INDUCTION OF GUINEA PIG B-CELL LYMPHOKINE SYNTHESIS BY MITOGENIC AND NONMITOGENIC SIGNALS TO Fc, Ig, AND C3 RECEPTORS

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Definition of the differences in mechanisms of activation of subpopulations of T and B lymphocytes is needed to further our understanding of their role in immunological reactions. Sensitized thymic-derived (T) cells when stimulated with specific antigen or nonspecific mitogens produce lymphokines thought to be responsible for the manifestations of cellular immunity (1). One of these mediators is a monocyte (MNL)[1] chemotactic factor (CTX) (2, 3) which has been shown to be produced even in the complete absence of bone marrow-derived (B) cells (4). However, B cells have also been shown to produce various lymphokines (references 5–7, and footnote 2) including MNL CTX. We have therefore used this very sensitive indicator of lymphocyte activation, namely production of MNL CTX, to study the mechanism of activation of B cells. The major distinction between the response of these two classes of lymphocytes is that B lymphocytes could not be activated by the same stimulants as T cells to produce MNL CTX (8), suggesting that although the activators for each of these populations are unique they result in the release of the same mediator. Furthermore, this implies that selective activation of T or B cells depends upon the nature and localization of the stimulus. With the recent development of techniques to isolate relatively pure populations of T and B cells, we have further investigated various stimulants which activate purified B lymphocytes to proliferate and produce MNL CTX. Recent reports have suggested that only B-cell mitogens can activate polyclonal antibody synthesis by B cells and that this activation is not dependent upon cross-linking nor does it involve the immunoglobulin receptors (9). In contrast, we find that the signal for stimulating B cells to produce MNL CTX need not be mitogenic and appears to be dependent upon a cross-linking phenomenon. Furthermore, not only is the immunoglobulin

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[1] Abbreviations used in this paper: Agg Ig, aggregated γ-globulin; Con A, concanavalin A; CTX, chemotactic factor; DNP-HSA, DNP human serum albumin; DNP-OA, DNP ovalbumin; E, erythrocytes; [³H]TdR, tritiated thymidine; LPS, lipopolysaccharide; MNL, monocyte; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; POL, polymerized flagellin.

[2] Mackler, B. F., L. C. Altman, D. L. Rosenstreich, and J. J. Oppenheim. 1974. Induction of lymphokine production by EAC and of blastogenesis by soluble mitogens during human B-cell activation. Nature (Lond.). 249:834.

receptor involved in such B-cell activation, but several other receptors described on the cell surface appear to have functional significance in B-cell activation as well.

Materials and Methods

Animals and Immunization. Male Hartley guinea pigs (400–500 g) were immunized with foot pad injections of 50 μg dinitrophenylated ovalbumin (DNP$_{14}$-OA) in complete Freund's adjuvant (H37Ra, Difco Laboratories, Detroit, Mich.) as previously described (3). The cells used in these studies were all from immunized animals, unless otherwise specified, and obtained 2–4 wk postimmunization.

Cell Collection and Preparation. Spleens were excised from immunized animals and carefully minced in RPMI 1640 (Grand Island Biological Co., Grand Island, N. Y.) to obtain a single cell suspension. Contaminating red cells were lysed with isotonic ammonium chloride (10) and the washed cells passed through sterile gauze to remove debris. The cells were resuspended in serum-free RPMI 1640 containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine.

Guinea pig T lymphocytes spontaneously rosette with rabbit erythrocytes (E) (11, 12) and by repeated (two times) removal of the rosettes (T cells), a relatively pure B-cell population could be isolated. Spleen cells prepared as described above (50–100 × 10^6) were incubated in 10 ml RPMI 1640 with 1 × 10^9 fresh, washed rabbit E. After a 15-min incubation at 37°C, the cells were centrifuged 5 min at 200 g and without disturbing the pellet, placed on ice (4°C) for 60 min. The pellets were then gently resuspended and layered on Ficoll-Hypaque gradients (13) which were centrifuged at 800 g for 30 min at 4–6°C. The nonrosetting cells at the gradient interface were harvested, washed several times, and again incubated with rabbit E at 37°C for 15 min, centrifuged, and placed on ice for 60 min. After separation on a second Ficoll-Hypaque gradient the nonrosetting cells were collected, washed three times, and pooled. The E-rosette-forming cells (T) were also pooled, washed, and the red cells lysed. In addition, enriched B-cell populations were obtained off nylon wool columns by the method of Julius et al. (14), and also by recovering EAC-rosetted lymphocytes (15).

Identification of B Lymphocytes. A sample (0.1 ml) of the isolated B cells was incubated at 37°C with an equivalent volume of RPMI 1640 containing 1 × 10^9/ml sheep E sensitized with the 19S fraction of rabbit antisheep hemolysin (1:200, Cordis Laboratories, Miami, Fla.) and guinea pig complement (C) (1:150, Texas Biological, Ft. Worth, Texas) (5). The percentage of these cells forming EAC rosettes was determined by counting in a hemocytometer.

Fluorescein-conjugated goat antiguinea pig Ig (Cappel Laboratories, Downingtown, Pa.) was dissolved in a 0.1% sodium azide solution before use. The antiserum was periodically ultracentrifuged (100,000 g, 60 min) to remove aggregates. A sample (0.1 ml) containing 1 × 10^6 cells was incubated at 4°C with 100 μl of the fluoresceinated antibody for 30 min. After three washes at 4°C, the cells were mounted in suspension on glass slides with Vaseline-sealed coverslips and viewed in a fluorescence microscope. Lymphocytes were first viewed under bright-field illumination and the same field then examined by ultraviolet light so that the percentage of fluorescence-positive cells could be determined.

MNL CTX Production. 1 × 10^6 cells of the isolated cell populations or whole spleen cells were cultured in 1-dram glass vials in 1 ml serum-free media containing antibiotics. After 48-h incubation at 37°C in 5% CO$_2$ in humidified air, the cultures were centrifuged (1,000 g, 15 min) and the cell-free supernates assayed for chemotactic activity. Appropriate amounts of antigens, mitogens, or other test materials were added at the end of culture to reconstitute the control cultures which had been incubated in the absence of such stimulants.

Chemotactic Assay. The culture supernates were assayed for chemotactic activity in modified Boyden chambers as previously described (3). Briefly, nonimmune guinea pig peritoneal exudate cells induced with 1% shellfish glycogen 4 days earlier were placed on the top of a 5 μm polycarbonate filter (Nuclepore Inc., Wallabs, San Rafael, Calif.) and the substance to be tested on the opposite side of the filter in the lower chamber. After 90-min incubation in humidified air at 37°C, the filters were removed from the chambers, fixed, stained, and quantitated. Chemotactic activity is defined as the mean number of macrophages which have migrated through the filter in each of 20 oil immersion fields in triplicate filters ± 1 SE.

Determination of Lymphocyte Proliferation. Parallel lymphocyte cultures to those described for

CTX production were established in duplicate and pulsed with 1 μCi tritiated thymidine ([³H]TdR) (sp act 6.0 Ci/mmol, Schwarz/Mann Div., Becton, Dickinson & Co., Orangeburg, N. Y.) 4 h before harvesting for determination of DNA synthesis. The cultures were processed with a modified automatic harvester as previously described (16) and the counts per 10⁶ cells determined in a Packard Scintillation counter (Packard Instrument Co., Downers Grove, Ill.).

Mitogens and Antigens. Antigens used in these experiments included DNP-OA and purified protein derivative of tuberculin (Connaught Medical Research Labs, Toronto, Canada) which were added at 10 μg/ml. Mitogens included concanavalin A (Con A, Calbiochem, San Diego, Calif.), phytohemagglutinin (PHA, Burroughs-Welcome & Co. Inc., Research Triangle Park, N.C.), lipopoly-saccharide (LPS) from *Escherichia coli* (055:B5, Difco Laboratories), and the lipid A fraction of LPS obtained by acid hydrolysis (17) of *Salmonella typhimurium* LPS (Difco Laboratories). Polymerized flagellin (POL) was prepared from *Salmonella adelaide* as described by Nossal and Ada (18). DNP conjugates of POL were synthesized using dinitroflorobenzene as coupling agent (19).

Antisera. The Ig fraction of rabbit antiguinea pig Ig was obtained from Cappel Laboratories. The antiserum was initially absorbed with guinea pig thymocytes before use. Since this absorption did not influence the activity, this procedure was discontinued. In certain experiments the anti-Ig was passed over an immunoabsorbant consisting of Sepharose beads that had been conjugated with DEAE-cellu-lose purified guinea pig Ig (20) to remove specific antiguinea pig Ig antibody. Fab fragments were prepared by papain digestion of this rabbit antiserum (21). The undigested components were sepa-rated from the digested frgments by passage through an Amicon X-100 filter (Amicon Corp., Lex-ington, Mass.) which did not allow passage of molecules greater than 100,000 mol wt. Completeness of digestion was assayed by immunoelectrophoresis.

The Ig fraction of a goat antirabbit Ig obtained from Cappel Laboratories was adsorbed with Sepharose beads conjugated with DEAE-cellulose purified guinea pig Ig to remove any cross-reacting antibodies.

Antigen-Antibody Complexes. The γ_1-fraction of guinea pig antiserum to dinitrophenylated bovine gamma globulin was obtained from Dr. A. L. Sandberg (NIH, Bethesda, Md.). Immune com-plexes of this antibody and dinitrophenlyated human serum albumin (DNP-HSA) were formed at equivalence in the presence of EDTA, washed, and resuspended in saline containing 0.1% sodium azide.

Aggregated γ-globulin (Agg Ig). The 7S fraction of guinea pig γ-globulin (Schwarz/Mann Div., Becton, Dickinson & Co., Rockville, Md.) was dissolved in phosphate buffered saline (PBS) at 50 mg/ml, and heated at 63°C for 15 min (22, 23). The aggregates were suspended in PBS and centrifuged at 1000 g for 30 min. The supernate containing the aggregates was stored until used.

Results

Isolation and Activation of T and B Lymphocytes to Produce MNL CTX. In initial experiments it was evident that LPS could induce cultures of unfrac-tionated spleen cells to produce a lymphokine with the ability to attract homologous monocytes. Therefore, we separated the spleen cells into populations of T and B lymphocytes. T lymphocytes activated by the specific antigen DNP-OA, and the T-cell mitogens PHA and Con A, produced MNL CTX, but did not respond to LPS (Table I). In order to investigate whether B cells could produce this lymphokine, we tried various means of isolating highly purified populations of B cells. Initially we tried procedures based on the presence of a C receptor on B cells (15) which allows them to bind EAC making it possible to separate them from other cells. However, cells obtained by this method when placed in culture consistently generated high levels of chemotactic activity even in the absence of exogenous stimulants (Table II).

The second method of obtaining B cells utilized a modification of the nylon wool column isolation of mouse lymphoid populations described by Julius et al.

TABLE I

Activation of T Lymphocytes to Produce Monocyte Chemotactic Factor

Stimulant	Chemotactic activity*
DNP-OA‡	48 ± 1
Con A	64 ± 2
PHA	50 ± 1
LPS	9 ± 2
None	8 ± 2

* Chemotactic activity is expressed as the mean number of migrating macrophages per oil immersion field of triplicate samples ±1 SE.

‡ Nonadherent T lymphocytes were eluted from nylon wool columns and cultured at 1×10^6/ml for 48 h with 10 µg/ml DNP-OA, 5 µg/ml Con A, 1 µg/ml PHA, or 100 µg/ml LPS. The supernates were then tested for chemotactic activity. Activation of sensitized T cells by DNP-OA required that macrophages (1×10^5) be added to the purified cells.

TABLE II

Nonspecific Activation of B Lymphocytes by Separation Techniques to Produce MNL CTX

Treatment	Chemotactic activity
EAC rosettes*	37 ± 3
Nylon column‡	40 ± 3
Negative selection§	6 ± 3
DNP-OA	3 ± 1
Con A	6 ± 1
LPS	66 ± 3

* B cells were obtained by forming rosettes with EAC and fractionation by centrifugation on Ficoll-Hypaque gradients. E were lysed and the B cells cultured without stimulants. 48-h supernates were assayed for MNL CTX.

‡ Spleen cells were fractionated on a nylon wool column and the adherent B cells obtained by this technique incubated without additional stimulants for 48 h. The supernates were then assayed for MNL CTX.

§ The non-E-rosetted fraction (B cells) was obtained by removal of the T cells as described in Materials and Methods. After incubation at 1×10^6 cells/ml for 48 h without stimulants the supernates were assayed for MNL CTX. Additionally, these cells were stimulated with 10 µg/ml DNP-OA, 5 µg/ml Con A, or 100 µg/ml LPS and the 48-h supernates assayed for chemotactic activity.

(14). Again, although >90% of the lymphocytes stained with fluoresceinated Ig, they also produced significant amounts of chemotactic activity without further stimulation (Table II). Consequently, we sought to isolate B cells with minimal manipulation by selectively removing the T cells twice by rosetting them with rabbit E, centrifugation on Ficoll-Hypaque, and then utilizing the nonrosetting

lymphocytes. After the first exclusion of E rosettes, the number of fluorescein-positive cells increased from 35–40% in unseparated spleen to 60–65% in the nonrosetted fraction. Repetition of T-cell removal increased the purity of the remaining nonrosetted cells to greater than 95% Ig-positive cells. In addition to being relatively pure, these B cells were not activated during the separation and had low background levels of chemotactic activity in their culture supernates (Table II). Subsequent experiments utilized cells obtained by this procedure. When incubated with LPS, these B cells readily produced MNL CTX (Table II), however, they were not stimulated to produce mediator by the specific antigen DNP-OA nor by the T-cell mitogen, Con A (Table II).

Activation of B lymphocytes to Proliferate and Produce MNL CTX.   Because LPS was such a potent mitogen and stimulator of MNL CTX production by B cells, it was of interest to determine whether other molecules with B-cell mitogenic properties might similarly induce mediator synthesis. The mitogenic component of LPS, lipid A, (24, 25) could similarly activate B cells (Table III). Moreover, POL, a T-independent antigen with multivalent determinants, also induced the appearance of significant MNL CTX. Although significant mediator synthesis was still detected at doses of POL lower than those required for the induction of measurable blastogenesis, a direct correlation between the ability of POL and the other mitogens to initiate B-cell proliferation and B-cell mediator production was demonstrable (Fig. 1). DNP-POL was also found to induce both proliferation and mediator production by B cells from unimmunized guinea pigs (Table III). This suggests that the cells were activated via the mitogenic POL component of the conjugate rather than by a preferential binding of the DNP-POL to DNP specific cells. Furthermore, the lack of response of sensitized

TABLE III

Activation of Guinea Pig B Lymphocytes by Mitogens to Proliferate and to Produce a Chemotactic Lymphokine

Stimulation*	[³H]TdR incorporation (E/C)‡	Chemotactic activity§
LPS	2.07	84 ± 7
Lipid A	14.49	83 ± 1
POL	11.59	88 ± 2
DNP-POL	4.25	83 ± 3
None	1.00	9 ± 3

* 1 × 10⁶ B lymphocytes/ml were cultured with optimal doses (10 µg/ml) of LPS, lipid A, POL, or DNP-POL in serum-free medium for 48 h. The supernates were then tested for chemotactic activity.

‡ Parallel cultures incubated for 48 h were pulsed with [³H]TdR 4 h before harvest and assayed for [³H]TdR incorporation. Ratio of mean cpm of experimental to control cultures is shown. The cpm of replicate cultures varied by less than 15% from the mean and more than twofold differences are significant.

§ Chemotactic activity is expressed as the mean number of migrating macrophages per oil immersion field of triplicate samples ± 1 SE.
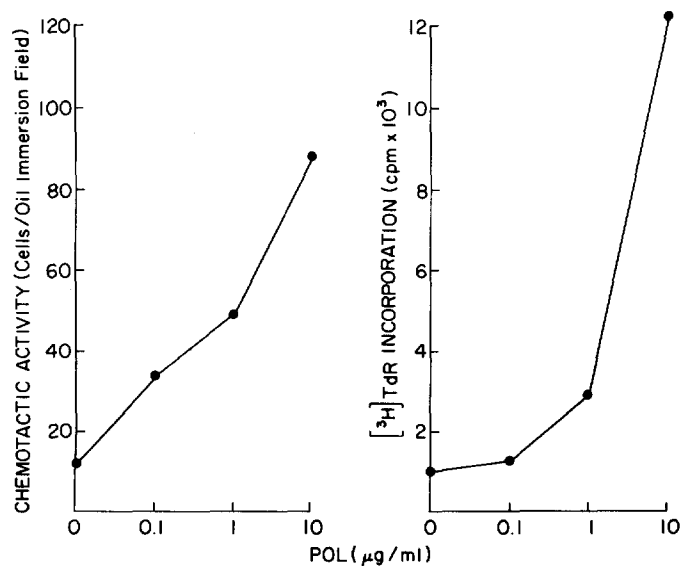
FIG. 1. Purified B lymphocytes were stimulated with POL in the indicated concentrations and the supernates assayed after 48 h for chemotactic activity. In parallel cultures 1 μCi [³H]TdR was added 4 h before harvest and the amount of [³H]TdR incorporated/10⁶ cells determined (cpm).

B cells to DNP-OA (Table II) also suggests that the binding of DNP to specific B-cell membrane Ig receptors is not an adequate stimulus for triggering proliferation and mediator synthesis.

B-Cell Activation by Nonmitogenic Interaction of EAC with the C Receptor. In our preliminary attempts to isolate B cells we observed that B cells which had been separated by rosette formation with EAC had significant levels of MNL CTX in their supernates even in the absence of any added stimulants. Therefore, we tested more directly whether the interaction of the EAC with the C receptor on B-cell surfaces was instrumental in triggering these cells to produce MNL CTX. The addition of these antigen-antibody-C complexes, namely EAC, to cultures of B cells from unsensitized guinea pigs which were separated by negative selection did indeed initiate MNL CTX (Table IV). In contrast, the antigen, E, alone or antigen coated with 19S antibody (EA) were unable to activate the B cells suggesting that modified C3 was involved in this type of activation. EAC failed to stimulate T cells (Table V). Whereas B-cell mitogens such as LPS and POL initiated both proliferation and mediator synthesis, the interaction of EAC with B cells although triggering MNL CTX production did not induce significant B-cell proliferation (Table IV) after 48-h incubation.

B-Cell Activation at the Fc Receptor. Activation of the B cells to synthesize a chemotactic lymphokine via their C3 receptors suggested that other receptors on the B-cell membrane might provide additional sites of activation. Antigen-antibody complexes which are known to interact with the B-cell Fc receptor (26, 27) were therefore formed with DNP-HSA and anti-DNP antibodies. These complexes were added in varying concentrations to the B cells in serum-free media and the culture supernates assayed for the presence of MNL CTX. As seen

in Table VI the preformed complexes were effective in activating B cells to produce MNL CTX, but did not initiate significant B-cell proliferation. The antigen or antibody by themselves were ineffective in triggering B cells to synthesize MNL CTX or to proliferate. Activation of the B cells through the Fc receptor by antigen-antibody complexes occurred both in immunized and nonimmunized animals.

Agg Ig also has been shown to bind specifically to the Fc receptor (22). Significant CTX mediator was also detected in cultures incubated with up to

TABLE IV

Activation of B Lymphocyte Proliferation and Production of MNL CTX by EAC Binding to the C3 Receptor

Stimulant*	[³H]TdR incorporation (E/C)‡	Chemotactic activity
None	1.00	6 ± 4
E	1.12	8 ± 2
EA	1.35	9 ± 2
EAC	1.80	59 ± 2

* Representative experiment in which 1×10^6 cells were incubated for 48 h with media alone, 1×10^8 sheep E, E coated with 19S antihemolysin (EA), or EA incubated with guinea pig C (EAC) and the supernates assayed for chemotactic activity.

‡ Parallel cultures incubated for 48 h were pulsed with [³H]TdR 4 h before harvest and assayed for [³H]TdR incorporation. Ratio of mean cpm of experimental to control cultures is shown (E/C).

TABLE V

Treatment of Purified Guinea Pig T Lymphocytes with B-Cell Stimulants

Stimulant*	[³H]TdR incorporation (E/C)	Chemotactic activity
PHA	17.06‡	55 ± 3§
POL	≤1	8 ± 2
EAC	≤1	10 ± 3
Agg Ig	≤1	9 ± 2
Anti-Ig	≤1	6 ± 1
None	1.00	5 ± 1

* Each stimulant was added to the purified T cells over a large dose range and the doses of LPS (10 µg/ml), POL (1 µg/ml), anti-Ig (1:100), and Agg Ig (1:20) giving maximum [³H]TdR incorporation and chemotactic activity are presented.

‡ Cultures were pulsed with 1 µCi [³H]TdR 4 h before harvest and assayed for thymidine incorporation. Ratio of mean cpm of experimental to control cultures is shown.

§ Chemotactic activity is expressed as the mean number of migrating macrophages per oil immersion field of triplicate samples ±1 SE.

1:200 dilutions of such aggregates (Table VI). In contrast, purified T cells were not activated by antigen-antibody complexes or Agg Ig (Table V).

Immunoglobulin Receptor as a Site of B-Cell Activation. Exposure of isolated B lymphocytes to divalent antiguinea pig Ig which binds to B-cell Ig receptors resulted in triggering of the cells to release MNL CTX. This activation occurred over a wide concentration (Table VII). Anti-Ig seldom induced a significant degree of proliferation of the purified B cells over the dose range used

TABLE VI

Immune Complex and Aggregated Gamma Globulin Activation of B Cell Production of MNL CTX

Stimulant	[^{3}H]TdR incorporation (E/C)	Chemotactic activity
DNP-HSA anti-DNP complexes*		
0.1 μg/ml	0.85‡	13 ± 2
1.0 μg/ml	1.24	21 ± 2
10.0 μg/ml	1.80	47 ± 4
20.0 μg/ml	1.93	58 ± 1
DNP-HSA		
10 μg/ml	1.07	3 ± 2
20 μg/ml	1.15	12 ± 4
Anti-DNP antibody		
10 μg/ml	1.37	4 ± 1
20 μg/ml	2.00	12 ± 4
Agg Ig§		
1:10	0.95	56 ± 3
1:50	0.85	44 ± 2
1:100	1.10	43 ± 5
1:200	0.87	31 ± 3

*Preformed immune complexes or the antigen or antibody alone were added at the indicated concentrations to 1 × 10⁶ B lymphocytes from DNP-OA-sensitized or -nonsensitized guinea pigs. Supernates were assayed 48 h later for chemotactic activity.

‡ Parallel cultures incubated for 48 h were pulsed with [^{3}H]TdR 4 h before harvest and assayed for [^{3}H]TdR incorporation. Ratio of mean cpm of experimental to control cultures is shown (E/C). The cpm of duplicate cultures varied by less than 15% from the mean and more than twofold differences are significant.

§ Heat Agg Ig prepared as described in Materials and Methods was added to the B cells as indicated.

in these studies. The specificity of this reaction was shown by removing the specific antiguinea pig Ig antibodies from the Ig fraction. This absorbed material no longer activated the cells whereas the specific antibody eluted from the Ig-Sepharose column did activate the cells. Normal rabbit Ig added to cultures failed to activate B lymphocytes. Addition of anti-Ig to purified T cells induced no mediator production nor proliferative response (Table V). In order to determine whether cross-linking was in fact required for activating B lympho-

TABLE VII

Anti-Immunoglobulin Activation of B Lymphocytes to Produce MNL CTX

Stimulant	[³H]TdR incorporation (E/C)	Chemotactic activity
Rabbit antiguinea pig Ig*		
1:50	0.81‡	89 ± 5
1:100	0.65	60 ± 3
1:200	0.99	35 ± 2
1:400	1.05	37 ± 2
1:800	0.97	16 ± 3
Normal rabbit Ig		
1:50	—	4 ± 1
1:100	1.03	3 ± 1
1:200	1.18	5 ± 2
1:400	0.94	5 ± 1
1:800	1.00	5 ± 2
None	1.00	9 ± 1

* Representative experiment in which rabbit antiguinea pig Ig was diluted in RPMI 1640 media and added in the indicated concentrations to 1 × 10⁶ B lymphocytes/ml. Ig from rabbits not sensitized to guinea pig Ig was added to additional cultures of B lymphocytes. 48-h supernates were assayed for chemotactic activity which is represented as the mean number of migrating macrophages per oil immersion field ±1 SE.

‡ Parallel cultures received 1 μCi [³H]TdR 4 h before harvest to determine [³H]TdR incorporation. Ratio of mean cpm of experimental to control cultures is shown.

cytes, we prepared Fab monomers from the anti-Ig which bind to but do not bridge the Ig receptors. When B cells were incubated with the Fab fragments, no detectable mediator synthesis was obtained (Table VIII). However, addition of an anti-Fab antiserum previously absorbed with guinea pig Ig to B cells that have bound Fab monomers initiated MNL CTX production (Table VIII). The anti-Fab by itself failed to activate B cells.

Discussion

Activation of B lymphocytes to produce a chemotactic lymphokine appears to occur subsequent to interactions with a variety of membrane-associated receptors. Receptors that have been detected on B-cell membranes include receptors which bind the Fc region of Ig molecules providing a site for adherence of antibody molecules, antigen-antibody complexes (26, 27), or Agg Ig (22); surface Ig receptors for specific antigens (28); and receptors for the C3 component of C (29, 30). While Ig receptors bear specific binding sites for antigen which can lead to induction of antibody synthesis (31), the functional significance of the other

TABLE VIII

Receptor Cross-Linking as a Stimulus for Chemotactic Lymphokine Synthesis

Treatment	Chemotactic activity
Rabbit antiguinea pig Ig*	
1:100	59 ± 6
Rabbit Fab antiguinea pig Ig	
1:100	17 ± 3
Rabbit Fab antiguinea pig Ig (1:50)‡	
+ goat antirabbit Ig	
1:50	38 ± 1
1:100	56 ± 3
1:200	50 ± 3
Goat antirabbit Ig	
1:50	13 ± 3
1:100	21 ± 2
1:200	10 ± 2

* Rabbit antiguinea pig Ig or its Fab fragments were added to 1×10^6 B cells/ml and the 48-h supernates assayed for chemotactic activity.

‡ Goat antirabbit Ig adsorbed with intact guinea pig Ig was added to B cells exposed to Fab fragments of antiguinea pig Ig. The goat antirabbit Ig was also added alone to B-cell cultures. After 48-h incubation, the supernates were assayed for chemotactic activity.

receptors is less clear. The initial reaction of a variety of molecules with the lymphocyte takes place via binding to these cell surface receptors. However, binding of stimulants to the receptors although necessary may not be sufficient for B-cell activation as measured by lymphokine synthesis, antibody synthesis, and/or proliferation. The mitogens, Con A and PHA, which are T-cell specific bind to B cells (32, 33) yet do not activate them to make MNL CTX. Rather it has been suggested that membrane activation and consequent cellular responses must involve configurational changes in receptor molecules (34). Evidence that conformational changes in the cell receptors may trigger mediator synthesis was initially obtained with B-cell mitogens and T-independent antigens. LPS, lipid A, and POL are polymers with multiple side chains which bind simultaneously to multiple sites on the B-cell membrane. These molecules induce mediator synthesis implying that the molecular structure of the mitogens is important and that it is the polymeric structure with repeating units which is essential for activation.

The induction of MNL CTX can also involve the C receptor as shown by EAC activation. This involved a direct interaction of modified C3 with the C3 receptor as a primary stimulus. In fact it has been suggested that all mitogenic activation of B cells is totally dependent on C components (35). However, our findings indicate that activation at C3 receptors may not even lead to proliferation. Furthermore, our data suggest that the C components presumably play no role in activation of B cells by soluble immune complexes, Agg Ig or anti-Ig because cell

cultures were maintained in the absence of serum eliminating any exogenous source of C. Although C synthesis or release by cells present in the culture is not excluded, it is unlikely that activation of B cells via the Ig or Fc receptors to produce MNL CTX required the interaction of activated C3 with the C3 receptor. Our data does not conflict with the possibility that a second signal provided by bound C3 may be a requirement for antibody synthesis (35) at least for T-dependent antigens (36). Although the suggestion that B cells can be activated at the C3 site (35) is supported by some of our data, there are many other pathways that achieve the same results.

The Fc and C3 receptors have been postulated to be responsible for antigen trapping or localization in lymphoid tissue and may thus indirectly be involved in regulating antibody synthesis. The binding of complexes to these receptors may coincidently initiate chemotactic mediator synthesis and recruit macrophages to the site which can in turn, by participating in T-cell triggering (37) enhance the immune response. Furthermore, it has been suggested that the Fc receptor is important in antibody-mediated target cell destruction by B cells (38) and in fact it has been recently shown that blockade of the Fc receptor with Agg Ig does indeed impair antibody-mediated B-cell target killing (39). Our data indicate that the interaction of antibody-coated target cells with the Fc receptor would elicit macrophages to migrate to such a site through production of MNL CTX. Moreover, since antibody is essential for activation of B cells by immune complexes, this suggests that B cells are involved in effector mechanisms primarily in secondary or hyperimmune responses.

When cells were exposed to divalent anti-Ig the B cells were activated via the Ig receptors to synthesize MNL CTX. This is in accord with previous reports that anti-Ig molecules could activate unfractionated lymphocytes in the rabbit (40) and chicken (41, 42) to proliferate. However, in mouse, humans, and guinea pigs lymphocytes proliferate only minimally when exposed to anti-Ig (43). The ability of an intact anti-Ig antibody to bind and activate lymphocytes and the failure of the monovalent Fab fragments from the same source to do so suggested that receptor cross-linking was instrumental in triggering the B cells. In addition, when bivalent antibody directed to the Fab was added to the B cells coated with Fab, mediator synthesis was again triggered. Lymphocyte activation by anti-Ig, antigen-antibody complexes, and Agg Ig is probably dependent upon subsequent cross-linking or bridging of the surface receptors. A similar requirement for cross-linking has also been described for the induction of the proliferative response of rabbit lymphocytes (44, 45) and for the induction of interferon production (46). It thus appears that interaction of appropriate molecules with the Fc, C3, or Ig receptors (Fig. 2) on the B-cell surface is a prerequisite for lymphocyte activation as measured by lymphokine production. A basic pattern emerges underlying the triggering of these cells to produce mediators which focuses on the ability of the interacting agents to connect adjacent receptors. This receptor linking then proceeds by some as yet unknown mechanism to lymphocyte activation.

Another point of interest is the lack of correlation between mediator synthesis and proliferation. While a similar dichotomy has been noted for T cells (4, 47) it is not clear whether different subpopulations of B cells participate in mediator
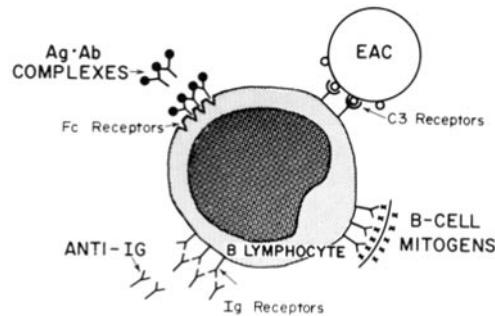
FIG. 2. Pathways for activating B cells to produce a chemotactic lymphokine. Interaction of appropriate molecules with the Ig receptors, C3 receptors, or the Fc receptors on the cell surface results in triggering of the cell providing that a minimal degree of cross-linging of receptors occurs. Molecules which can interact with the appropriate receptors to facilitate receptor bridging include Agg Ig or antigen-antibody complexes, antigen-antibody-C complexes (EAC), anti-Ig, or T-independent antigens (B-cell mitogens).

synthesis and proliferation or whether the stimulus for mediator synthesis requires a lower threshold than that for proliferation. The crucial first step in B-cell activation by antigens (48) and B-cell mitogens (49) appears to be the cross-linking of surface receptors by polymeric molecules. Possibly, stimulation of mediator synthesis may require a lesser number of cross-links of membrane receptors than proliferation. B-cell mitogens may provide their own second signal by direct interaction with the B cell, whereas antigen-antibody complexes or antigen-antibody-C complexes may be unable to achieve this additional signal. In any case our observations suggest that the concept that mitogens nonspecifically activate B cells must be enlarged in scope to include the fact that nonmitogenic stimulants can also activate B cells to make the lymphokine MNL CTX.

Summary

This study shows that bone marrow-derived lymphocytes of guinea pigs if appropriately activated produce a monocyte chemotactic factor (MNL CTX). Activation of B lymphocytes to produce a chemotactic lymphokine occurs subsequent to interactions with a variety of membrane-associated receptors. Polymeric B-cell mitogens with multiple binding sites, polymerized flagellin and lipopolysaccharide, initiated mediator synthesis. Furthermore, interaction of antigen-antibody complexes or aggregated gamma globulin with the Fc receptor and binding of antigen-antibody-complement complexes at the C3 receptor can effectively facilitate mediator production in the absence of a significant proliferative response. Additionally, intact anti-immunoglobulin but not its Fab fragments activated the B cells. An anti-Fab effectively converted the inactive Fab-bound B cells into producers of MNL CTX, suggesting that the basic mechanism of activation depended upon cross-linking of receptors. Thus, interaction of B-cell surface receptors such as Fc, Ig, and C3 sites with mitogenic

as well as nonmitogenic molecules capable of bridging the receptors appears to trigger B-cell mediator production.

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