

ACTIVATION OF MOUSE LYMPHOCYTES BY ANTI-IMMUNOGLOBULIN

I. Parameters of the Proliferative Response

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Although thymus-independent (B) lymphocytes of mice bear large amounts of membrane immunoglobulin (Ig) (1), it has been difficult to demonstrate directly that membrane Ig is involved in the "signal generation" which leads to proliferation and differentiation of B lymphocytes. One of the chief arguments against membrane Ig playing a role in such "signaling" has been the apparent failure of anti-Ig reagents to cause thymus-independent activation of B lymphocytes (2). Most investigators have failed to detect proliferation of mouse, rat, or guinea pig lymphocytes in response to anti-Ig antiserum (2-4). However, it is known that anti-Ig can initiate proliferative responses in lymphocytes from rabbits (5), chickens (6, 7), pigs (8), and humans (9-12) in at least some cases. Recently, two groups have reported initiation of proliferative responses by mouse B lymphocytes under special circumstances. Parker (13) reported that polyacrylamide beads coated with anti-Ig, but not soluble anti-Ig, caused uptake of ¹²⁵I-deoxyuridine by splenic B lymphocytes. Weiner et al. (14) have recently described stimulation of splenic lymphocytes from mice older than 5 mo of age by anti-Ig antiserum, but have failed to obtain proliferative responses with spleen cells from normal young adult mice. In the Parker system, no synthesis of Ig as a result of anti-Ig stimulation could be detected.

Because of the potential importance of anti-Ig as a probe to explore requirements for B-cell activation, we have reinvestigated this problem using highly purified goat antibodies directed at mouse μ - and κ -determinants. Our results demonstrate that such reagents, particularly anti- μ , are potent mitogenic stimulants for lymphocytes from young adult mice. In this communication we describe the purity of the reagents and the parameters of stimulation. In a forthcoming paper, we will show that this is a T-independent response and that it very likely involves a subset of mature B lymphocytes.

Materials and Methods

Animals. A/J, AKR/J, BALB/cJ, (C57BL/6 \times DBA/2J)F₁ (BDF₁/J), C57BL/6J, C3H/HeJ, CBA/J, and SJL/J mice were obtained from The Jackson Laboratory, Bar Harbor, Maine. CBA/N, (CBA/N \times DBA/2N)F₁, C57BL/10ScCr, and (C57BL/6 \times DBA/2N)F₁ (BDF₁/N) mice were obtained from the Division of Research Services, National Institutes of Health. Mice were used at 2-4 mo of age unless noted otherwise.

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Antisera. G615 antiserum was raised by intramuscular immunization of a goat with 100 μ g MOPC 104E (μ , λ) in complete Freund's adjuvant, followed 4 wk later by a second immunization with 100 μ g of Mc471B (μ , κ) in complete adjuvant. The goat was bled each week beginning 5 wk after the second immunization. G125 antiserum was raised by immunization of a goat with MPC 25 (γ_1 , κ). The goat was immunized twice with 100 μ g of protein in complete Freund's adjuvant, and exsanguinated 6 wk after the last injection. A rabbit anti-mouse κ -antiserum was a generous gift of Dr. Rose Mage, Laboratory of Immunology, National Institutes of Health. Goat anti-ferritin antiserum was kindly provided by Dr. Fred Weinbaum, Laboratory of Microbial Immunity, National Institutes of Health. All antisera were absorbed with CBA/J and BDF₁ spleen, thymus, and liver tissue before use in culture or further purification on affinity columns.

Preparation of Purified Antibody. Antibodies specific for mouse heavy (H)¹ and light (L) chains were isolated on Sepharose 2B affinity columns of purified mouse myeloma proteins. The myeloma proteins were isolated from 50% ammonium sulfate fractions of ascitic fluid by preparative electrophoresis in gel slabs (15) with 1% agarose (Indubiose A-37, L'Industrie Biologique, Francaise, 92- Genevillers) to prevent contamination with mitogenic factors present in conventional agar preparations (16). Myeloma proteins or other proteins were coupled to cyanogen bromide-activated Sepharose 2B (17). Serum from germ-free mice was coupled to cyanogen bromide-activated Sepharose 2B (2 ml per ml Sepharose) after dialysis against 0.05 N NaHCO₃-0.075 N NaCl.

Anti-Ig antibodies were purified on immunoabsorbent columns as previously described (18). Whole antiserum, previously cleared by centrifugation at 10,000 rpm for 15 min, was passed over the columns, followed by a wash with borate-buffered saline (0.005 M borate buffer in 0.15 M NaCl, pH 8.4) and a sodium-potassium phosphate buffer (0.1 M, pH 6.49). Antibody was eluted with a sodium acetate buffer (0.1 M, pH 3.6) and 0.1 M acetic acid (pH 3.2). The eluates were immediately neutralized in borate buffer followed by vacuum dialysis against 0.15 M NaCl. Preparations were sterilized before use in tissue culture by passage through a washed 0.22- μ Millipore filter (Millipore Corp., Bedford, Mass.).

Antisera and purified antibodies were analyzed for specificity by immunoelectrophoresis in 1.5% agar gel (Agar, Nobel; Difco Laboratories, Detroit, Mich.) against a panel of BALB/c myeloma ascites. G615 antiserum and purified anti- μ antibody precipitated MOPC 104E (μ , λ) and Mc471B (μ , κ) but failed to precipitate IgG₁, IgG_{2a}, IgG_{2b}, or IgA myeloma proteins. On the other hand, G125 antiserum and purified anti- γ_{2a} , κ antibody precipitated κ -containing myeloma proteins of all classes. Neither reagent precipitated nonimmunoglobulin proteins from ascitic fluid.

The purified antibody preparations were also analyzed for specificity by immunoprecipitation of radiolabeled membrane proteins of BDF₁ spleen cells. The latter were ¹²⁵I-labeled by the lactoperoxidase technique, extracted with Nonidet P40, (Particle Data Laboratories Ltd., Elmhurst, Ill.) and then incubated with 10 μ l of various anti-Ig antisera or 10 μ l of affinity column purified antibody (125 μ g/ml). The immune complexes were precipitated with Cowan I strain of *Staphylococcus aureus* as previously described (19). After washing, the complex was solubilized and reduced by boiling in 2% sodium dodecyl sulfate (SDS) with 5% 2-mercaptoethanol and analyzed by electrophoresis on 10% polyacrylamide gels in 0.1% SDS (20). As shown in Fig. 1 A, G615 antiserum, before affinity column purifications, predominantly precipitates Ig containing μ -H-chains. However, a significant peak with the mobility of the putative mouse δ -chain is also precipitated by G615, suggesting that in addition to anti- μ , G615 antiserum contained anti-Fab or anti-L chain activity. After double passage over Sepharose 2B affinity columns bearing germ-free mouse serum, HOPC 1 (γ_1 , λ) and MOPC 173 (γ_{2a} , κ) myeloma proteins, only the predominant μ -chain and light chain peaks were visible (Fig. 1 A). This serum was then passed over a TEPC 183 (μ , κ) affinity column, after which no precipitating activity for μ was detectable (Fig. 1 B). The precipitating activity for μ was recovered in the acid eluate from the TEPC 183 column (Fig. 1 B). Thus purified G615 can be regarded as specific for μ -chain. The mitogenic potential of these preparations will be discussed in the Results section. For comparison, the precipitating activity of affinity column purified G125 antibody is shown in Fig. 1 C. G125 precipitates Ig containing μ - and δ -chains in similar proportions to that precipitated by a rabbit anti- κ antiserum (Fig. 1 D).

¹ Abbreviations used in this paper: FCS, fetal calf serum; H, immunoglobulin heavy chains; [³H]TdR, tritiated thymidine; L, immunoglobulin light chains; LPS, lipopolysaccharide; 2-ME, 2-mercaptoethanol; SDS, sodium dodecyl sulfate.

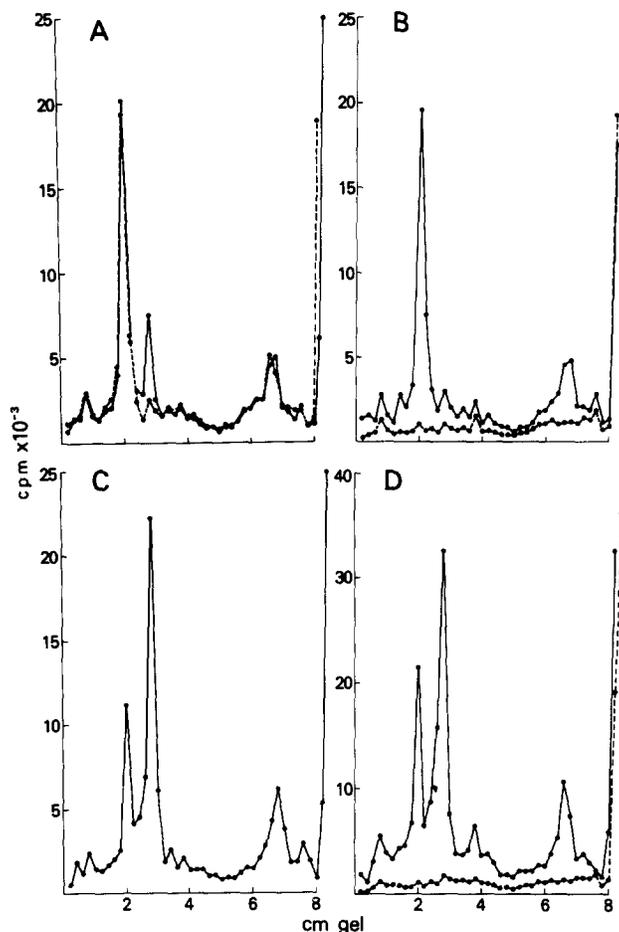


FIG. 1. SDS polyacrylamide gel electrophoresis of ^{125}I -BDF₁ membrane proteins precipitated by anti-Ig antisera or purified antibody, as described in A-D. Precipitates were reduced by boiling in 2% SDS and 5% 2-mercaptoethanol. The reduced samples were subjected to electrophoresis in 10% polyacrylamide gels with 0.1% SDS. 2-mm slices were cut and radioactivity measured in a Beckman Gamma Counter. A. Nonabsorbed G615 antiserum (—) and G615 antiserum after double passage over germ-free serum, HOPC 1 and MOPC 173 affinity columns (----); B. G615 antiserum after passage through a TEPC 183 affinity column (----); and acid eluate from this affinity column (—); C. G125 antibody purified on MOPC 173 affinity column; D. Rabbit anti- κ antiserum (—) and normal goat serum (----).

Test For Endotoxin Contamination. G615 serum (diluted 1:10) and purified antibody (at 124 $\mu\text{g}/\text{ml}$) were tested for endotoxin contamination by the *Limulus* amoebocyte lysate assay (21). We are grateful to Dr. Ronald Elin, Clinical Pathology Department, Clinical Center, National Institutes of Health, for making this measurement. Both preparations were negative after chloroform extraction under conditions in which 10 ng of reference endotoxin per ml could have been detected.

Cell Culture and Assay For (Methyl ^3H) Thymidine (^3H TdR) Incorporation. Spleen cells (5×10^5 unless noted otherwise) were cultured in a modified Mishell-Dutton medium (22) containing 0.016 M HEPES buffer (CMEM) 5×10^{-5} 2-mercaptoethanol (2-ME) and 10% fetal calf serum (Rehatuin, Armour Pharmaceutical Co., Phoenix, Ariz.) or in a modified Click medium (23)

without nucleic acid precursors (EHAA) in a vol of 0.2 ml in flat bottom microtiter plates (Falcon Micro-test II, Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) in a 5% CO₂ atmosphere. 1 μ Ci of [³H]TdR (5 Ci/mmol, Amersham Corp., Arlington Heights, Ill.) was added at 48 h. After an additional 16 h of culture, incorporation of [³H]TdR was measured by harvesting the cells onto glass fiber filters using a semiautomated harvester (Mash II, Microbiological Associates, Walkersville, Md.). Results are expressed as the geometric mean of triplicate cultures \pm the standard error of the mean based upon a log normal distribution (24). The standard error was calculated as follows:

$$SE = \bar{x} \sqrt{\frac{e^{n(SE_{\bar{x}})^2} - 1}{n}}$$

where \bar{x} is the geometric mean, n is the number of samples, and $SE_{\bar{x}}$ is the standard error of the logarithmic mean. Results are also expressed as the difference between the geometric means of the experimental and control groups \pm the standard error of the difference of the means calculated by a standard form.

Ultracentrifugation of Purified Anti-Ig. Purified antibody was centrifuged in a Beckman Airfuge (Beckman Instruments, Inc., Spinc Div., Palo Alto, Calif.) for 1 h at 165,000 g_{max} . Pools of the top 0.1 ml and the bottom 0.07 ml of several tubes were adjusted to equivalent protein concentrations based on optical density measurements.

Mitogens. Lipopolysaccharide (LPS) (*Escherichia coli* 0111:B4, Westphal, Difco Laboratories) was used at 50 μ g/ml unless noted otherwise.

Pepsin Digestion and Preparation of F(ab')₂. A 50% ammonium sulfate fraction of G615 (20 mg/ml) was incubated with 2% pepsin (Sigma Chemical Co., St. Louis, Mo.) for 48 h at 37°C in a 0.1 M sodium acetate:acetic acid buffer (pH 4.0). The digest was dialyzed against phosphate-buffered saline and chromatographed on Sephadex G150. Fractions containing F(ab')₂ were examined for purity by immunoelectrophoresis against rabbit anti-goat IgG, anti-Fab, and anti-Fc (generously provided by Dr. Michael Mage, National Cancer Institute, National Institutes of Health). Fractions containing anti-Fab reactivity but lacking anti-Fc reactivity were pooled. On rechromatography on Sephadex G150, they emerged with an estimated molecular size of ~90,000 daltons.

Results

The Proliferative Response to an Anti-Ig Antiserum and Isolation of the Mitogenic Anti-Ig on Ig Affinity Columns. Whole serum from a goat immunized with mouse IgM myeloma proteins (G615) was found to be mitogenic in vitro for mouse spleen cells (Fig. 2). Proliferative responses in the range of 20–100,000 cpm have been obtained at a serum concentration of 1:200 in cultures of adult BDF₁ spleen cells. Three separate bleedings from this goat have been shown to be active. In addition, spleen cells from many other mouse strains, including A, AKR, BALB/c, C57BL/6, CBA/J, DBA/2, C3H/HeJ, and SJL were responsive to G615.

The specificity of the mitogenic activity in the antiserum was investigated by a series of absorptions on Ig affinity columns. As shown in Fig. 3, the antiserum was found to retain its mitogenic potential after double passage through (a) a Sepharose column bearing serum from germ-free mice; (b) a Sepharose column bearing HOPC 1 (γ_1 , λ) and (c) a Sepharose column bearing MOPC 173 (γ_{2a} , κ). All of the stimulatory activity was removed by a single passage through a TEPC 183 (μ , κ) column. These results suggested that the mitogenic activity was associated with the anti- μ antibody present in the antiserum. This conclusion was further supported by the demonstration of mitogenic activity in the acid eluates from the IgM column. The eluates proved to be highly stimulatory at concentrations ranging from 5 to 250 μ g/ml (Fig. 3). As was shown

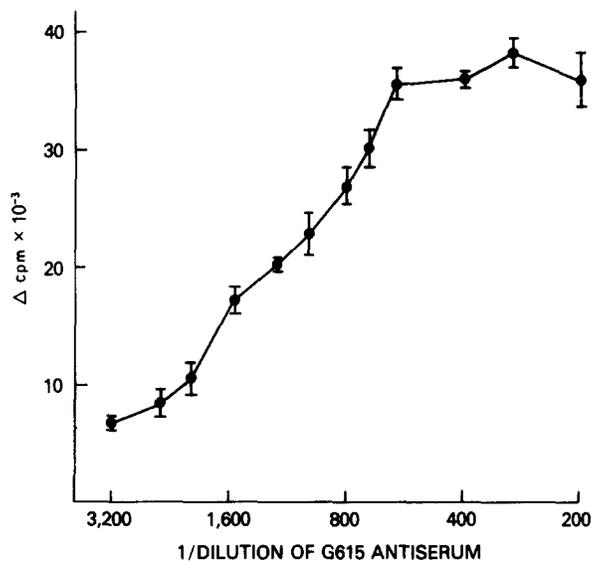


FIG. 2. Stimulation of [^3H]TdR uptake by cultures of 10 wk old BDF₁/J spleen cells with G615 antiserum. Cells (5×10^5) were cultured for 48 h with various dilutions of G615 antiserum in CMEM. Cultures were then assayed for thymidine uptake after a 16–17 h pulse of [^3H]TdR. Control cultures responded to 50 $\mu\text{g}/\text{ml}$ LPS with $74,794 \pm 746$ cpm per culture. Medium control was 6025 ± 495 cpm per culture.

in Fig. 1 and discussed in the Materials and Methods section, immunoelectrophoretic and immunoprecipitation analyses of the eluted material demonstrated only anti- μ activity. This stimulation appears to be specific in that a goat anti-ferritin antibody purified on an affinity column in a similar manner did not stimulate a proliferative response over a wide concentration range (Fig. 4). We also observed that a goat anti- γ , κ antibody (G125) and a rabbit anti-mouse κ -antibody both purified on a MOPC 173 affinity column were also stimulatory in the range of 5–500 $\mu\text{g}/\text{ml}$, with an optimal response at 250 $\mu\text{g}/\text{ml}$ (Fig. 5). These preparations displayed a much lower stimulatory capacity for BDF₁ spleen cells than did purified anti- μ antibody. These results suggest that anti- κ antibodies can also be stimulatory, although contributions by anti- γ antibodies in these preparations have not been ruled out.

On the other hand, preliminary experiments indicate that several other rabbit and goat monospecific and polyvalent antisera do not have such stimulatory activities. In one case, specifically purified antibody prepared from a nonstimulatory rabbit anti-mouse IgM antiserum caused proliferative responses of 30,000 cpm at concentrations of 250 $\mu\text{g}/\text{ml}$. Thus, the inability of certain antisera to display mitogenic potential may be due to inhibitors in these antisera.

Parameters of the Proliferative Response. The proliferative response to anti-Ig in spleen cell cultures of various cell densities is shown in Fig. 6. The response to anti- μ antiserum and to anti- γ , κ purified antibody occurred over a wide range of cell densities. Maximal responses to anti- μ and to anti- γ , κ required a higher cell density than that required for LPS. The difference

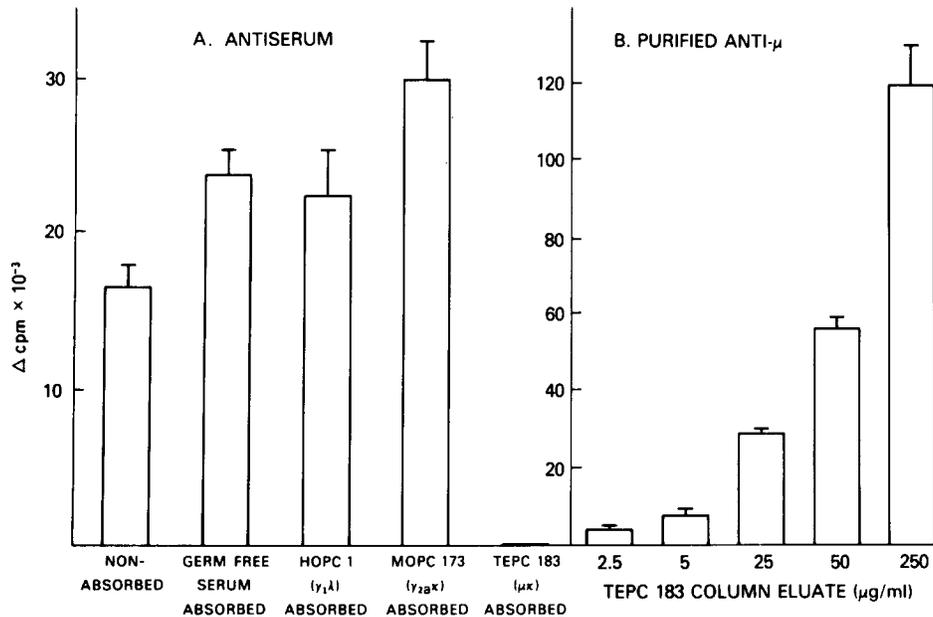


FIG. 3. Specific absorption and elution of stimulatory goat anti- μ antibody from an IgM affinity column. Panel A shows the mitogenic effects of G615 antiserum at 1:100 in cultures of 5×10^6 BDF₁ 10 wk old spleen cells, before and after absorption on germ-free serum, HOPC 1 or MOPC 173 affinity columns (double passage on each column) and a TEPC 183 affinity column. Panel B shows the mitogenic effects of material eluted from the TEPC 183 column at pH 3.6. Protein concentrations are based on optical density measurements. LPS stimulation was $81,141 \pm 1,624$ cpm per culture and medium control was $4,357 \pm 419$ cpm per culture. All determinations represent the geometric mean of three experimental cultures—the geometric mean of three control cultures \pm the computed standard error based on a lognormal distribution.

appeared to be absolute and not dependent upon the stimulant concentration, since the optimal cell density for responsiveness to anti- μ remained the same when the concentration of anti- μ was diminished 10-fold.

The kinetics of the proliferative response to anti-Ig and LPS are shown in Fig. 7. The responses to both anti- μ antiserum and anti- γ , κ purified antibody were delayed and longer lasting than the response to LPS under the culture conditions used in this study. The LPS response peaked on day 2 and was only minimal on day 3, whereas the response to anti- μ was high on days 2, 3, and 4. Response to anti- μ also occurred in EHAA medium in the absence of FCS, although the magnitude of the response was somewhat lower. In contrast to cultures in complete medium containing FCS, the maximum response to anti- μ in EHAA medium was obtained on day 2 and responses diminished rapidly thereafter. These results suggest that optimal culture conditions and serum proteins may be needed to maintain anti- μ induced proliferation at a maximal level, but these are not required for induction of the anti- μ responses. It should be noted that if 2-ME was omitted from serum-free culture medium, no proliferative response to anti- μ was obtained.

Further Evidence for Specificity of Anti-Ig Stimulation. Specific affinity

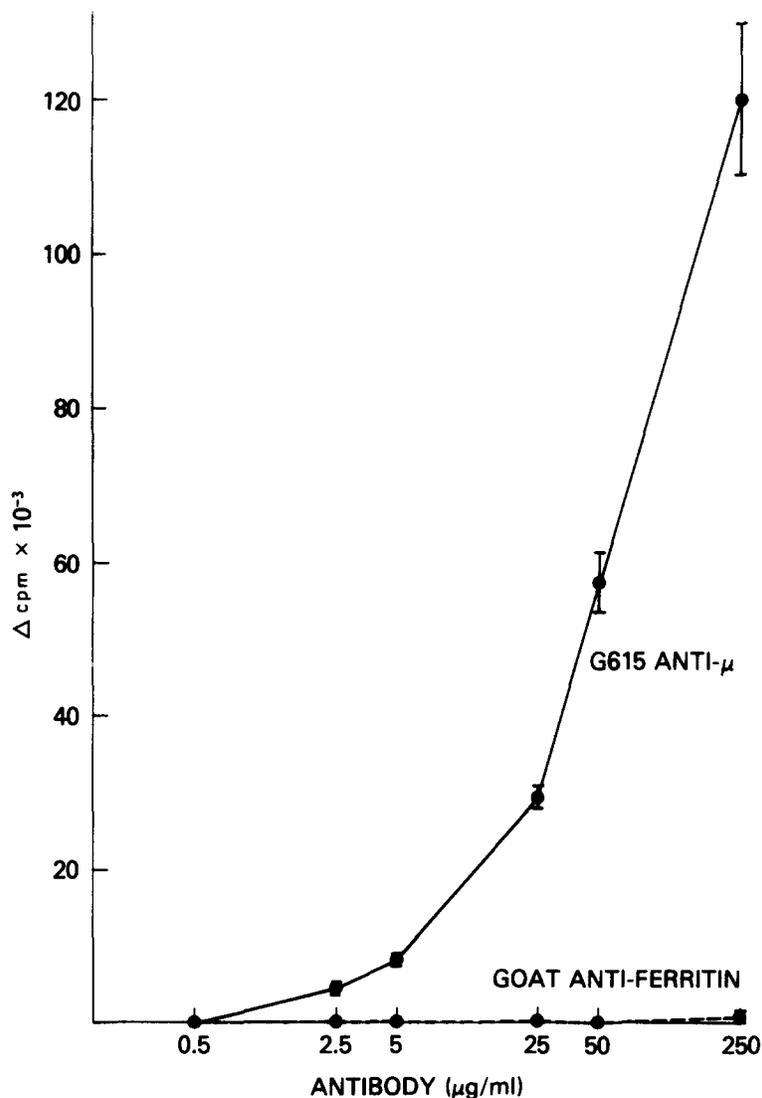


FIG. 4. Stimulation of BDF₁ spleen cells with affinity column purified G615 anti- μ antibody or affinity column purified G846 anti-ferritin antibody.

column absorption and elution of the mitogenic factor in the anti-Ig antisera seemed to rule out the possibility that stimulation was solely due to a contamination with a nonspecific mitogen such as bacterial LPS. Furthermore, as described in Materials and Methods, the G615 antisera and purified antibody were negative for LPS contamination when tested by the *Limulus* amebocyte lysate assay.

In addition, two mouse strains which are unresponsive or poorly responsive to endotoxin, C3H/HeJ and C57BL/10ScCr, respond to anti- μ , and one strain, CBA/N, which is responsive to LPS is unresponsive to anti- μ . Responses of the latter two strains are shown in Fig. 8. The significance of the unresponsiveness

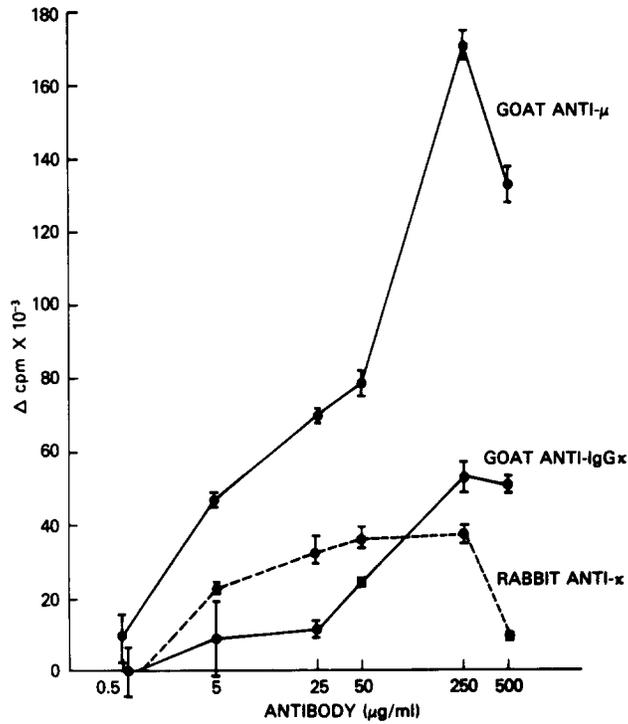


FIG. 5. Stimulation of BDF₁ spleen cells (17 wk old) with G615 anti- μ , G125 anti- γ , κ , and rabbit anti- κ affinity column purified antibodies. The goat anti- μ is a pH 3.6 eluate from a TEPC 183 column. The goat anti- γ , κ and rabbit anti- κ are eluates from a MOPC 173 column at pH 3.6.

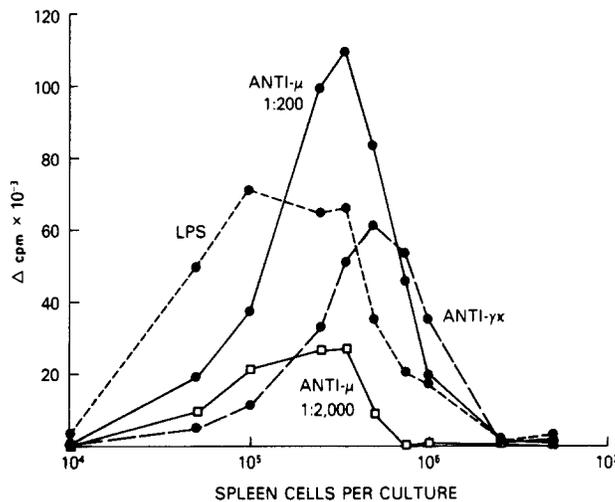


FIG. 6. The proliferative response as a function of spleen cell density in culture. Cultures of 15 wk old CBA/J spleen cells at various concentrations were stimulated with G615 antiserum at a 1:200 dilution or a 1:2,000 dilution, G125 purified anti- γ , κ at 250 μ g/ml, or with LPS at 50 μ g/ml.

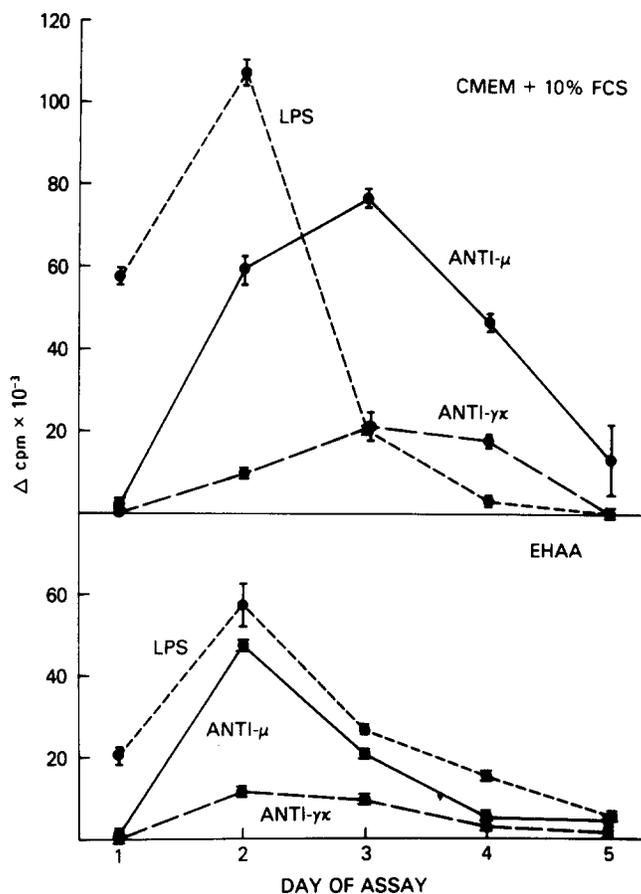


FIG. 7. Kinetics of the proliferative response to a 1:200 dilution of G615 anti- μ antiserum, G125 anti- γ , κ purified antibody (250 $\mu\text{g}/\text{ml}$), and LPS (50 $\mu\text{g}/\text{ml}$) in cultures of 5×10^5 CBA/J spleen cells. The stimulation of cultures in complete medium with 10% FCS (top) and in EHAA medium without FCS (bottom) was measured each day after a 4 h pulse of [^3H]TdR.

of CBA/N spleen cells to anti- μ will be discussed in a subsequent paper. Finally, F_1 female mice from a cross between CBA/N females and C57BL/10ScCr males respond well to both LPS and anti- μ .

Role of Aggregated Anti-Ig in Stimulation. To determine whether the stimulatory activity of anti- μ was due to the presence of soluble aggregates within the preparation, the purified anti- μ was centrifuged at 160,000 g for 60 min. The supernate and pelleted fractions were compared to the starting preparation for stimulatory activity (Fig. 9). When compared on a weight basis, the three preparations were indistinguishable in their ability to stimulate proliferation of BDF $_1$ spleen cells. Similar results were obtained with the purified G125 (anti- γ , κ) preparation. These results strongly suggest that large aggregates of anti-Ig cannot be exclusively responsible for proliferative responses and that buoyant lipoproteins or high molecular weight endotoxins are not critical to responsiveness.

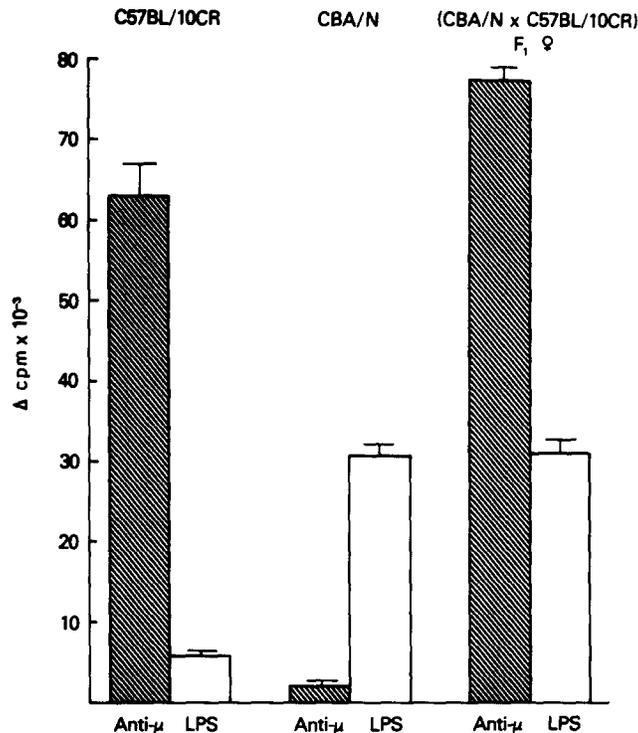


FIG. 8. Separate genetically controlled restrictions on the ability to respond to anti-Ig and LPS. Anti- μ antiserum was used at 1:200 and LPS at 5 μ g/ml in cultures of 10 wk old spleen cells from female C57BL/10ScCr, CBA/N, or (CBA/N \times C57BL/10ScCr) F_1 mice.

Stimulation by $F(ab')_2$ Fragments of Anti- μ . The proliferative response obtained with purified anti- μ antibodies suggests that modulation of surface Ig receptors has some functional role in lymphocyte activation. However, in view of the presence of Fc receptors on B lymphocyte membranes (25, 26) and their possible role in controlling B lymphocyte function (27-29), it was important to determine whether or not the Fc portion of the anti- μ antibodies is required for their mitogenic activity. Pepsin fragments of the IgG fraction of G615 antiserum were prepared and assayed for their stimulatory potential. The results in Fig. 10 show that the $F(ab')_2$ molecules are just as active as the whole molecule based upon molar concentration. These results strongly suggest that the stimulation obtained with anti- μ is independent of the presence of the Fc portion of the molecule and Fc receptor binding of the goat antibody. Thus, stimulation is most probably a result of the binding of anti- μ to the membrane Ig receptors via the antigen combining site of the goat anti-mouse- μ .

Discussion

The experiments presented in this study demonstrate that lymphocytes from young adult mice can be activated to synthesize DNA by culture with purified antibodies specific for either μ - or κ -determinants. Because of the many previous reports that anti-Ig antibodies fail to activate mouse lymphocytes and

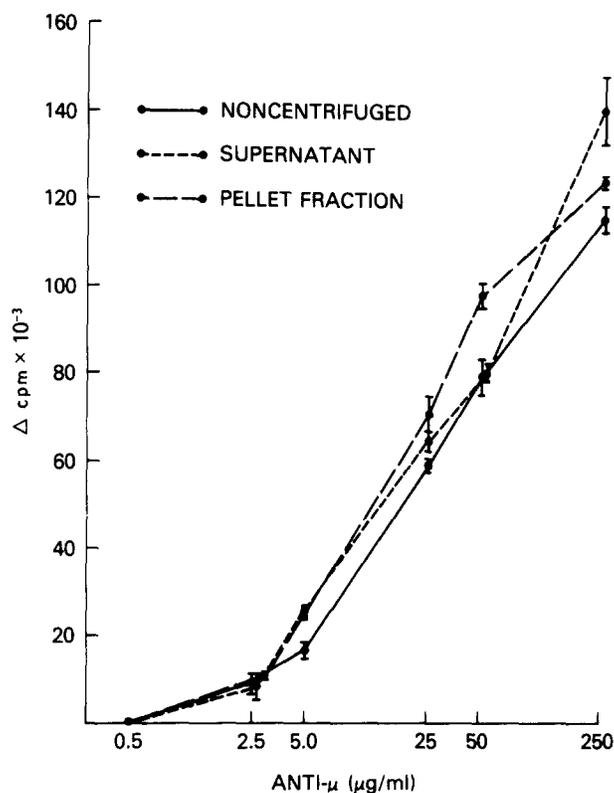


FIG. 9. Ultracentrifugation of G615 purified antibody. The ability of noncentrifuged, supernatant fraction, and pellet fraction of G615 anti- μ , ultracentrifuged as described in the Materials and Methods section, to stimulate BDF₁ spleen cells was measured.

of differences between our results and those of both Parker (13) and Weiner et al. (14), who have described successful activation under more rigorous conditions, we took considerable care to insure the specificity of our reagents. As described, the anti- μ reagent was rendered specific by passage over both $\gamma_{1,\lambda}$ and $\gamma_{2a,\kappa}$ columns as well as a column bearing germ-free mouse serum proteins. It was then specifically purified on a μ,κ immunoadsorbent column and analyzed by immunoelectrophoresis and by immunoprecipitation of radioiodinated membrane proteins. In the latter procedure, analysis of the immunoprecipitated material by SDS-polyacrylamide gel electrophoresis revealed only two principle radioactive peaks, one corresponding to μ -H chains and one to L chains. The absence of a peak corresponding to the putative mouse δ -H chain, in this very sensitive assay, indicated that all antibodies specific for Fab or L-chain determinants had been removed. The absence of any other principle radioactive peak on electropherograms of anti- μ precipitated membrane proteins suggests that antibodies to other lymphocyte membrane components were not present in appreciable quantities. Thus, we consider it unlikely that antibodies to β_2 microglobulin, which have been reported to be mitogenic (30), could be responsible for the activity of our anti-Ig preparation. It has been reported that carbohydrate-specific antibodies can activate lymphocytes (31)

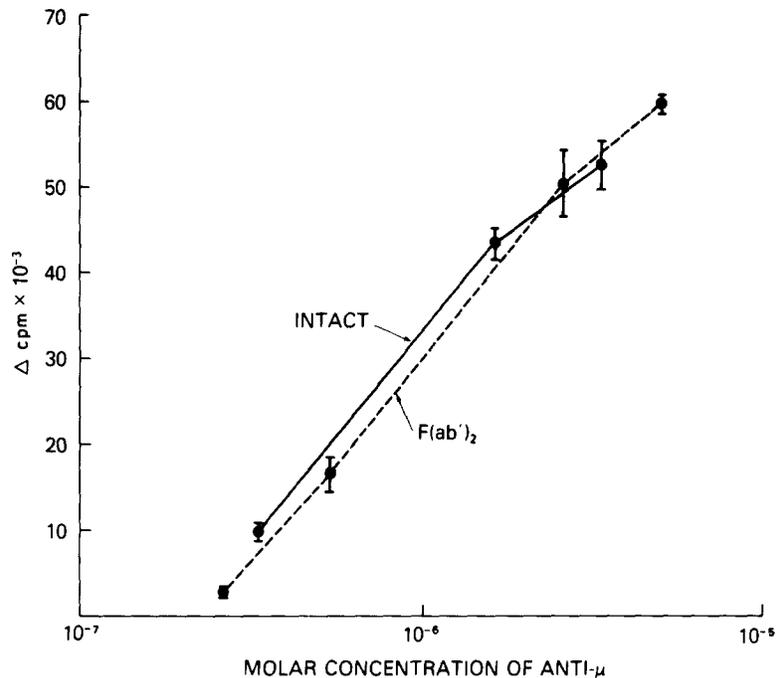


FIG. 10. Stimulation of BDF₁ spleen cells with G615 IgG or with F(ab')₂ fragments of G615 IgG. Molar concentrations are based upon estimated mol wt of 150,000 daltons for whole IgG molecule and 92,000 daltons for F(ab')₂.

and since IgM is a glycoprotein, it is still possible that the stimulatory activity we have observed is reacting through sugar specificities. The more important question is whether this anti- μ antibody reacts with other membrane glycoproteins or glycolipids bearing cross-reactive determinants and the binding to these moieties is what really activates the lymphocytes. Although we regard this as unlikely, since we do not observe any precipitation of radioiodinated membrane molecules other than IgM, it can not be entirely excluded.

A second major concern in any study involving activation of mouse lymphocytes is the possibility that contaminating bacterial LPS in the anti-Ig preparation might be wholly responsible for the stimulatory activity. This seems most unlikely for several reasons. First, an assay for LPS contamination by the *Limulus* amoebocyte lysate test revealed no detectable contamination of the goat anti- μ antiserum or of the purified antibody. Second, the anti- μ preparation stimulated a response by lymphocytes from two mouse strains (C57BL/10ScCr and C₃H/HeJ), which are hypo- or unresponsive to LPS (32, 33), while it failed to stimulate a response by CBA/N lymphocytes, although the latter cells respond to LPS. Consequently, we feel confident that anti- μ is not simply a passive diluent for LPS in the stimulation of the lymphocyte DNA synthesis.

Parker (13) previously reported that anti-Ig conjugated to polyacrylamide beads stimulated lymphocyte DNA synthesis, while soluble anti-Ig did not. This raises the possibility that anti-Ig in a highly aggregated form might be required for activation. We see that as unlikely, since ultracentrifuged anti-Ig, which should be free of any large aggregates, has the same stimulatory capacity as

the unseparated preparation. Of course, we cannot exclude the possibility that anti-Ig aggregates form during the culture period or that anti-Ig is displayed in an aggregated form on the surface of an auxiliary cell such as a macrophage.

The data presented here do not yet allow us to comment on the mechanism by which anti-Ig activates B lymphocytes; however, one striking finding is that there is a dose-dependent increase in responsiveness up to 250 $\mu\text{g/ml}$. Such concentrations appear to be far in excess of what should be required to saturate membrane Ig receptors and to induce capping of membrane Ig components. It is very likely that simple occupation of antigenic sites on the surface Ig molecules is not solely responsible for stimulation. On the other hand it is possible that the stimulatory antibodies represent a minor fraction of the anti- μ molecules, perhaps specific for certain critical determinants on membrane IgM. Indeed, it could be proposed that the failure of many other laboratories to obtain activation of B lymphocytes by anti-Ig might reflect an absence of such antibodies in some anti- μ antisera. Since F(ab')_2 fragments of anti- μ are as active as whole molecules, binding of anti-Ig to membrane Fc receptors does not appear to be required. However, additional unknown interactions may be needed for induction of cell proliferation, and these may involve specific patterns of cross-linkage of membrane components.

Finally, our preliminary results indicate that anti- μ fails to stimulate Ig synthesis by cell populations which proliferate in response to this reagent. This is consistent with a previous report using anti-rabbit Ig antibodies (34) and with Parker's results using anti- κ -polyacrylamide beads (13). Indeed, anti- μ antibodies, (35), including G615 (D. G. Sieckmann, unpublished observations), block in vitro antibody responses of mouse spleen cells to sheep erythrocytes and other antigens. It has also been reported that anti-Ig will block Ig synthesis in response to LPS (36). This suggests that the simple interaction of anti- μ with the cell can cause proliferative responses but may block differentiation into an antibody secreting state. However, Kishimoto and Ishizaka (34) have shown that addition of a soluble supernate from lymphoid cells primed to and challenged with *Ascaris* antigens can cause anti-Ig treated rabbit cells to synthesize Ig, suggesting that, under appropriate conditions, anti-Ig can provide at least one of the "signals" required for differentiation into an antibody secreting cell. The availability of a simple lymphocyte activation system using a reagent which interacts with membrane receptors for antigen should provide a powerful model for the study of B lymphocyte activation. In a forthcoming paper² we will describe the cellular basis for this response.

Summary

Spleen cell cultures from young adult mice of a variety of strains were stimulated to incorporate tritiated thymidine (^3H TdR) by a goat anti-mouse IgM antiserum and by purified anti- μ antibodies prepared from this serum. This stimulation was shown to depend upon the anti- μ activity of the antiserum. In addition, ultracentrifuged anti- μ and F(ab')_2 fragments of anti- μ were shown

² D. G. Sieckmann, I. Scher, R. Asofsky, D. Mosier, and W. E. Paul. Activation of mouse lymphocytes by anti-immunoglobulin. II. Requirement for a mature subset of B lymphocytes. Manuscript in preparation.

to be stimulatory. The anti- μ preparation lacked detectable endotoxin contamination and was also shown to stimulate response by two strains (C57BL/10ScCr and C3H/HeJ) which are unresponsive to the mitogenic effects of endotoxin, while it failed to stimulate a response by cells from a mouse strain (CBA/N) which responds to endotoxin. In addition purified goat anti-mouse γ , κ antibodies and rabbit anti-mouse κ -antibodies stimulated uptake of [3 H]TdR by mouse spleen cells, although to a lesser degree than the anti- μ preparation. The cell density, culture requirements, and kinetics of the response are presented.

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