PEC) were washed twice at 4°C, suspended in ice-cold serum-free medium, the number of nucleated cells counted, and appropriately diluted. This procedure yielded 30-50 × 10<sup>6</sup> PEC per mouse of which 75% was morphologically typical macrophages. Noninduced resident peritoneal cells were harvested by sterilely washing out the peritoneal cavity with 8 ml of ice-cold serum-free medium.

*Adherent Cell Depletion by Sephadex G-10 Columns.* The preparation of adherent cell-depleted mouse spleen or lymph node cell suspensions by passage through columns of Sephadex G-10, was based upon the technique of Ly and Mischell (22). 2 ml of 1 × 10<sup>8</sup> washed spleen or lymph node cells/ml in McCoy's 5A Modified Medium containing 5% fetal calf serum (FCS; Flow Laboratories, Inc., Rockville, Md.) was gently pipetted into 10-ml syringes containing 10 ml of autoclaved and washed Sephadex G-10 beads (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) which was warmed to 40°C. After disappearance of the cell suspension beneath the surface of the Sephadex, 5 ml of Hanks' balanced salt solution containing 5% FCS was added and the nonadherent filtrate cells sterilely collected, washed three times, and counted. Cells recovered in the column filtrate constituted 30-40% of the initial unseparated spleen or lymph node cell suspension. Determination of residual macrophages in the adherent cell-depleted lymphoid suspensions was performed by staining with neutral red (23) the cells immediately after depletion or those cells remaining adherent to plastic culture dishes after 4-5 days of incubation. The extremely low incidence of macrophages seen after the short in vitro culture period excluded the presence of nonadherent macrophage precursors.

*Separation of Phagocytic Cells by Carbonyl Iron Ingestion.* 1 × 10<sup>7</sup> mouse spleen cells were incubated in 3.0 ml McCoy's 5A Modified Medium containing 10% FCS and 250 mg of dry-heat sterilized carbonyl iron (Atomergic Chemetals Corp., Plainview, N. Y.) in sterile glass Leighton tubes. After incubation at 37°C for 45 min with gentle agitation every 5 min, the tubes were placed flat-side down on a large magnet and the contents were carefully eluted. 2 ml of fresh medium was added to the tubes, the process repeated, and the contents were pooled with the previous cells. These cells were placed into clean Leighton tubes and again situated on the magnet to remove residual iron-containing cells. After this procedure, the cells were washed three times in McCoy's medium containing 15% FCS and appropriately diluted. Spleen cells prepared in this manner, though constituting only 40-50% of the original cell suspension, were devoid of macrophages as determined by neutral red staining.

*Soft Agar B-Lymphocyte Cultures.* The basic culture methodology was essentially as described

earlier (24). Briefly, various numbers of adult mouse spleen, lymph node, or neonatal spleen cells were cultured in 0.3% Bacto-agar in McCoy's 5A Modified Medium containing 15% FCS and supplemented with additional essential and nonessential amino acids, glutamine, asparagine, and sodium pyruvate. 2-Mercaptoethanol (2-ME) was incorporated into the agar-cell medium at a final concentration of  $5 \times 10^{-5}$  M and 1 ml of the agar-cell mixture dispensed in 35-mm Petri dishes (Lux Scientific Corp., Newbury Park, Calif.) and allowed to gel. Colonies were scored after 5-7 days of incubation at 37°C in a humidified atmosphere of 10% CO<sub>2</sub> in air, using an Olympus dissecting microscope (Olympus Corporation of America, New Hyde Park, N. Y.) at 25 times magnification. In particular experiments, 20 µg lipopolysaccharide (LPS) from *Salmonella typhosa* (W0901, Difco Laboratories) or 0.05 ml of a 10% vol/vol washed SRBC suspension (Grand Island Biological Co., Grand Island, N. Y.) were included in the soft agar cultures.

The effects of diffusible factors elaborated by an adherent population of peritoneal macrophages on B-lymphocyte colony formation was investigated in a two-layer soft agar culture system which consisted of a 1-ml 0.3% agar matrix containing spleen or lymph node cells suspended over underlayers of adherent macrophages. Various numbers of either thioglycollate-induced PEC or noninduced peritoneal washout cells were allowed to adhere to 35-mm Petri dishes in McCoy's medium containing 15% FCS. After 1.5 h at 37°C, the nonadherent cells of the exudate (comprising mostly lymphocytes and polymorphonuclear leukocytes) were vigorously washed off. To the peritoneal macrophages remaining adherent to the culture dishes was added a 1-ml cell-free 0.5% agar layer in complete McCoy's medium. The 0.5% agar layer constituted a physical separation between the adherent macrophages and the overlying layer of spleen or lymph node cells suspended in 1 ml of 0.3% soft agar medium. Control underlayers containing only cell-free 0.5% agar in McCoy's medium (i.e., no macrophages) were prepared at the same time and were used to control for the additional nutrients provided by the extra culture medium. Indomethacin (Sigma Chemical Co., St. Louis, Mo.), a known inhibitor of prostaglandin synthesis, was added to both control and adherent macrophage underlayers.

The adherent components of thioglycollate-induced PEC consisted mainly of macrophages as determined by their ability to stain with the vital lysosomal stain, neutral red, and comprised 20-30% of the initial exudate population. Noninduced peritoneal adherent macrophages comprised 10-15% of the initial peritoneal washout population.

*Preparation of Macrophage-Conditioned Medium.* To the various numbers of adherent peritoneal macrophages was added 1 ml of McCoy's medium containing 15% FCS and  $5 \times 10^{-5}$  M 2-ME in the absence and presence of LPS, SRBC, and indomethacin. The supernatant media were harvested 48 h later, centrifuged at 5,000 rpm for 15 min, Millipore filtered (a 0.45-µm pore size, Millipore Corp., Bedford, Mass.), and assayed for B-lymphocyte colony-stimulating activity at various volume percents in cultures of either unseparated or Serhadex G-10 passaged lymph node cells.

*Conventional Mitogen-Stimulated Lymphocyte Culture.* Sephadex G-10 column-purified lymph node cells were cultured in the absence and presence of varying numbers of adherent peritoneal macrophages and tested for their responsiveness to a number of mitogens. Briefly, various numbers of thioglycollate-induced PEC were allowed to adhere in Microtest wells (Falcon Plastics, Div. of BioQuest, Oxnard, California) for 1.5 h and the nonadherent cells removed. The adherent macrophages were vigorously washed and 0.2 ml of  $2 \times 10^6$  or  $2.5 \times 10^5$  Sephadex G-10-passaged lymph node cells/ml in McCoy's 5A Modified Medium containing 15% FCS was added per well. Mitogens used for stimulating these lymphocyte cultures were LPS from *S. typhosa*, 5 µg/culture; dextran sulfate (DS;  $5 \times 10^4$  mol wt, Pharmacia Fine Chemicals, Inc.), 20 mg/culture; concanavalin A (Con A; Pharmacia Fine Chemicals, Inc.), 5 µg/culture; and agar-derived mitogens (AM) prepared as previously described (17), 25 µg/culture. Cultures were initiated either with or without FCS to investigate serum-dependence of mitogenesis. After 72 h of incubation at 37°C in 7% CO<sub>2</sub> in humidified air, 0.2 µCi [<sup>125</sup>I]iododeoxyuridine ([<sup>125</sup>I]IUdR; sp act 200 Ci/mM, New England Nuclear, Boston, Mass.) was added to the culture wells for an additional 3 h. The cells were harvested by a semi-automatic cell harvester, deposited on filter paper, washed with saline and 10% trichloroacetic acid (TCA), and the incorporation of [<sup>125</sup>I]IUdR counted in a gamma counter, as previously described (17).

## Results

*Macrophage Requirement for B-Lymphocyte Clonal Proliferation.* The re-

TABLE I  
*Macrophage Requirement for B-Lymphocyte Colony Formation by CBA/Cum Adult Spleen, Lymph Node, and Neonatal Spleen Cells\**

Number of PEC used as source of adherent MØ‡	Adult spleen			Adult lymph node		Neonatal spleen
	Unseparated	Sephadex G-10 separated	Carbonyl iron separated	Unseparated	Sephadex G-10 separated	Unseparated
None	310 ± 21	120 ± 17	98 ± 4	31 ± 2	4 ± 1	0
5 × 10 <sup>3</sup>	288 ± 18	221 ± 20	187 ± 12	68 ± 1	64 ± 4	32 ± 4
1 × 10 <sup>4</sup>	316 ± 18	310 ± 18	325 ± 18	164 ± 8	169 ± 6	81 ± 6
5 × 10 <sup>4</sup>	295 ± 12	420 ± 18	405 ± 13	258 ± 13	286 ± 6	118 ± 10
1 × 10 <sup>5</sup>	240 ± 17	364 ± 21	372 ± 11	213 ± 16	266 ± 14	133 ± 8
5 × 10 <sup>5</sup>	148 ± 17	230 ± 17	232 ± 6	147 ± 8	162 ± 12	36 ± 7

\* 5 × 10<sup>4</sup> nucleated cells per culture.

‡ PEC harvested 4 days post-intraperitoneal injection of thioglycollate medium in CBA/Cum mice.

quirement for macrophages in B-cell colony formation and the ability of our two-layer culture system to satisfy this requirement was initially tested by using B lymphocytes from various tissue sources (Table I). B-lymphocyte colony formation by neonatal spleen and adult lymph node cells was consistently and markedly potentiated by culture underlayers containing an optimal number of peritoneal macrophages. Similar numbers of macrophages only occasionally increased numbers of colonies developing in cultures of adult spleen, though in most cases, the presence of macrophages markedly increased mean colony size. When suspensions of adult spleen or lymph node were depleted of phagocytic or adherent cells by carbonyl iron or passage over Sephadex G-10, respectively, the incidence of colony-forming cells was significantly decreased. Colony number was restored to normal or supranormal levels, however, when macrophage-depleted suspensions were cultured over underlayers containing various concentrations of macrophages.

These observations indicate that macrophages are required for B-cell colony formation and that this requirement can be satisfied by diffusible macrophage-derived factors. Newborn spleens, and to a lesser extent, adult spleens, contained appreciable numbers of granulocyte-macrophage progenitor cells which underwent clonal proliferation in the presence of CSF produced by macrophages in the underlayers. Such myeloid colonies were, however, distinguished from B-lymphocyte colonies by their gross morphology, lack of sensitivity to anti-Ig, and by conventional histochemical differentiation of the cells comprising the colonies. Macrophage-depleted lymph node cells contained no granulocyte-macrophage progenitor cells which could respond to macrophage-derived CSF, and as a practical matter we therefore employed lymph node cells as targets for macrophage regulation in all subsequent studies of B-cell activation.

*Role of Macrophage-Derived Prostaglandin in the Inhibition of B-Lymphocyte Clonal Proliferation.* As shown in Table II, increasing the numbers of macrophages in culture underlayers invariably depressed colony formation. We have

TABLE II  
*Effect of Combinations of Peritoneal Macrophages,  
 Lipopolysaccharide, and SRBC on the Frequency of B-  
 Lymphocyte Colony Formation*

Addition*	Number B-lymphocyte colonies per $7.5 \times 10^4$ lymph node cells + SEM
None*	0 ± 0
MØ‡	109 ± 9
LPS§	72 ± 3
SRBC	35 ± 2
MØ + LPS	247 ± 6
MØ + SRBC	193 ± 11
LPS + SRBC	212 ± 15
MØ + LPS + SRBC	311 ± 19

\* Cell-free 0.5% agar culture underlayer.

‡ Adherent macrophages from  $1 \times 10^8$  thioglycollate-induced PEC incorporated in culture underlayer.

§ 20 µg LPS.

|| 0.1 ml of 10% washed SRBC.

previously reported that B-lymphocyte colony formation by adult spleen cells can be effectively inhibited by concentrations of prostaglandin E (PGE<sub>1</sub> and PGE<sub>2</sub>) as low as  $10^{-10}$ – $10^{-12}$  M (24), and that macrophages represent a major source of PGE within the hemopoietic system (25, 26). We therefore tested the effect of the prostaglandin synthetase inhibitor, indomethacin (27), on the diminution of B-lymphocyte colonies observed in cultures containing an excessive number of macrophages (Fig. 1). Cultures of lymph node cells depleted of adherent cells by prior passage through Sephadex G-10 formed very few colonies. Colony number was increased  $\cong$  14-fold by an optimal number ( $0.05 \times 10^6$ ) of peritoneal macrophages, whereas an excess of macrophages significantly suppressed colony formation. Note that in the presence of adherent macrophages derived from  $1 \times 10^6$  PEC, <10% of the clonable B-cell population commenced proliferation to form colonies. However, the inclusion of indomethacin into the cultures permitted a clonal efficiency identical to that observed under peak control conditions. Indomethacin had no effect on B-lymphocyte colony formation in the absence of peritoneal macrophages, thus indicating a role for macrophage-derived prostaglandin in the suppression of B-cell clonal proliferation. Note that at macrophage concentrations of  $1 \times 10^6$  and greater, the effectiveness of indomethacin to prevent inhibition decreased, suggesting that the accumulation of other macrophage products, in addition to prostaglandin, may be involved in the suppression of B-cell proliferation.

*Comparison of Macrophage-Containing Underlayers with Other Potentiators of B-Cell Colony Formation.* The B-cell colony assay as originally described (20) is characteristically nonlinear; i.e., colony number is not a linear function of the number of B cells placed in culture. Colony formation is mitogen dependent (17) and the addition of the B-cell mitogen LPS, or SRBC enhances colony formation, particularly when few B cells are cultured (20). We therefore compared the dose response of lymph node cells cultured with or without these

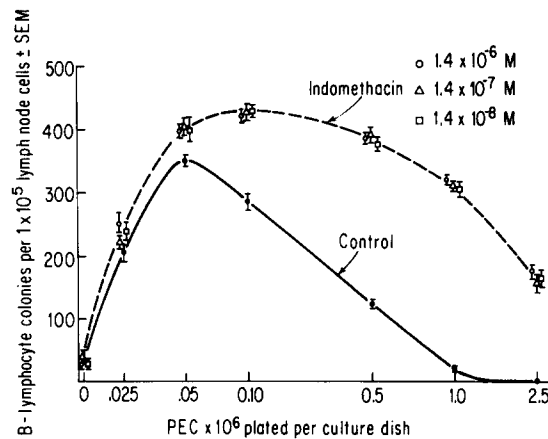


FIG. 1. Effect of indomethacin on the inhibition of B-lymphocyte colony formation by high numbers of peritoneal macrophages.  $1 \times 10^5$  Sephadex G-10-passaged B6D2F<sub>1</sub> lymph node cells/ml were cultured over agar underlayers containing adherent macrophages from various numbers of B6D2F<sub>1</sub> thioglycollate-induced PEC. Macrophage-containing and cell-free underlayers were prepared in the absence (control) and presence of indomethacin 24 h before the addition of the lymph node cells.

agents. Based on the observations described in Fig. 1,  $1 \times 10^5$  peritoneal macrophages and  $1.4 \times 10^{-7}$  indomethacin, were selected as conditions which ensure sufficient macrophage-derived B-cell stimulating factor(s) and prevent prostaglandin-mediated suppression. Both macrophage underlayers and LPS resulted in linear dose-response curves (Fig. 2), which were calculated statistically by linear regression analysis to possess  $r^2$  of 1.0, and have nearly equal slopes. Analysis of SRBC-stimulated colony formation also demonstrated a linear dose-response ( $r^2 = 1.0$ ), but the slope of this relationship was significantly greater than that of macrophage- or LPS-stimulated cultures. At any given concentration of lymph node cells cultured under the varied conditions, different numbers of colonies resulted.

To determine if these potentiating agents act via a common mechanism to augment colony formation by the same or different lymphocyte subpopulation, we prepared lymph node cell cultures in the absence and presence of all possible combinations of these agents (Table II). Greater numbers of colonies were obtained in cultures containing two or more of these potentiating agents than with any one alone. When all three potentiators were used together,  $\cong 13\%$  of the B cells proliferated sufficiently to form colonies and a larger number formed clusters (not shown). The observed increase in colony formation in the co-presence of the potentiating agents was slightly greater than additive, suggesting that each may only induce a particular subpopulation of B cells to undergo clonal proliferation. Alternatively, LPS and SRBC may have an effect on augmenting the elaboration of stimulatory factors by those macrophages present in the lymph node suspension or in the culture underlayer.

*Requirement for Macrophages in Mitogen-Stimulated Liquid Cultures.* We tested the ability of macrophages to potentiate or inhibit B-cell proliferation in conventional liquid cultures of mitogen-stimulated lymphocytes.  $4 \times 10^5$  Seph-

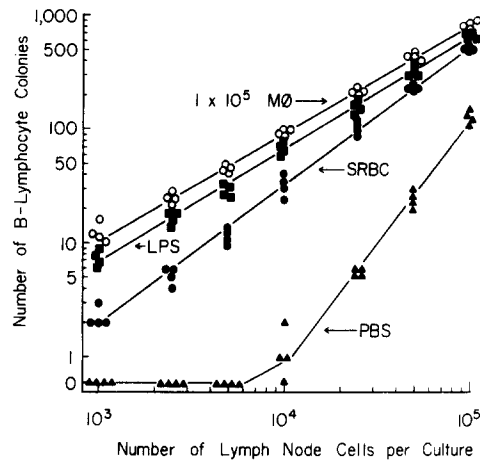


FIG. 2. Comparative potentiation of lymph node B-lymphocyte colony-forming ability by peritoneal macrophages (M $\phi$ ), endotoxin, and SRBC. Various numbers of B6D2F<sub>1</sub> lymph node cells were cultured in soft agar medium over cell-free or  $1 \times 10^5$  peritoneal exudate macrophage-containing underlayers in the presence of  $1.4 \times 10^{-7}$  M indomethacin. SRBC, LPS, or phosphate-buffered saline (PBS) were included in cultures prepared over cell-free underlayers.

adex G-10-passaged lymph node cells were added to culture wells to which different numbers of peritoneal macrophages were previously allowed to adhere. Significant proliferative responses, (as assayed by [ $^{125}$ I]IUDR incorporation on day 3) to the B-cell mitogens, DS, LPS, and AM, and the T-cell mitogen Con A, were observed in cultures initiated in the absence of additional macrophages (Fig. 3A). Stimulation with DS was variable and occasionally augmented by macrophages, and optimal responsiveness to Con A always occurred when low numbers of macrophages were present. When the ratio of initially added PEC to lymph node cells exceeded 1:10, marked inhibition of all responses was observed. Essentially the same results were obtained in serum-free cultures, except that no lymphocyte stimulation was observed with the serum-dependent mitogen, Con A (Fig. 3B).

These observations are consistent with previously reported findings (12-15) and were obtained using relatively high initial cell densities. Since we were unable to demonstrate macrophage potentiation of B-cell responses to LPS and AM in these cultures, and other studies suggest that the lymphocytes which respond in high density liquid culture and semisolid culture are different (26), we tested the effects of macrophages on liquid cultures prepared at relatively low cell densities; i.e., conditions more nearly approximating those which occur in the semisolid cultures. An eightfold reduction in the number of lymphocytes cultured reduced the magnitude of the responses, but the effect of macrophages was not significantly different (not shown). That is, no potentiation of the responses was observed and an excess of macrophages completely prevented lymphocyte stimulation. In other experiments, the inhibition of B-cell responses to mitogens in liquid cultures by macrophages was not reversible with indomethacin (not shown). In contrast to the dramatic potentiation of B-cell clonal proliferation in semisolid cultures by macrophage-derived factors, we were

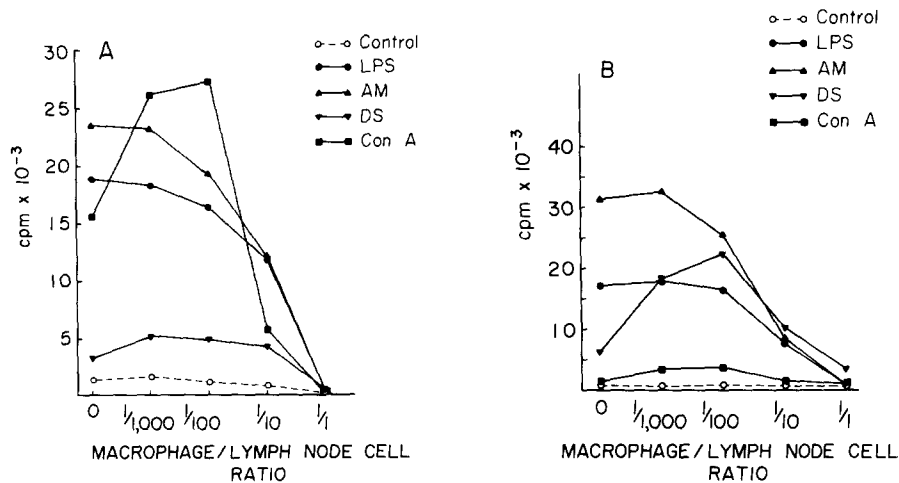


FIG. 3. Effect of various numbers of adherent macrophages on the mitogen-responsiveness of macrophage-depleted lymph node cells in liquid cultures performed in serum-containing (A) or serum-free (B) medium. Proliferative responses were assessed by the amount of [<sup>125</sup>I]IUDR incorporation (counts per minute) after 72 h of culture, as described in Materials and Methods.

unable to demonstrate this activity in conventional liquid cultures. Furthermore, since indomethacin did not prevent high dose macrophage inhibition in liquid cultures, macrophages may be able to suppress B-cell activation through a contact-mediated mechanism, independent of prostaglandin. Thus, the semi-solid culture system is uniquely suited to detecting both macrophage-derived stimulating and inhibiting principles.

*Detection of B-Lymphocyte Colony-Stimulating Activity in Peritoneal Macrophage-Conditioned Medium.* Since the stimulatory and inhibitory actions of adherent macrophages on the clonal proliferation of B lymphocytes in semisolid culture involve the elaboration of factors which can diffuse through an agar layer separating the macrophages from the responding B cells, we attempted to obtain a cell-free conditioned medium which could exhibit these activities. 48-h conditioned medium from  $1 \times 10^5$  thioglycollate-induced macrophages exhibited appreciable B-cell colony-stimulating activity at 25% vol/vol, but colony numbers declined at higher concentrations (Fig. 4). In contrast, medium conditioned by noninduced macrophages was significantly more effective in stimulating B-lymphocyte clonal proliferation and no decline in colony numbers was observed at correspondingly high concentrations. Thus, in the case of thioglycollate-induced macrophages, both stimulatory and inhibitory principles were detectable in conditioned medium.

*Effect of LPS and SRBC on Macrophage Production of B-Lymphocyte Colony-Stimulating Activity.* Since both LPS and SRBC potentiate B-cell clonal proliferation (Fig. 2) we investigated whether this effect may in part reflect an action on the elaboration of stimulatory factors from macrophages. Conditioned media from  $1 \times 10^5$  thioglycollate-induced macrophages prepared in the presence and absence of LPS or SRBC were assayed for stimulating activity in cultures of Sephadex G-10-passaged lymph node cells, as before. To



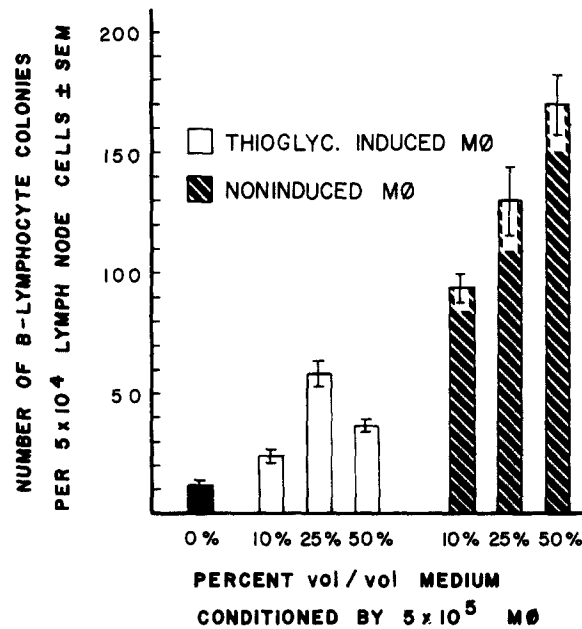


FIG. 4. Effect of thioglycollate-induced and noninduced peritoneal macrophage-conditioned medium on lymph node B-lymphocyte colony formation. 48-h conditioned media from the adherent macrophages (MØ) of  $5 \times 10^5$  B6D2F<sub>1</sub> thioglycollate-induced and noninduced PEC were incorporated in soft agar cultures of  $5 \times 10^4$  B6D2F<sub>1</sub> lymph node cells.

control for the direct stimulatory actions of LPS, 20  $\mu$ g of LPS was incubated for 48 h in macrophage-free medium (LPS medium). All concentrations of the different supernates possessed demonstrable but varying levels of B-lymphocyte stimulatory activity (Fig. 5). Macrophage-conditioned medium prepared in the presence of LPS potentiated colony formation to the extent that would be predicted from the independent effects of LPS and macrophage supernate alone. However, when higher concentrations of conditioned medium were tested, it was apparent that the supernate generated in the presence of both macrophages and LPS contained significant quantities of inhibitory activity. Therefore, the net effect of LPS was to augment inhibitor production by the macrophages. Medium conditioned by SRBC alone had no effect on B-cell colony formation (not shown). However, macrophage-conditioned medium prepared in the presence of SRBC had more enhancing activity than medium conditioned by macrophages alone.

Therefore, both LPS and SRBC can modulate the production of immunoregulatory substances by macrophages. Under the conditions employed in these experiments, LPS augmented inhibitor production and SRBC enhanced the release of B-cell potentiating factors by macrophages. In other experiments (not shown), indomethacin largely prevented the pronounced suppressor activity of high numbers of LPS-treated macrophages. This protective effect of indomethacin was independent of a direct action on the B-lymphocyte, but was active only when continuously present during conditioning of medium by macrophages.

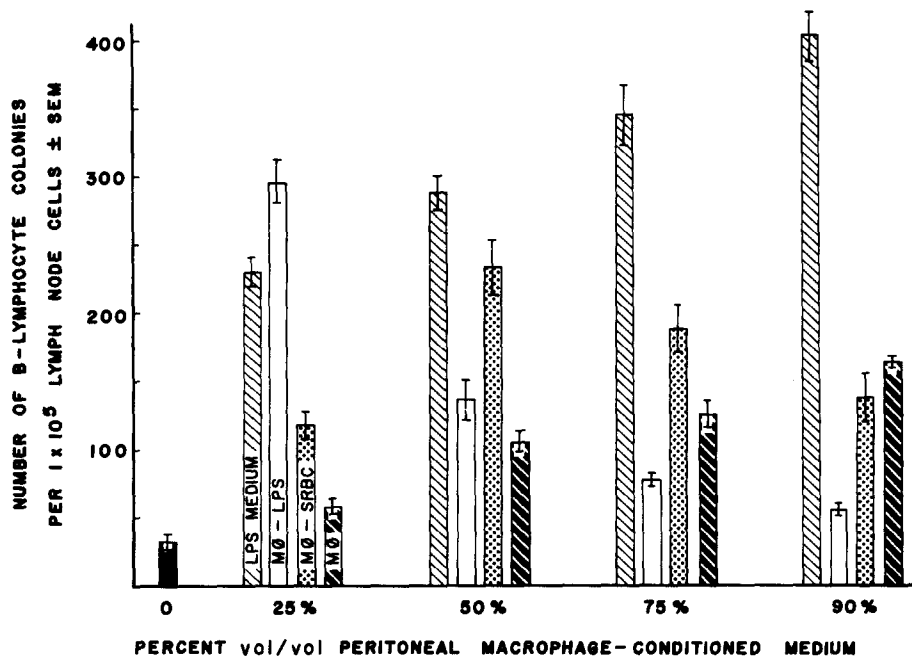


FIG. 5. Effect of endotoxin and SRBC on peritoneal macrophage (MØ) production of B-lymphocyte colony-stimulating factor. Various volume percents of 48-h conditioned media of  $1 \times 10^5$  B6D2F, thioglycollate-induced peritoneal macrophages prepared in the absence and presence of  $20 \mu\text{g}$  LPS and 0.05 ml of 10% SRBC were incorporated into soft agar cultures of  $1 \times 10^5$  Sephadex G-10-passaged B6D2F, lymph node cells. Macrophage-free medium containing only LPS and incubated for 48 h, served to control for the direct stimulatory effects of LPS.

### Discussion

The results reported here indicate that low cell density semisolid cultures are ideally suited to studying the role of macrophages in mitogen activation of B lymphocytes *in vitro*. Very few proliferating foci result in cultures containing B lymphocytes prepared from newborn spleen, adult lymph node, or macrophage-depleted adult spleen cell suspensions. By incorporating a critical number of adherent peritoneal macrophages within a culture underlayer, B-lymphocyte colony formation was markedly augmented; an effect which was mediated by the elaboration of one or more diffusible substances of macrophage origin. The magnitude of potentiation of B lymphocyte colony formation increased when the target lymphoid cell suspensions were previously depleted of adherent or phagocytic cells, indicating that a macrophage population indigenous to adult lymph node or spleen plays an accessory cell role in B-cell activation *in vitro*.

When the number of macrophages exceeded that required for optimal potentiation, B-cell clonal proliferation was markedly suppressed. This suppressive function of macrophages was largely prevented by incorporating the prostaglandin synthetase inhibitor, indomethacin (27), into the culture underlayers. The interpretation of a role for macrophage-derived prostaglandin in the suppression

of B-cell activation is consistent with our previous observations that the synthetic E-series prostaglandins are efficacious inhibitors of B-lymphocyte clonal proliferation in spleen cell cultures (24), and that the mononuclear phagocyte is the principal hemopoietic cell source of PGE (25, 26). Both B-lymphocyte stimulatory and inhibitory principles could be demonstrated in macrophage supernates, the net levels of which were dependent upon the numbers of macrophages permitted to condition medium, and the concentration of conditioned medium added to the B-lymphocyte cultures.

When macrophage influence was optimized by the use of culture underlayers containing an ideal concentration of both macrophages and indomethacin, the number of proliferating B-lymphocyte colonies was a linear function of the number of rigorously macrophage-depleted lymph node cells added to the cultures. Similar potentiation was observed in such cultures by the addition of LPS or intact SRBC, and it can thus be argued that macrophages may not be obligate to B-cell activation by mitogens. In this regard, since the number of colonies resulting in cultures containing more than one of these agents was equal to or greater than the sum of those in cultures potentiated by any one alone, it is possible that different B-cell subpopulations are detected under the different conditions. If this is so, they must be closely related since none are functional in CBA/N mice (28). In addition to possible direct effects that LPS and SRBC might have on B lymphocytes, these substances modulate the elaboration or regulatory factors by macrophages. In the presence of low numbers of macrophages, SRBC facilitated the production and/or release of the B-cell stimulating activity. Conversely, when added to high concentrations of macrophages, SRBC, and to a greater extent, LPS, promoted the elaboration of inhibitory factors. This heightened suppressor activity of macrophages exposed to LPS was again largely indomethacin-sensitive and we have found in other experiments that LPS is a potent stimulator of PGE production by macrophages.<sup>2</sup>

We have not as yet characterized the macrophage-derived factor(s) which augment B-cell activation and we do not know if it plays a role in immune responsiveness *in vivo*. However, since we have been unable to demonstrate this activity in media conditioned by a variety of other cell types, it seems unlikely that it is merely a nutritional factor. Furthermore, since the addition of macrophages or macrophage-conditioned medium does not replace the need for 2-ME, these must subserve two entirely different functions in potentiating B-cell proliferation. There is certainly precedence in the literature for a role for adherent cells or their products in a variety of *in vitro* immune responses. For example, macrophages provide factors necessary for T-cell activation in response to mitogens (2) and histocompatibility antigen (4), augment the *in vitro* antibody response of B cells to SRBC (6), and substitute for a T-helper cell in the B lymphocyte response to T-dependent antigens (11). Alternatively, the macrophage-derived B-cell colony-stimulating factor may be one of the neutral proteases released by adherent cells which potentiates *in vitro* phytohemagglutinin and purified protein derivative responses (29), or may be similar to the factor described by Namba and Hanoaka (30) which is produced by adherent spleen cells and a phagocytic cell line and which stimulates the proliferation of

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<sup>2</sup> J. I. Kurland and R. Bockman. Manuscript submitted for publication.

an IgM myeloma cell line. Similarly, Metcalf (31) described a factor which potentiates the cloning of murine plasmacytoma cells *in vitro*, and Hamburger and Salmon (32) found that conditioned medium of oil-primed murine spleen cells is required for colony formation by primary human myeloma cells.

The capability to document a requirement for macrophages for B-cell activation in semisolid culture is in contrast with previously reported observations (12-15) that T-cell, but not B-cell responses in mitogen-stimulated liquid cultures are augmented by the presence of macrophages. Our findings confirm that in conventional liquid cultures, macrophage enhancement of Con A, but not LPS or agar-mitogen responses, is demonstrable. What then may account for the different macrophage requirements in the two B-cell proliferative assays? In contrast to conventional liquid cultures, the focal proliferation of B cells in semisolid medium occurs under extremely low cell densities ( $10^3$  cells/mm<sup>3</sup>) in which the cells are separated in three dimensions. In liquid cultures, cells settle to the bottom of the culture well and the actual cell densities are much greater ( $1.45 \times 10^4$ /mm<sup>2</sup>). Reduction of cell numbers in liquid cultures eightfold ( $1.8 \times 10^3$  cells/mm<sup>2</sup>) such that normal but not CBA/N cell mitogen responses occur, did not permit a macrophage requirement to be demonstrable. It is therefore possible that conventional mitogen-stimulated liquid cultures prepared from even the most meticulously purified lymphocyte suspensions facilitates the crucial interaction between lymphocytes and contaminating macrophages, and that this cell-to-cell contact must be prevented in order to demonstrate a macrophage requirement. Other differences exist between the liquid and semisolid culture systems. Responses in liquid cultures were assessed after 72 h, whereas focal B-cell proliferation in semisolid medium is optimally observed after 6 days of culture. Colony formation is dependent upon 2-ME (16), mitogens native to laboratory grade agar (17) and serum, whereas B-cell activation by LPS in liquid cultures is demonstrable in the absence of 2-ME and serum (P. W. Kincaide, unpublished observations). B-cell clonal expansion in soft agar medium is probably more fastidious than short-term liquid culture responses and the semisolid colony assay may be uniquely suited to studying the role of accessory cells because macrophage-lymphocyte, as well as lymphocyte-lymphocyte contact is prevented.

It tends to be the rule that whenever macrophages are found to promote some aspect of immune function, they can also inhibit that particular function. In cases where both stimulatory and inhibitory activities are observed, inhibition is consistently viewed as the result of excess macrophages (12, 33-37). The interpretation that this inhibitory effect is due to an accumulation of excess helper factors is conjectural, since macrophages may elaborate separate factors with enhancing or suppressive activities. In our studies this is clearly the case, and the inhibitory activity has been putatively defined as PGE. Similarly, depressed immune status may result from an accentuated inhibitory role of the macrophage. This is indicated by the recent reports of a suppressor macrophage in the peripheral blood of patients with multiple myeloma who are unable to generate a polyclonal immunoglobulin synthetic response to pokeweed mitogen (38, 39). In this context, it has been reported by Gailani et al. (40) that bone marrow from patients with multiple myeloma releases large amounts of prostaglandin during culture; an observation which is given particular rele-

vance in view of our present findings that macrophage-derived prostaglandin exerts a profound inhibition on B-cell activation. Similarly, the recently described inhibition of human T-cell colony formation by a dialyzable factor from adherent human spleen cells and monocytes (41) may also be a prostaglandin-mediated event, whose pathoclinical correlate may be the marked suppression of T-cell proliferation observed in patients with Hodgkin's disease (42) or solid tumors (43). Derangements in the otherwise physiologic functions of a regulatory cell could then result in accentuated immunopotentiality or significant immunosuppression.

We have examined the role of accessory cells in B-cell activation by exploiting a simple culture system in which humoral regulation of B lymphocytes may be selectively studied. This report clearly documents the ability of macrophages to either potentiate or inhibit the clonal proliferation of B cells in semisolid medium. The dissociation of stimulatory and inhibitory mechanisms, by incorporating an inhibitor of prostaglandin synthesis into the two-layer culture system, suggests that each may operate via separate macrophage-derived factors.

### Summary

A functional subpopulation of murine B lymphocytes proliferate in semisolid agar culture in the presence of 2-mercaptoethanol to form colonies. The effects of diffusible macrophage-derived factors on this focal proliferation was investigated using a two-layer culture system which prevented macrophage-lymphocyte contact and permitted B-cell activation to be critically assessed under conditions of extremely low cell densities. Adherent peritoneal macrophages incorporated within underlayers of spleen or lymph node cell cultures potentiated both the number and size of developing B-cell colonies. These effects were most striking when low numbers of spleen or lymph node cells, or macrophage-depleted lymphoid cell suspensions were used. Thus, macrophage-depleted lymph node cells gave rise to virtually no colonies, but colony-forming ability was restored by the presence of an optimal number of macrophages. When the number of macrophages exceeded that required for optimal stimulation, colony formation was suppressed; an effect which was largely prevented by indomethacin, an inhibitor of prostaglandin synthesis. Under these conditions, stimulation and inhibition of B-cell activation by macrophages could be dissociated, indicating that each signal is selectively controlled by individual molecules elaborated by the macrophage. With an appropriate number of macrophages required for B-cell activation, and sufficient indomethacin to inhibit the accumulation of macrophage-derived prostaglandin, B-lymphocyte clonal proliferation was a linear function of the number of B cells placed in culture. In the absence of macrophages, B-cell colony formation was potentiated by both lipopolysaccharide and intact sheep erythrocytes through a mechanism different from that of the macrophage-derived stimulatory factor. In addition to their direct stimulatory effect on B-cell proliferation, lipopolysaccharide and sheep erythrocytes were each capable of modulating the production and/or release of B-cell stimulatory and inhibitory factors by the macrophage. Parallel studies of conventional mitogen-stimulated lymphocyte cultures did not show a

requirement for macrophages and confirm that the semisolid assay is uniquely suited to studies on the regulatory role of the macrophage in B-cell activation.

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