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Immunology

Control of proliferation and differentiation in B lymphocytes by anti-Ig antibodies and a serum-derived cofactor

(lymphocyte proliferation/serum factors/B lymphocyte function)

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ABSTRACT **The effects of various anti-Ig antibodies on different B lymphocyte functions were investigated. With the proper accessory cofactor(s) derived from serum, anti-IgM antibodies induced a vigorous proliferative response in normal adult murine B cells, while polyspecific anti-Ig and anti-IgD had no effect. Without the required cofactor, all three anti-Ig antibodies were inhibitory for mitogenic responses. All three anti-Ig antibodies were also inhibitory for mitogen-induced antibody responses with or without the cofactor. Even with the required cofactor, neonatal B cells as well as adult C3H/HeJ B cells were not triggered into proliferation by anti-IgM. Finally, the cofactor required for anti-IgM-triggered mitogenesis was shown to be generated from serum by 2-mercaptoethanol and to be approximately 65,000 in molecular weight. These results indicate that, for at least some responses, B lymphocyte surface IgM molecules are involved in both triggering and suppression, depending both on the developmental state of the B cell and the presence or absence of accessory influences. In these experiments, IgD gave evidence only of being suppressive.**

Although the surface Ig molecules of B lymphocytes are known to be the B cells' antigen receptors, the precise role that these molecules play in controlling B cell functions is not well established. Thus, much work has been devoted to investigating the results of interactions of anti-Ig antibodies with surface Ig molecules. In some species (most strikingly, the rabbit), anti-Ig antibodies by themselves induce extensive cell proliferation (1) and, when applied in the proper sequence with supernatants from other cells, also lead to B cell differentiation into Ig-secreting plasma cells (2). With most other species, however, and in particular with the mouse, these effects so far have not been observed. The overall result of work with murine cells is that anti-Ig antibodies generally have suppressive effects on development (3, 4), proliferation (5–9), and differentiation to plasma cells (10, 11). In only two rather specialized cases have anti-Ig antibodies been shown, up to now, to induce proliferation in murine B cells: one with anti-Ig antibodies bound to beads (12) and the other with soluble anti-IgM and cells from aged mice (13).*

This report shows that soluble anti-Ig antibodies, with the proper accessory cofactor(s), can both trigger and inactivate normal adult murine B lymphocytes. Moreover, the various potential responses of B cells are under distinctly different control mechanisms and are influenced differently by interactions with various classes of surface Ig molecules (IgM versus IgD).

MATERIALS AND METHODS

Mice. Mice of the C57L/J and C3H/HeJ strains were bought from the Jackson Laboratory, Bar Harbor, ME, while C57BL/6 mice were from our own breeding facility. Unless otherwise stated, C57L/J mice were used, and in most experiments the mice were used between 2 and 6 mo of age. Spleens and thymi were removed and teased into the standard medium of Hanks' balanced salt solution (HBSS) (Microbiological Assoc., Bethesda, MD) containing 2 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (Hepes) (Microbiological Assoc.) plus 5% fetal calf serum (FCS) (Associated Biomedic Systems, Buffalo, NY).

Cultures. For assays of proliferation, duplicate cultures were set up in round-bottom microtiter plates (Linbro Scientific Co., Hamden, CT) in a total volume of 200 μl per well. Each well contained 2 or 5×10^5 responding cells (spleen cells unless otherwise stated) and various concentrations of antibodies as described in the text and figure legends. [An inoculum of 2×10^5 cells was determined to be optimal for cultures containing 2-mercaptoethanol (see below) and 5×10^5 cells in cultures without mercaptoethanol.] The culture medium was RPMI-1640 (Microbiological Assoc.) containing 10 mM Hepes, 50 μg of penicillin per ml, 50 international units of streptomycin per ml, 2 mM L-glutamine, and FCS and mercaptoethanol as indicated. "Basic" and "enriched" media in the text refer to media containing 5% FCS, or 10% FCS with 50 μM mercaptoethanol, respectively. Mitogenesis was also evaluated using *Escherichia coli* lipopolysaccharide (LPS) 0111:B4 Westphal type (Difco Laboratories, Detroit, MI) at 50 μg/ml. Cultures were incubated in a humidified 5% CO_2 atmosphere at 37°. After 2 days, each well received 0.2 μCi of [^{3}H]thymidine (2 Ci/mmol; New England Nuclear, Boston, MA) in 10 μl of RPMI-1640. After 1 more day, the cultures were harvested on a multiple automated sample harvester (MASH-II; Microbiological Assoc.), and the ^{3}H incorporated into trichloroacetic acid-insoluble material was determined.

The antibody-secreting, plaque-forming cell (PFC) assays were done on cultures similar to the proliferative cultures described above, with the following differences: (*i*) spleen cells (2×10^5) were cultured with 6×10^5 nylon-wool-purified thymocytes as feeder cells; (*ii*) these cultures were incubated for 5 days with or without LPS and then harvested. On the fifth day, the cells in duplicate wells were combined, washed once, and resuspended in 250 μl. The number of direct PFC was determined on duplicate slides of fluorescein-conjugated sheep red blood cells (FITC-SRBC), using 50 μl of the cultured cells per slide. Results were expressed as PFC per culture well.

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Abbreviations: C, complement; FCS, fetal calf serum; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LPS, lipopolysaccharide; M_r, molecular weight; PFC, plaque-forming cell.

* During preparation of this manuscript, Sieckmann *et al.* generously showed us a preprint of their work (14). These authors report findings very similar to those shown here, that anti-IgM mitogenically stimulates young adult murine B cells.

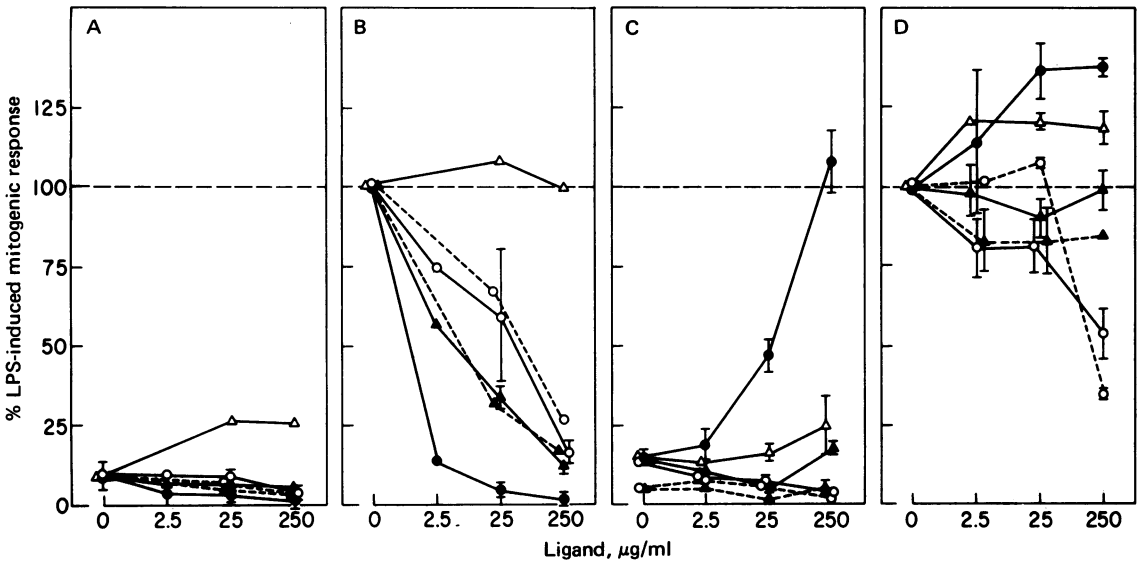

FIG. 1. Effects of anti-Ig antibodies on mitogenesis. (*A*) Inoculum of 5×10^5 cells in basic medium without LPS; (*B*) 5×10^5 cells in basic medium with 50 μg of LPS per ml; (*C*) 2×10^5 cells in enriched medium without LPS; and (*D*) 2×10^5 cells in enriched medium with 50 μg of LPS per ml. The responses are expressed as percentages of the LPS-stimulated mitogenic response of cultures containing no anti-Ig antibodies (4063 cpm in *A* and *B*; 26,370 cpm in *C* and *D*). Error bars indicate SEM. ●—●, C57L/J cells with anti-IgM; ▲—▲, C57L/J cells with anti-Ig; ○—○, C57L/J cells with anti-IgD; △—△, C57L/J cells with normal rabbit IgG; ▲- - -▲, C57BL/6 cells with anti-Ig; and ○- - -○, C57BL/6 cells with anti-IgD.

For both mitogenic and PFC data, the values reported represent the mean plus or minus the standard error of the mean from two to five separate experiments.

Antibodies. IgG fractions were obtained from all antisera by ammonium sulfate fractionation and then DEAE-cellulose columns (for rabbit globulins) or preparative starch block electrophoreses (for mouse globulins). We used:

(*i*) Normal rabbit IgG, prepared from normal rabbit serum.

(*ii*) A polyspecific rabbit anti-mouse Ig (anti-Ig) prepared from rabbits repeatedly immunized with isolated mouse IgG. The main determinants recognized by this antiserum were light chains and IgG heavy chains.

(*iii*) An anti-IgM prepared from rabbits repeatedly immunized with purified MOPC-104E myeloma protein (IgM, λ1). After purification, the IgG was passed over columns of Sepharose-4B coupled to purified myeloma proteins RPC-20 (λ1), MOPC-300 (IgG1, κ), MOPC-195 (IgG2b, κ), and UPC-10 (IgG2a, κ). The resulting antibody reacted only with IgM proteins, as tested by Ouchterlony analysis.

(*iv*) An anti-IgD prepared by the method of Goding *et al.* (15), against allotypic determinants, by repeated immunizations of CBA/J spleen cells intraperitoneally into C57BL/6 recipients. The resulting sera contain anti-H-2^k as well as anti-IgD^a allotypic antibodies. Accordingly, C57L/J mice (H-2^b, Ig^a) were used as the basic mouse strain in these experiments in order to be affected by only the anti-IgD^a and not the anti-H-2^k antibodies. When tested by ^{51}Cr release cytotoxicity with complement, this anti-IgD killed up to 25–35% of C57L/J spleen cells. By sodium dodecyl sulfate gel electrophoresis, it was specific for IgD and did not precipitate IgM.

(*v*) An anti-θ antiserum prepared from AKR mice repeatedly injected with C3H thymi.

RESULTS

Effects of Anti-Ig Antibodies on Proliferation and Differentiation. Initial experiments testing the effects of various anti-Ig antibodies on mitogen-induced proliferation and differentiation were done in RPMI-1640 medium supplemented with 5% FCS and no mercaptoethanol (basic medium). Consistently, all three anti-Igs used (polyspecific, anti-IgM, and anti-IgD) did not stimulate DNA synthesis by themselves (Fig. 1*A*) and, furthermore, inhibited the mitogenic (Fig. 1*B*) and antibody responses to LPS. Anti-IgM was the most inhibitory of the three. Strikingly different results were obtained when the cells were cultured in medium with 10% FCS plus 50 μM mercaptoethanol (enriched medium). In this case, anti-IgM antibodies produced a vigorous proliferative response by themselves (Fig. 1*C*) and slightly enhanced the response to LPS (Fig. 1*D*). Polyspecific anti-Ig and anti-IgD did not cause proliferation (Fig. 1*C*). The small inhibition of the LPS response

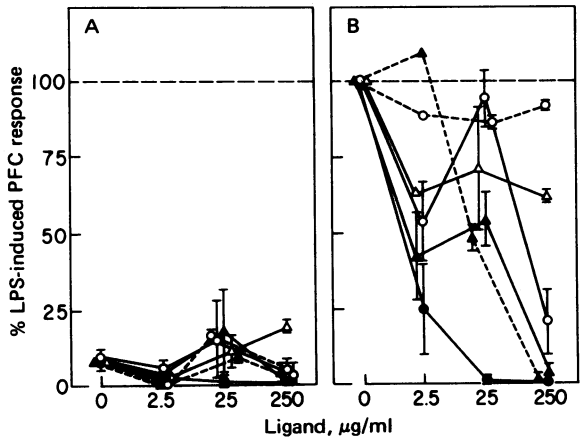

FIG. 2. Effects of anti-Ig antibodies on PFC development. Samples of 2×10^5 cells plus 6×10^5 nylon-wool-purified thymocytes were cultured without (*A*) or with (*B*) 50 μg of LPS per ml in enriched medium. The responses are expressed as percentages of the LPS-stimulated PFC response of cultures containing no anti-Ig antibodies (498 PFC per well). The symbols used are identical to those of Fig. 1.

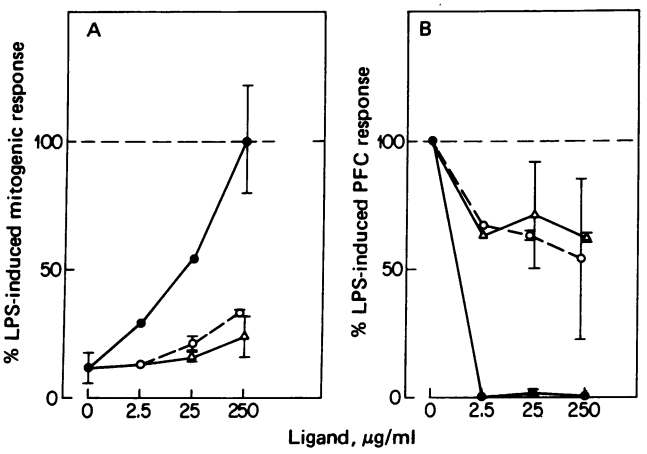

FIG. 3. Absorption of anti-IgM activity. (*A*) Mitogenic response of 2×10^5 cells in enriched medium, as in Fig. 1*C* (100% LPS-induced response = 28,994 cpm) and (*B*) PFC response of 2×10^5 cells plus 6×10^5 nylon-wool-purified thymocytes in enriched medium with 50 μg of LPS per ml, as in Fig. 2*B* (100% LPS-induced response = 413 PFC). ●—●, Anti-IgM; ○- - -○, IgM-Sepharose-4B-depleted anti-IgM; and △—△, normal rabbit IgG.

seen with anti-IgD (Fig. 1*D*) was apparently due to nonspecific effects of mouse globulin, because cells without the proper IgD target allotype (C57BL/6) also experienced the inhibition.

With regards to the PFC response induced by LPS in the enriched culture medium, we observed about 25–30% inhibition with all ligands, including normal rabbit IgG. However, the three anti-Igs were more inhibitory (up to 100%), with anti-IgM being the most active (Fig. 2*B*). Inhibition by anti-IgD was noted at high doses and was specific in that anti-IgD had no effect on the irrelevant target B cell (C57BL/6). None of the antibodies tested induced PFC by themselves (Fig. 2*A*).

In experiments to be reported elsewhere, we mixed polyspecific anti-Ig together with anti-IgM and found the mixture to be as stimulatory for DNA synthesis in enriched medium as anti-IgM alone. Thus, polyspecific anti-Ig was not nonstimulatory because of an inhibitory effect but most likely because it did not bind to a critical site of the surface IgM of B cells.

Specificity of Anti-IgM Effects. In order to be certain that the observed effects of anti-IgM antibody were truly directed at IgM determinants on the spleen cells, the specific anti-IgM antibodies were removed by absorption. Fig. 3 shows that absorption over an IgM-Sepharose column removed both the mitogenic-stimulatory effects and the PFC-inhibitory effects of anti-IgM, tested in enriched medium. In one experiment (data not shown), absorption of anti-IgM against thymus cells had no effect on these activities.

Cellular Target of the Anti-IgM Antibodies. It was expected that the anti-IgM stimulatory effects on proliferation would be exerted on B cells, and direct proof of this was obtained in the experiments shown in Fig. 4. Spleen cells depleted of B or T cells by treatment with anti-Ig and complement (C) or anti-θ and C, respectively, were examined for the proliferative response to various ligands in the enriched medium. Thymus cells did not respond to LPS or to anti-IgM but only to concanavalin A. Spleen cells responded to all three agents. However, after their treatment with anti-Ig and C, the responses to LPS and anti-IgM were reduced, but that to concanavalin A was maintained. On the other hand, treatment with anti-θ and C abolished the concanavalin A response, while it did not affect the LPS and anti-IgM responses. These findings show that it is the B cell which is responding to the anti-IgM. (The incomplete abolition obtained with anti-Ig and C is in accord with the previously observed difficulty in killing 100% of B cells with anti-Ig and C and with the small but measurable response of Ig-negative cells to LPS.)

Response of B Cells from Neonatal Mice and from C3H/HeJ Mice. We have evaluated whether immature B cells, particularly from young mice, which are highly sensitive to tolerence induction (16, 17) and which do not re-express Ig receptors after capping (18), could proliferate in response to anti-IgM antibodies. We also tested the effects of anti-IgM on spleen cells from C3H/HeJ mice, which are relatively unresponsive to LPS (19). First, neonatal mouse spleen cells, which contain only a restricted subset of immature B cells, were not

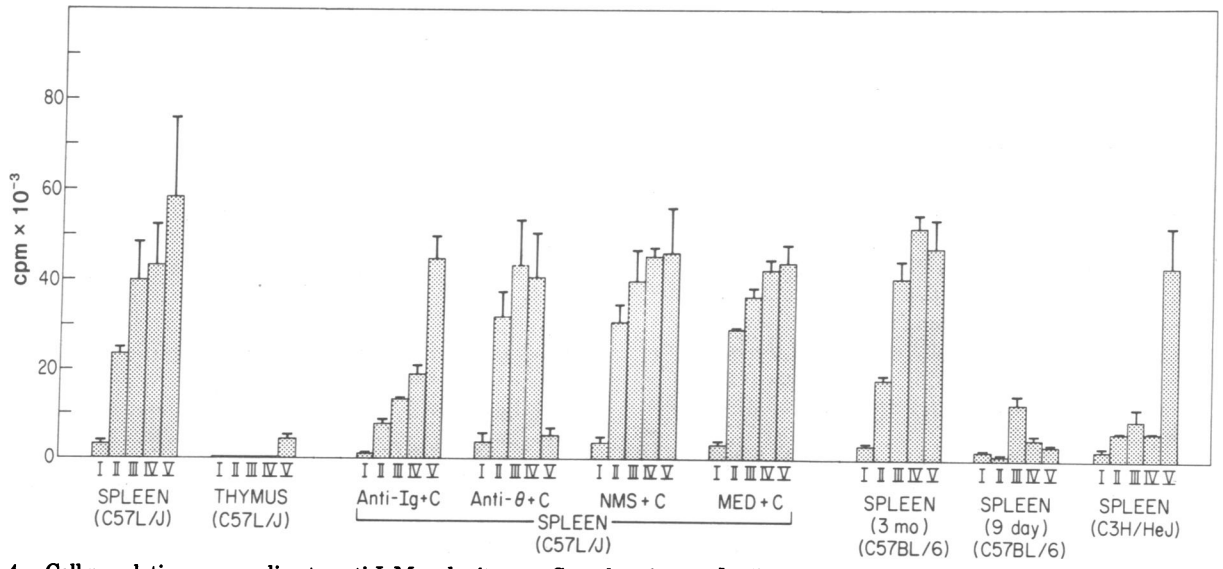

FIG. 4. Cell populations responding to anti-IgM and mitogens. Samples of 2×10^5 cells were cultured in enriched medium containing antibody or mitogen. For purified cell populations, spleen cells were treated with anti-Ig (100 μg/ml), anti-θ (1:10 serum), normal mouse serum (NMS) (1:10), or medium alone for 30 min at 4°, washed twice, resuspended in 1:10 guinea pig serum as a complement source for 45 min at 37°, and washed five times. I, Medium alone; II, anti-IgM (100 μg/ml); III, LPS (50 μg/ml); IV, anti-IgM (100 μg/ml) plus LPS (50 μg/ml); and V, concanavalin A (2 μg/ml).

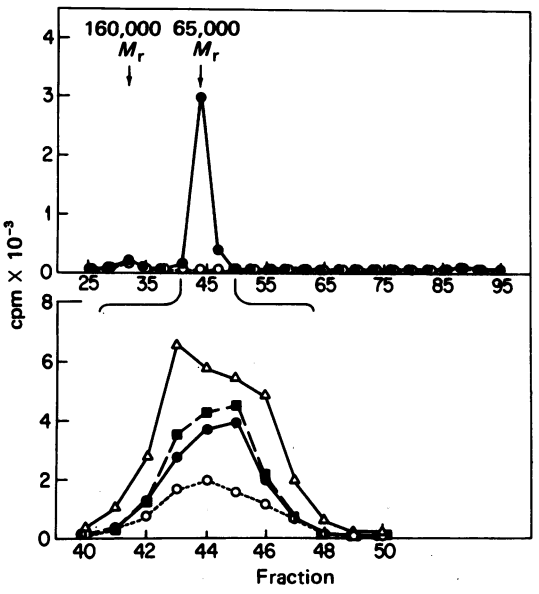

FIG. 5. Fractionation of mercaptoethanol-treated FCS. Three milliliters of FCS was treated with 1 mM mercaptoethanol for 20 hr at 37° and fractionated on a 2.5 × 100 cm Sephadex G-100 column, run at 17 ml/hr and collected in 5-ml fractions. Elution positions of serum globulin (M_r 160,000) and albumin (M_r 65,000) are indicated by arrows. (*Upper*) Fractions (●—●) tested at 25% vol/vol on 2×10^5 spleen cells in basic medium plus anti-IgM (100 μg/ml) and LPS (50 μg/ml). Also shown is a similarly fractionated FCS not treated with mercaptoethanol (○- - -○). In a further experiment (*Lower*), fractions 40 to 50 of the mercaptoethanol-treated FCS from above were reanalyzed at 40% vol/vol in basic medium with various ligands: ○- - -○, medium alone; ●—●, anti-IgM (100 μg/ml); △—△, LPS (50 μg/ml); and ■- - -■, anti-IgM (100 μg/ml) plus LPS (50 μg/ml).

stimulated by anti-IgM and, in fact, were significantly inhibited instead, even though they responded to LPS at least as well as adult cells on a per-B-cell basis. Second, the LPS-unresponsive strain C3H/HeJ also proved to be unresponsive to anti-IgM (Fig. 4).

Mechanisms of the Mercaptoethanol Effect. In experiments to be reported separately, we found that mercaptoethanol without serum had only minimal effects on the mitogenic response to anti-IgM in the optimal culture conditions used here. Thus, in these culture conditions, it is likely that mercaptoethanol acts by generating an active ingredient from an inactive precursor in serum. In fact, the proliferative response to anti-IgM was supported by FCS treated with 1 mM mercaptoethanol and then extensively dialyzed. Also, in other experiments not shown, FCS treated with 0.1 mM mercaptoethanol and dialyzed also supported anti-IgM-induced mitogenesis, as did FCS treated with mercaptoethanol and then with iodoacetamide to block reduced sulfhydryl groups. We have attempted to characterize the active principle by passing mercaptoethanol-treated FCS over a Sephadex G-100 column and testing the fractions. As shown in Fig. 5, the potentiating activity was found in a narrow band coincident with albumin at about 65,000 molecular weight (M_r).

DISCUSSION

The first main result of this paper is that various anti-Ig antibodies can markedly affect the functions of B lymphocytes in either a positive or a negative manner. Thus, the B cell's surface Ig molecules have an active role in controlling B cell activities and do not serve merely to passively focus antigens onto the B cell, as has been postulated by some (20). Here, without a factor generated from serum by mercaptoethanol, anti-IgM and polyspecific anti-Ig antibodies inhibited B cell mitogenesis. Anti-IgD showed no specific inhibitory influence. Second, with or without the mercaptoethanol-generated serum factor, anti-IgM, polyspecific anti-Ig, and anti-IgD were inhibitory for PFC development (in decreasing potency of inhibition). Finally, the mercaptoethanol-generated serum factor changed the anti-Ig effects on mitogenesis in two ways: first, it allowed anti-IgM itself to stimulate vigorous mitogenesis in the absence of LPS; second, it converted the effects of anti-IgM and polyspecific anti-Ig from inhibitory signals for LPS-induced mitogenesis into noninhibitory influences. Thus, in the presence of the mercaptoethanol-generated serum factor, anti-IgM both caused proliferation and inhibited LPS-induced differentiation. This shows the possible multiple effects of a single ligand–receptor interaction and further emphasizes the distinction between the proliferative and differentiative pathways upon which B cells can embark.

Another major area in which these findings have relevance is the current question of the relative roles of surface IgM and IgD in B cell physiology (21). In the studies reported here, IgM mediated both positive and negative signals, depending on the function assayed and the accompanying cofactors, while the only measurable role IgD had was inhibitory. These data are difficult to reconcile with the theory of IgM being the inhibitory receptor and IgD being the stimulatory one (21). An alternative interpretation of the significance of IgD might be that different B cells are preprogramed for different functions independently or by some means other than their IgM and IgD representation. Perhaps IgD is present on a B cell that is preprogramed to be activated into a certain pathway but is not the mechanism by which activation occurs. This possibility is consistent with the data presented here. Neonatal B cells, which have mainly IgM on their surfaces, are not stimulated mitogenically by anti-IgM, while adult B cells are. It thus appears likely that adult B cells, most of which have both IgM and IgD on their surfaces, are responding positively and perhaps preferentially to IgM interactions. Experiments using purified cell populations should be informative.

It should also be noted that this analysis, that adult B cells bearing both IgM and IgD are responding positively to anti-IgM interactions, does not exclude the possibility that these cells might also respond to anti-IgD interactions. No such positive response was seen in these experiments, using an allo-anti-IgD antibody preparation. However, the concentration of anti-IgD needed to specifically inhibit LPS-induced PFC responses was nonspecifically inhibitory for LPS-induced mitogenic responses. This could potentially have masked an otherwise stimulatory effect of anti-IgD on mitogenesis.

The last major points raised by these experiments concern the generation and action of an accessory material induced by mercaptoethanol from a serum precursor and shown here to be a discrete molecular species of about 65,000 M_r. First, in contrast to merely enhancing another proliferative or differentiative stimulus, mercaptoethanol acts here to convert an inhibitory signal (anti-IgM) into a fundamentally opposite stimulatory signal. Second, as in another report concerning the macrophage-replacing effects of mercaptoethanol, the effect on B cell mitogenesis described here can also be mediated by serum exposed to mercaptoethanol and then dialyzed (22). In these experiments, the active component was shown to be coincident with the major serum albumin fraction at approximately 65,000 M_r. However, the paper (22) showing the effects of mercaptoethanol-treated serum on replacing macrophages in a primary *in vitro* response reported (without data) that the

active ingredient was in the range of ovalbumin (45,000 M_r) rather than bovine serum albumin (65,000 M_r). Except for this confusion in apparent sizes, the means of generation and observed effects are comparable between our data and this previous report. An intriguing possibility is that, because the mercaptoethanol-generated FCS factor can act to replace macrophages in some systems (22), perhaps *in vivo* the macrophage provides the physiological analogue to the mercaptoethanol effects reported here, in acting as an accessory influence to modulate B cell function.

Previous reports from other investigators have shown that, in the absence of mercaptoethanol, anti-IgM antibodies can stimulate mitogenesis in aged (over 7 mo) but not in normal adult (6 mo or less) mouse spleen cells (13). It is interesting to note that, in the B cell blasts resulting from stimulated aged spleen cells, Fc receptors no longer co-cap with surface Ig molecules as they do in normal B cells (23). Also, we have previously shown that surface Ig interactions occurring simultaneously with Fc receptor interactions are together the most potent inactivating signal for B cells (9). Perhaps the material derived from FCS by mercaptoethanol acts here to convert inhibitory into stimulatory interactions by interfering with normal Ig–Fc receptor interaction. Such a defective interaction may also be the basis for the easy activation of aged cells.

Finally, could the stimulation observed here with anti-IgM be due to LPS contamination? This is extremely unlikely for three reasons. First, the stimulatory activity was specifically removed by passage over IgM-Sepharose columns. Second, in experiments not shown in this report, $F(ab')_2$ fragments of anti-IgM have been shown to be as stimulatory as intact antibodies. The preparation of these $F(ab')_2$ fragments involves a Sephadex G-100 size fractionation, which should remove most LPS contaminants, which are usually present as hydrophobic aggregates much larger than $F(ab')_2$ fragments. Last, the biological activities of our anti-IgM preparations do not match with those of LPS. Two examples of this are the findings that (*i*) LPS stimulates neonatal B cells but anti-IgM does not; and (*ii*) LPS stimulates the PFC response while anti-IgM inhibits it.

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