CONTROL OF B-LYMPHOCYTE FUNCTION

I. Inactivation of Mitogenesis by Interactions with Surface Immunoglobulin and Fc-Receptor Molecules

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The role of surface immunoglobulin (Ig) in triggering B-lymphocyte responses is not yet clear. For several years it has been known that anti-Ig antibodies can interfere with the activation of B cells by mitogens (1–5). Whether this block is external, interfering with the mitogenic receptor, or internal, affecting deeper features of the cells' metabolism, is not yet established.

Anti-Ig antibodies have a particularly deleterious effect on the immature B lymphocyte (6), suggesting that surface Ig may control the state of differentiation and activation of the B cell. First, immature B cells do not re-express their surface Ig molecules after capping and clearance with anti-Ig antibodies even though they do so after the removal of surface Ig from the membrane by enzymes (7). Second, after interaction with anti-Ig antibodies, immature B cells can no longer respond mitogenically to lipopolysaccharide (LPS)[1] (7). If surface Ig molecules are required for the action of mitogens, the immature B cells' lack of surface Ig re-expression could explain their insensitivity to LPS after anti-Ig treatment. Alternatively, anti-Ig treatment could have changed the activation potential of the immature B cells in some other way.

The present studies were undertaken in hopes of elucidating the nature and mechanism of the anti-Ig-induced blockage of mitogen responsiveness. We have designed experiments to examine the action of LPS on adult mouse B cells in the few hours after exposure to anti-Ig antibodies when surface Ig has been capped and cleared but before new surface Ig is expressed (8). This paper reports that rather than merely being the initial target of mitogen action, surface Ig molecules in cooperation with Fc receptors are fundamentally involved in controlling the internal state of activation of the B lymphocyte.

Materials and Methods

Cells. Adult (6-wk to 6-mo old) male and female C57BL/6 mice were used, either raised in our own facilities or purchased from The Jackson Laboratories, Bar Harbor, Maine. Their spleens were removed and teased into the standard medium of Hanks' balanced salt solution (BSS) (Microbiological Associates, Inc., Bethesda, Md.) plus 1% *N*-2-hydroxyethylpiperazone-*N*-2-ethane sulfonic acid (HEPES) (Microbiological Associates) plus 5% fetal calf serum (FCS) (Associ-

[1] *Abbreviations used in this paper:* ALG, rabbit anti-mouse lymphocyte antibodies; BSS, balanced salt solution; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; GAMG, goat anti-mouse Ig; GARG, goat anti-rabbit Ig; HGG, human IgG; LPS, lipopolysaccharide; NWSM, nocardia water-soluble mitogen; PPD, purified protein derivative; RAMG, rabbit anti-mouse Ig; RGG, rabbit IgG.

ated Biomedic Systems, Inc., Buffalo, N. Y.). The cells were washed three times before the experimental manipulations.

Immunoglobulins. The following antibodies were used: (*a*) rabbit anti-mouse Ig (RAMG), a polyvalent antibody obtained by repeatedly immunizing rabbits with isolated mouse Ig (9); (*b*) goat anti-mouse Ig (GAMG), obtained similarly to (*a*); (*c*) goat anti-rabbit Ig (GARG), obtained similarly to (*a*); rabbit IgG (RGG) was obtained from pooled normal rabbit serum; (*d*) rabbit anti-mouse lymphocyte antibodies (ALG), obtained by immunizing rabbits with two intravenous injections of mouse thymocytes (10); (*e*) antibodies to mouse histocompatibility antigens (anti-H-2) obtained by immunizing DBA/2 mice with C57BL/6 spleen cells (10). The serum contained high titers of antibodies to the specific $H\text{-}2^b$ haplotype.

The IgG was isolated from all of these antisera by ammonium sulfate fractionation and then a DEAE column (for rabbit antisera) or a preparative starch block electrophoresis. $F(ab')_2$ fragments of RAMG, GAMG, and GARG were prepared by digestion with pepsin (Worthington Biochemical Corp., Freehold, N. J.) and purified on Sephadex G-200 (11). They were tested for purity by Ouchterlony gel diffusion analysis. $F(ab')_2$ reagents were considered pure when antisera against the appropriate gamma globulin gave a spur with the undigested reagent which ran directly towards the well containing the digested reagent. Fab fragments of RAMG were prepared by papain (Worthington Biochemical) digestion and purified by carboxymethylcellulose (11). Finally, human IgG (HGG), obtained from Pentex Biochemical, Kankakee, Ill., was aggregated by heating for 1 h at 63°C.

Reagents. The following reagents were used: sodium azide (Matheson, Coleman, & Bell, Rutherford, N. J.); lipopolysaccharide-boivin, from *Escherichia coli* 0111:B4 (Difco Laboratories, Detroit, Mich.); nocardia water-soluble mitogen (NWSM) (12) obtained from Doctors Bona and Chedid of the Pasteur Institute in Paris, France; purified protein derivative (PPD) (Connaught Medical Research Laboratory, Toronto, Canada); trypsin (TR-TPCK, Worthington Biochemical); pronase (protease from *Streptomyces griseus*, Type VI) (Sigma Chemical Company, St. Louis, Mo.); and RGG and HGG (Pentex Biochemical).

Basic Experimental Procedure. The basic experimental design consisted of incubating cells at a concentration of 10^7/ml in standard medium or an antibody solution at 37°C for 1 h. (Unless otherwise stated, this initial incubation in anti-Ig was done with 200 μg per ml of RAMG.) The cells were washed three times at room temperature and resuspended in standard medium not containing any mitogen or containing 25 μg per ml of LPS (or optimal concentrations of other B-cell mitogens). After 1 h at 37°C, the cells were again washed three times at room temperature, counted, and cultured in triplicate in 12 × 75-mm plastic tissue culture tubes (Falcon Plastics, Oxnard, Calif.). Each tube contained 10^6 cells in 1.0 ml of RPMI-1640 with penicillin and streptomycin (Associated Biomedic Systems) plus 5% FCS and 2 mM L-glutamine. The tubes were incubated in a humidified 5% CO_2 atmosphere at 37°C. After 24 h, 1 μCi of [^{3}H]thymidine (2 Ci per mmol, New England Nuclear, Boston, Mass.) in 50 μl of RPMI-1640 was added. The incorporation of [^{3}H]thymidine into trichloroacetic acid-insoluble material was determined 24 h later. All figures represent average results of at least three different experiments.

Two-Step Antibody Treatments. For experiments in which cells were incubated successively in two separate antibodies before being incubated with a mitogen, the first antibody incubation was done at 4°C for 30 min. The cells were then washed three times in cold medium, and the second antibody solution was added. The mixture was then brought to 37°C for 1 h for the second antibody incubation. Further washes and manipulations were done as in the basic procedure described above. This procedure was used to prevent the cell from redistributing or clearing the first ligand before it was incubated in the second.

Fluorescence Studies. All antibodies used were tested by fluorescence for binding to spleen cells. The following were directly fluoresceinated and tested in a one-step reaction: RAMG, RAMG $F(ab')_2$, RAMG Fab, GAMG, GAMG $F(ab')_2$, aggregated HGG, and RGG-anti-RGG (both fluoresceinated). Two-step sandwich reactions were also used to detect the binding of unlabeled RAMG, RAMG $F(ab')_2$, RAMG Fab, and ALG (FITC-GARG used as second layer) and anti-H-2 antibodies (FITC-RAMG used as second layer). For these tests, aliquots of cells at a concentration of 10^7/ml were incubated with the first antibody (10–100 μg/ml) for 30 min at 4°C and then washed three times at 4°C. If necessary, a second incubation was done with a fluorescent anti-antibody (50–100 μg/ml) for 30 min at 4°C. After three final washes at 4°C, the cells were fixed by the addition of equal parts medium and 2% paraformaldehyde. After 15 min at room temperature, the cells

were washed once more and examined in a Leitz Ortholux fluorescent microscope. All anti-Ig antibodies, as well as heat-aggregated human gamma globulin (agg-HGG) and RGG-anti-RGG complexes, label 40–45% of spleen cells. Anti-H-2 and ALG antibodies, tested in a sandwich reaction, label 95–100% of the spleen cells.

To test the capping behavior of anti-Ig antibodies and their fragments, cells were incubated at 4°C for 30 min with 50 μg/ml of directly fluoresceinated antibody. After three washes at 4°C, the cells were warmed to 37°C for 10 min. They were then fixed with paraformaldehyde as above, washed, and scored for percentage capped. Intact and $F(ab')_2$ anti-Ig antibodies capped at least 75% of the stained cells, while Fab fragments capped less than 10%.

Enzymatic Removal of Surface Ig. Cells at a concentration of 10^7 per ml were washed once in BSS and HEPES and then exposed to 1.5 mg per ml pronase in BSS plus HEPES for 60 min at 37°C. They were then washed three times at room temperature in the standard medium. By fluorescence, this procedure reduced the percentage of spleen cells staining with FITC-RAMG from approximately 50 to 5%. The remaining 5% detectable were stained extremely faintly, unlike control cells which were very brightly stained.

Results

Phenomenon of Anti-Ig-Induced Inactivation. To study the relationship between surface Ig-anti-Ig interactions and the B-cell mitogenic response, we used a system in which cells were exposed to LPS in free solution for only a brief period of time. Spleen cells incubated with 25 μg per ml of LPS for 1 h at 37°C responded with 15,000–20,000 cpm of [^{3}H]thymidine incorporated into DNA. This response was maximal when assayed at 48 h and represented about one-quarter of the response of cells cultured continuously in LPS.

In agreement with others, we have found that anti-Ig inhibited the mitogenic response to LPS. Fig. 1 shows that the LPS response of cells pretreated with anti-Ig for 1 h was much less than that of control cells. Note also that the background (actually the basal mitogenic response to FCS) was similarly reduced in the anti-Ig-treated cells. Fig. 2 shows that the response to three other B-cell stimulators (two bacterial products – NWSM and PPD, and one protease – trypsin) was similarly reduced by the anti-Ig pretreatment. Thus, the action of all B-cell stimulators tested was similarly inhibited by anti-Ig. For the rest of this report, the response to LPS is considered as a model for all B-cell mitogens.

Kinetic experiments were then performed to determine how long lasting is the state of inactivation produced by anti-Ig and under what circumstances can it be produced. First, it was apparent that the inactivation by anti-Ig persisted in cells examined up to 1 wk (data not shown). Thus the inactivation was not accounted for by a simple shift in the kinetics of proliferation. Also, it was seen for pulses of LPS ranging in concentrations from 0.1 to 1,000 μg per ml (data also not shown). In the experiments shown in Fig. 3, the interval between the anti-Ig and LPS incubations was varied. The results show that the state of inactivation was quite long lasting, not being significantly reversed by 36 h after the anti-Ig treatment and still evident 48 h afterwards. In contrast, the B cells' surface Ig is essentially completely re-expressed by 12 h after the anti-Ig treatment (8).

In addition to pretreating cells with anti-Ig, the LPS mitogenic response was equally reduced by treating cells with anti-Ig after the LPS incubation (Table I). We then checked to see how long after the LPS pulse anti-Ig could be inhibitory. Fig. 4 shows that as the interval between the LPS and anti-Ig treatments was lengthened, the inhibitory effects of anti-Ig became less evident. Anti-Ig added

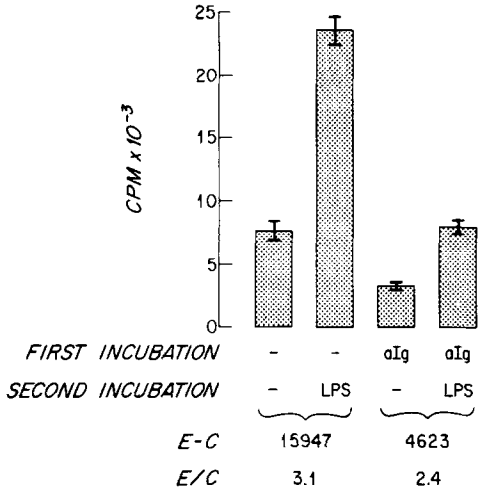

FIG. 1. Anti-Ig inhibition of basal proliferation and of LPS-stimulated mitogenesis. The basic experimental design is as detailed in Materials and Methods. In this and all subsequent figures and tables, values reported represent the combined means ± standard error of the mean from several individual experiments. E, experimental (LPS stimulated); C, control (non-LPS stimulated).

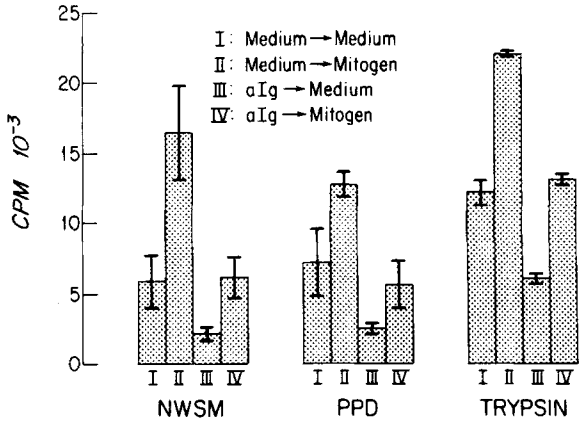

FIG. 2. Anti-Ig inhibition of basal and stimulated mitogenesis. These experiments were done as in Fig. 1, except that other B mitogens were used instead of LPS. NWSM, nocardia water-soluble mitogen (25 μg per ml); PPD, purified protein derivative (100 μg per ml); trypsin (100 μg per ml).

in the middle of the [^{3}H]thymidine exposure still partially suppressed the incorporation of the radioactive precursor, however.

Mechanism of Inactivation. A role for surface Ig molecules in LPS triggering has been proposed as a basis for the anti-Ig-induced inactivation of LPS mitogenesis (13). The simple removal from the membrane of the surface Ig molecules themselves does not appear to be the basis for this inactivation, however. Table I shows that stripping the cells of surface Ig by using the enzyme pronase did not block the subsequent stimulation by LPS.

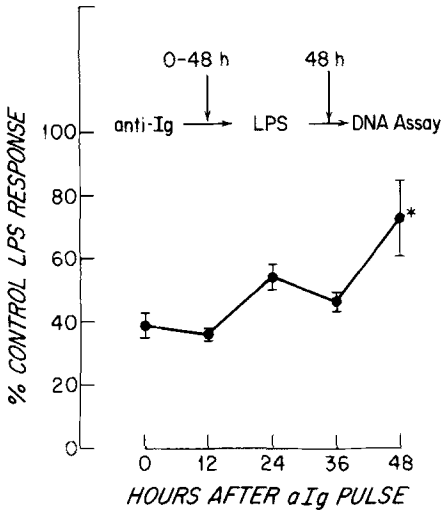

FIG. 3. Kinetics of recovery from anti-Ig-produced inhibition of mitogenesis. After a 1-h incubation with anti-Ig, cells were incubated up to 48 h before being pulsed with LPS for 1 h. They were then put into culture, pulsed with [^{3}H]thymidine 24 h later, and harvested 24 h later. The response of the anti-Ig-treated cells is represented as a percentage of the response of non-anti-Ig-treated cells manipulated in parallel as controls. (In this and Fig. 4 points with a star represent results of one experiment only.)

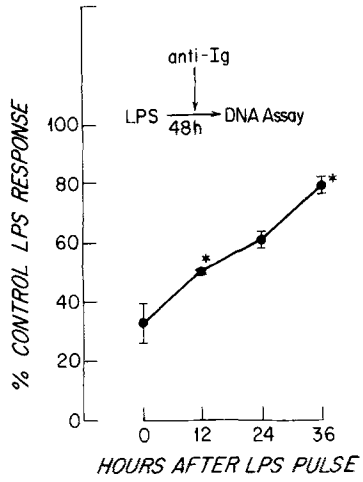

FIG. 4. Inhibition by exposing cells to anti-Ig after a pulse of LPS. After a 1-h incubation with LPS, cells were put into culture, pulsed with [^{3}H]thymidine 24 h later, and harvested 24 h later as described in Materials and Methods. 0, 12, or 24 h (i.e., immediately before the addition of [^{3}H]thymidine) after the LPS incubation, cell aliquots were incubated with anti-Ig for 1 h (cell concentrations were corrected to 10^6 per ml) and replaced in culture. For the 36 h point anti-Ig (for a final concentration of 200 μg per ml) was simply added to the tubes already containing [^{3}H]thymidine. The response of the anti-Ig-treated cells is reported as a percentage of the response of cells manipulated in parallel but exposed to medium not containing anti-Ig.

TABLE I
Relationship of Mitogenic Inhibition to Presence or Absence of Surface Ig

First incubation	Second incubation	Mitogenic response (cpm per 10^6 cells)
Medium	Medium	8,773 ± 1,769
Medium	LPS	25,082 ± 2,611
Anti-Ig	Medium	2,981 ± 583
Anti-Ig	LPS	7,664 ± 1,210
LPS	Medium	20,183 ± 5,632
LPS	Anti-Ig	5,905 ± 709
Pronase	Medium	6,308 ± 1,400
Pronase	LPS	19,965 ± 3,433

We next tested several other ligands for their effect on B-cell mitogenesis (Fig. 5). Of these, the most inhibitory was ALG which, at high concentrations, was almost as inhibitory as anti-Ig, although it was significantly less effective at lower concentrations. Immunoglobulin containing antibodies to the major histocompatibility antigens (anti-H-2) yielded a weak inhibition of borderline significance at high concentrations. Finally, neither normal rabbit gamma globulin (RGG, not shown), immune complexes (RGG-anti-RGG), nor agg-HGG gave any inhibition at any concentration tested. Thus, the inhibition of mitogenesis is clearly not achieved by any ligand binding to the cell surface, but is restricted to only some.

We then examined the contribution of the Fc portion of anti-Ig and the effects of cross-linking in this inactivation. Fig. 6 shows that, of various anti-Ig antibody fragments, intact anti-Ig molecules were by far the most inhibitory. $F(ab')_2$ fragments were weakly inhibitory, but about 20 times less so than intact molecules on a molar basis. No inhibition was seen with Fab fragments at any concentration tested.

The possible role of the classical sequence of patching, capping, and endocytosis of surface Ig-anti-Ig complexes in the inactivation process was then examined. Since in Fig. 6 it was shown that $F(ab')_2$ fragments of anti-Ig were much less effective inactivators than intact molecules, even though they capped and cleared the surface Ig equally as well (11; and confirmed in these experiments), capping of surface Ig alone could not be sufficient for this inactivation. Fig. 7 confirms that anti-Ig Fab and $F(ab')_2$ fragments (of RAMG) were not inhibitory by themselves. However, when cells that had been incubated with these antibody fragments at 4°C and washed were then incubated at 37°C with doses of GARG known to produce redistribution of (RAMG) Fab anti-Ig, the mitogenic response was deeply inhibited. In two other experiments (not shown), cells incubated first with anti-Ig (RAMG) $F(ab')_2$ were then incubated with intact or $F(ab')_2$ fragments of GARG. The result was that $F(ab')_2$ GARG fragments produced about 50% of the inhibition as compared to the effect of undigested GARG.

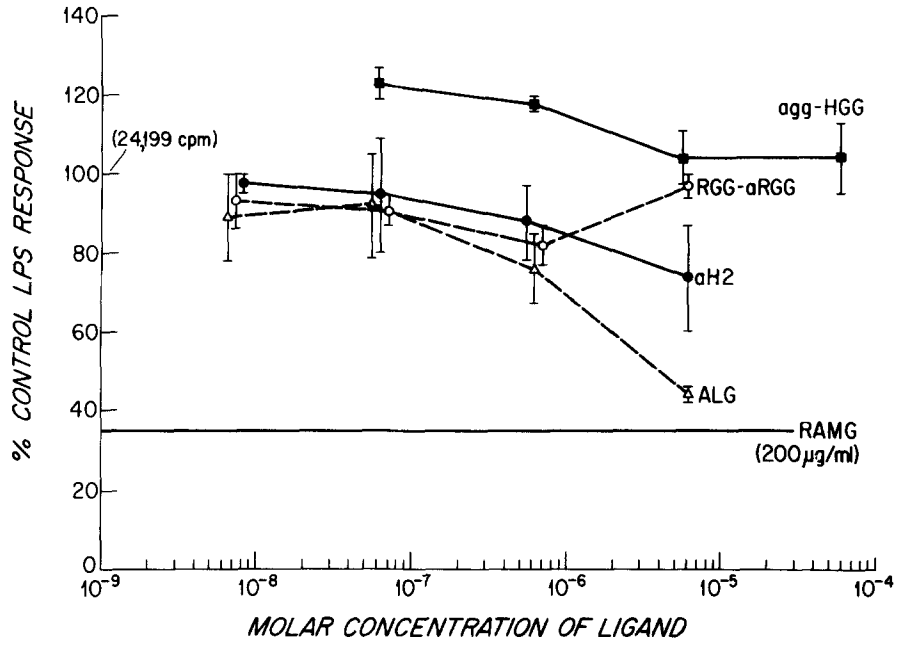

FIG. 5. Inhibition of mitogenesis by other ligands. Cells were pretreated with these ligands before an LPS pulse. ALG and anti-H-2 globulin were used at 1, 10, 100, and 1,000 μg per ml. Aggregated HGG was used at 10, 100, 1,000, and 10,000 μg per ml. Anti-RGG (GARG) (0.5, 5, 50, and 500 μg per ml) was combined with RGG at the optimum ratio for fluorescent staining of cells (one-tenth of the anti-RGG concentration, or 0.05, 0.5, 5, and 50 μg per ml. This approximates molar equivalence if one assumes that 1 out of 10 antibody molecules in the GARG preparation is specific anti-RGG). The mitogenic responses are expressed as a percentage of the LPS-stimulated mitogenic response of cells pretreated only with standard medium. The mol wt used for gamma globulins was 150,000 daltons.

Since capping of surface Ig was not sufficient to produce the inhibition of DNA synthesis, we next asked if the classical capping and clearing sequence was necessary to produce it. To do this, we let anti-Ig interact with cells in normal medium or in the presence of sodium azide, a reversible metabolic inhibitor which prevents capping and endocytosis of surface complexes (14). At the end of the incubation with anti-Ig, we stripped the cells of surface Ig-anti-Ig complexes by incubation with the proteolytic enzyme pronase (also in the presence of azide). Thus, the cells had interacted with anti-Ig in conditions that either did or did not allow capping and interiorization.

Fig. 8 presents the results of these experiments. Control cells first treated with azide and then with pronase were slightly inhibited. Cells treated with anti-Ig and then with pronase were as deeply inhibited as those treated with anti-Ig, but without the pronase step. When the cells were incubated with anti-Ig in the presence of azide and then exposed to pronase to remove the complexes before they could cap and endocytose, the inhibition observed was significantly deeper than that produced by anti-Ig alone. Capping and endocytosis of surface Ig-anti-Ig complexes was, therefore, neither sufficient nor even necessary to produce inhibition. In fact, preventing this normal sequence resulted in a deeper and more profound inhibition.

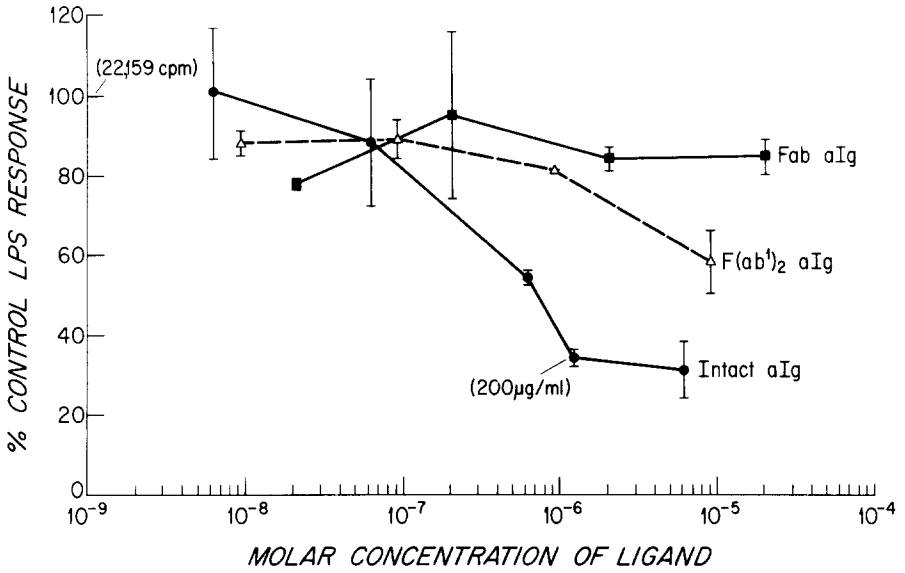

FIG. 6. Inhibition of mitogenesis by anti-Ig fragments. Before an LPS pulse, cells were pretreated with $F(ab')_2$ and Fab anti-Ig molecules (of RAMG and GAMG) at 1, 10, 100, and 1,000 μg per ml. Mol wts of 150,000, 106,000, and 56,000 daltons were used, respectively, to calculate the molar concentrations. The mitogenic responses are expressed as percentages as in Fig. 5.

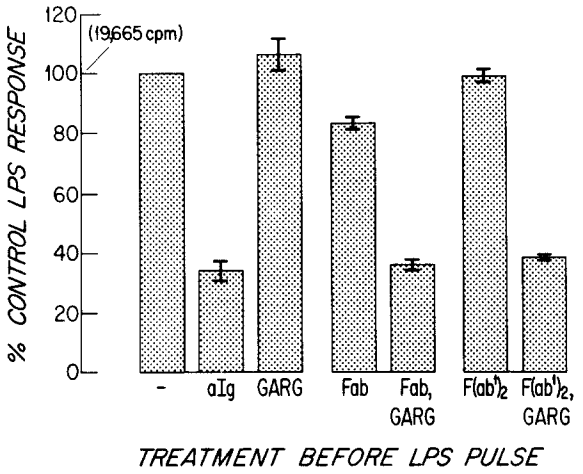

FIG. 7. Inhibition of mitogenesis by capped anti-Ig fragments. Except for anti-Ig (200 μg per ml), all reagents were used at 100 μg per ml. As described in Materials and Methods, the first of two preincubations was done at 4°C. The mitogenic responses are expressed as percentages as in Fig. 5.

These results suggest that an antibody Fc end, rather than capping and endocytosis of surface Ig-anti-Ig complexes, facilitates the anti-Ig-induced inhibition of B-cell mitogenesis. Our data present several arguments that surface Ig and Fc receptor interactions together result in optimal inhibition. First, in Figs. 5 and 6, it was shown that neither anti-Ig fragments lacking the Fc end, immune

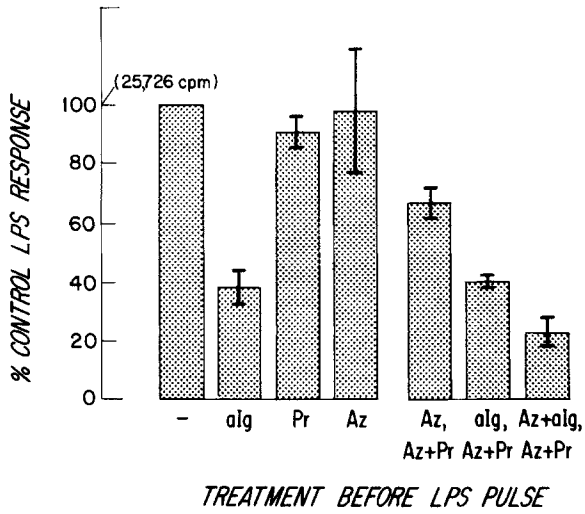

FIG. 8. Role of capping and endocytosis in anti-Ig inhibition of mitogenesis. Pr, pronase (1.5 mg per ml in BSS plus HEPES); Az, sodium azide (2×10^{-2} M in standard medium); Az + Pr (1.5 mg per ml pronase plus 2×10^{-2} M azide in BSS and HEPES); Az + aIg (2.5×10^{-2} M azide in standard medium for 15 min at 37°C then 200 μg per ml RAMG plus 2×10^{-2} M azide). All incubations shown were for 60 min at 37°C. Before any incubation with pronase, the cells were washed once in the same medium lacking pronase (i.e., BSS plus HEPES ± azide). Before the LPS incubation, all cells were washed twice, purified on Ficoll-Hypaque gradients, and washed three times more. Mitogenic responses are expressed as percentages, as in Fig. 5.

complexes, nor aggregated gamma globulins produced a very good inhibition by themselves. Secondly, the second (sandwich) layer of GARG antibody only yields a deep inhibition if it is added to the proper first layer of (anti-Ig) antibody. Fig. 9 shows that the same concentration of GARG which yielded the deep inhibition with anti-Ig Fab or $F(ab')_2$ (Fig. 7) did not give any additional inhibition with a moderately inhibitory dose of ALG as the first layer, even though by fluorescence it appeared that at least as much GARG had bound to ALG as to the anti-Ig fragments. (The moderately inhibitory dose of ALG was chosen because it should have very sensitively revealed any additional effect of the second layer of GARG.)

Finally, in two experiments (not shown), we have incubated cells simultaneously with anti-Ig $F(ab')_2$ antibody fragments and soluble immune complexes. No inhibition due to cooperation was observed. Thus, it is possible that both its surface Ig molecules and Fc receptors must be cross-linked or present in the same molecular complex to optimally inactivate the B cell.

Discussion

In the first section of this paper, we have shown that the classical surface and cytoplasmic cycle of binding, patching, capping, endocytosing, and finally resynthesizing and re-expressing surface Ig does not leave the B lymphocyte as it was initially. Even though it has regenerated its receptors and appears to be the same, it is no longer the cell it once was, as evidenced by the fact that it no longer responds well to a short exposure to mitogen. This state of inactivation is

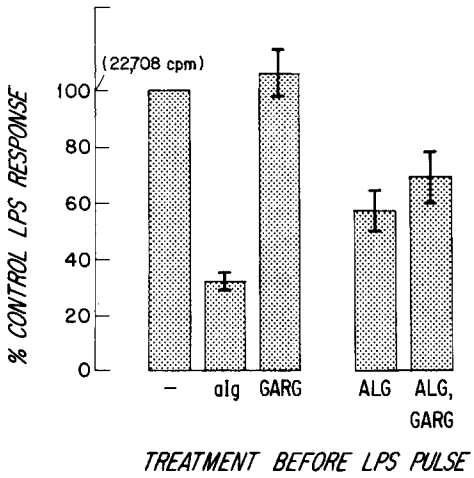

FIG. 9. Role of Fc in inhibition of mitogenesis. See Fig. 7 for experimental details.

quite long lasting, enduring for several days in vitro, beyond which it was not practical to carry out the experiments. In contrast, within 1 h at 37°C, the B lymphocyte completely clears its membrane of surface Ig-anti-Ig complexes and has fully re-expressed them in about 12 h (8).

Given the fact that a single cycle with anti-Ig severely and long lastingly inactivates a B cell, how can one reconcile previously published results that, after 1- or 24-h incubations with anti-Ig, normal adult B cells are still able to respond vigorously to LPS (3, 7)? In those past studies, cells were incubated with LPS continuously after the anti-Ig treatment, whereas here they were only given a limited exposure to LPS. In both protocols, all of the cells that were exposed to LPS had been reacted with anti-Ig and, presumably, inactivated, although a limited number were still able to divide. We suggest that the difference between the two systems occurred at this point, that the few new cells emerging from the first, limited round of division had little or no "memory" of the anti-Ig-induced suppression, and thus were able to proliferate normally or even better upon further LPS exposure, if it was still present to trigger them.

Certain earlier reports concerning the anti-Ig-induced inhibition of the mitogenic response to LPS have suggested that surface Ig molecules may be involved in cell triggering, or even that they are the mitogen receptors themselves (13). Thus the modulation of surface Ig by anti-Ig antibody would make the cells refractory to LPS stimulation. Four reasons make this unlikely, however. First, as has been previously reported (4), anti-Ig can block the LPS response even if given after the LPS exposure. Second, the direct removal of surface Ig by the enzyme pronase does not block the LPS response. Third, removal of surface Ig by anti-Ig $F(ab')_2$ fragments leaves the cell still functional. Fourth, anti-Ig inhibits the mitogenic response to all B-cell stimulators tested, even to the enzyme trypsin (see also 3–5). The fact that the response to different B-cell mitogens is independently genetically controlled, apparently at the membrane receptor level (15), implies that various mitogens have different membrane receptors. Surface Ig molecules could not be identical to a number of different mitogen receptors. For all these reasons, it appears that the anti-Ig-induced inhibition of

the B-cell mitogenic response occurs at some intracellular level and is independent of the presence or absence of surface Ig.

One of the major conclusions of this paper is that the classical B-cell reaction of capping and endocytosing surface Ig-anti-Ig complexes is not necessary for the delivery of the negative signal for mitogenesis. Indeed, the signal is delivered efficiently if capping and endocytosis are prevented. The fact that the inhibition still occurs even when the complexes are enzymatically stripped from the cell without allowing capping and endocytosis to occur at all proves that the negative signals are truly trans-membrane signals. Ligand-receptor complexes do not themselves have to be internalized to signal the cell that the interaction has taken place.

These findings also allow a new interpretation of the B cell's capping and endocytosing response to ligand interactions. In addition to other possible functions, we would like to suggest that this response is the B cell's attempt to rid itself of a membrane perturbation. If signals are delivered to the cell by activated (perhaps cross-linked) receptors on the cell surface, removing these activated receptors by capping and endocytosis might damp down and terminate the signal. Conversely, a ligand-receptor complex that is not readily capped and endocytosed might deliver the most powerful signal. The possibility that positive as well as negative signals are similarly affected (reduced) by the B cell's capping and endocytosing response might partially explain the special immunogenicity of macrophage-bound antigen (16), as well as the distinctly different effects on mouse B cells of the soluble vs. the insoluble forms of anti-Ig (17), antigen (18, 19), immune complexes (20), phytohemagglutinin (21), and concanavalin A (22).

The studies on ligand specificity reported here show that the optimal inactivation occurs with intact anti-Ig antibody or with anti-Ig fragments cross-linked by a second antibody containing an Fc end. (The inactivation with ALG at high doses remains unexplained. ALG is a crude antibody reacting presumably with various surface macromolecules, besides remaining surface bound for long periods of time.) This suggests that the B-cell Fc receptor may serve mainly a negative, or inhibitory, role. While no inhibition was seen with aggregated or antigen-complexed immunoglobulins, others have observed an inhibition of B-cell mitogenesis by insolubilized immune complexes (20). That system differed from the one reported here in that the immune complexes were immobilized onto plastic dishes (and thus were nonendocytosable by the B cells) and were present for the entire mitogen incubation. These factors may have augmented the Fc receptor interaction and allowed it alone to inhibit the mitogenic response in that system. In contrast, we have shown here that Fc receptor interactions delivered alone via soluble molecules are ineffective unless accompanied by surface Ig binding as well. It may be that bringing surface Ig molecules and Fc receptors into close proximity produces the maximum negative effect of the Fc receptors. Two possible mechanisms for this could be the production of an optimal cooperative association of Fc receptors themselves or a true two-molecule association between Fc receptors and surface immunoglobulins along the lines of the hormone receptor for insulin (23). In any case, these data emphasize the requirement for surface Ig binding in producing the optimal (Fc receptor ?)

inactivation. This is presumably the physiological situation occuring with antigen.

Other papers on the suppressive effects of anti-Ig antibody have shown that $F(ab')_2$ fragments were as effective as intact antibodies for the suppression of the PFC response (3) and for antibody production (24). In contrast, we show in our system that on a molar basis $F(ab')_2$ fragments are about 20 times less effective than intact molecules. Two points should be mentioned regarding these differences. First, those systems in which $F(ab')_2$ anti-Ig is equally effective are more complicated than the current one, involving cell differentiation and division, as opposed to just division. It may be that Fc receptor as well as surface Ig interactions are needed to inhibit B cell division, but that surface Ig interactions alone can inhibit certain differentiation processes. Second, even the fact that in our system of mitogenesis certain anti-Ig antibodies without Fc ends can have an inhibitory effect may still be due to an Fc receptor cooperation. It has been shown that the interaction of anti-Ig $F(ab')_2$ fragments with surface Ig molecules induces a degree of association with Fc receptors (11), presumably via interactions with the surface Ig's own Fc ends. Perhaps a strong enough anti-Ig binding, either with enough anti-Ig $F(ab')_2$ or with extra cross-linking such as with GARG $F(ab')_2$, even though without an external Fc end, can inactivate the B cell by using the Fc end of the surface Ig molecules themselves.

A number of facts concerning immunological tolerance are consistent with the two main considerations emphasized here, Fc receptors and capping/clearing behavior. First, it is known that gamma globulins used as antigens or carriers for haptens are among the most effective tolerogens (25). This could be due simply to the fact that they carry an Fc end of their own which can help inactivate specific B cells. Second, immune complexes have been reported to be both suppressive and stimulatory in the immune response (26). To be truly suppressive, the antibody involved has been shown to require an Fc end (27), indeed one that binds to Fc receptors (26). This suppression may, therefore, operate via the process discussed here. Finally, in certain systems of B-cell tolerance, antigens have been shown to remain on the cell surface for several hours before they are completely removed (POL [28]), or to remain essentially forever (D-GL [29]). In contrast, protein antigens are completely eliminated from the cell surface in a matter of minutes (8). Perhaps antigens like POL and D-GL are easily tolerogenic in part because they linger so long on the B-cell surface and at the proper dose are able to deliver a sustained negative signal, possibly utilizing the Fc end of the surface Ig as discussed above.

Our results should now be integrated with the broader field of B-cell triggering in general. In our view, the paramount fact of B-cell activation is that surface Ig molecules play a central role in controlling the cell's functions. This is in direct contrast to the view that surface Ig molecules merely passively "focus" antigens to the cell (30). By themselves, surface Ig interactions are either suppressive (for differentiation? as discussed above) or are stimulatory in a limited fashion, for mitogenesis only. (Rabbit B cells are mitogenically stimulated by soluble anti-Ig [31], as are mouse B cells by insolubilized anti-Ig [17].) As shown here, when combined with Fc receptor interactions, the surface Ig interaction is strongly inhibitory for mitogenesis. On the other hand, when

accessory cell activities are properly provided, the surface Ig interaction allows differentiation to secreting plasma cells (32). Thus, the end result of interactions with surface Ig molecules depends critically on the modulating influences of other cells and molecules.

Only a hint is yet possible of the temporal and sequential relationships of these various B-cell states. For activation to secreting PFC, accessory cell factors are optimally provided 24 h after anti-Ig (32), perhaps because the cells have developed receptors for these factors (33). During this sequence, enzymatic changes are then seen within hours (34) which indicate that the B cell has embarked on the differentiative pathway which, in several more days, will yield a PFC response. The data in this report have shown that the inactivated state brought about by a single cycle of anti-Ig antibodies is of long duration and is not indicated by any lack of surface Ig molecules. But is this cell permanently disabled or merely incompletely activated? B cells "tolerized" by D-GL (35) and SIII (36) are rescued and indeed activated to produce PFC by the proper action of T cells. It is possible that, for at least some period of time, B cells inactivated by the process described here are susceptible to rescue and further activation by accessory cell influences. The relationship of this inactivation process and state to the activation pathway is currently under investigation.

Summary

Mouse spleen cells were incubated with anti-Ig antibodies for 1 h, washed, exposed to LPS for 1 h, washed, and their DNA synthetic responses assayed 2 days later. It was shown that the 1-h incubation with anti-Ig antibodies produced a profound, internal, and long lasting state of inactivation in the B cells. Experiments with anti-Ig fragments and other ligands showed that the inactivation occurred optimally when both surface Ig molecules and Fc receptors were bound simultaneously. The role of the classical capping and clearing cycle was also investigated. It was shown that capping and clearing were neither necessary nor sufficient for the inactivation to occur, and that the signals, but only secondarily the ligands themselves, were transmitted across the membrane. Capping and clearing were viewed as a natural regulatory mechanism by which the B cell attempts to clear its membrane of perturbations and signals from the exterior.

Received for publication 24 May 1976.

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