

INHIBITION OF LYMPHOCYTE MITOGENESIS BY IMMOBILIZED ANTIGEN-ANTIBODY COMPLEXES

BY JOHN L. RYAN, ROBERT D. ARBEIT, HOWARD B. DICKLER, AND
PIERRE A. HENKART

(From the Immunology Branch, National Cancer Institute, National Institutes of Health, Bethesda,
Maryland 20014)

Certain subpopulations of lymphocytes bear on their surfaces receptors which specifically bind the Fc portion of antigen-antibody complexes or heat-aggregated IgG. These Fc receptors are expressed on B lymphocytes both in the mouse (1), and in man (2). In addition, they have been reported to be present on activated T cells (3) and on a subpopulation of normal mouse T cells (4). Macrophages also bear similar Fc receptors (5), but it is not known if such receptors are identical to those of lymphocytes.

The biological function(s) of lymphocyte Fc receptors remains unclear. This receptor is involved in antibody-mediated lymphocyte-dependent cytotoxicity (reviewed in reference 6), but the physiologic role of this type of cell killing has not yet been established. Studies on the ability of antigen-antibody complexes to induce DNA synthesis have produced conflicting results (7, 8). It has clearly been shown that antibody exerts a specific inhibitory effect on the humoral antibody response (9), and that this effect is dependent on an intact Fc portion of the antibody molecule (10). While it has been hypothesized that this effect is mediated by antigen-antibody complex feedback on Fc receptors (11), Möller and Coutinho (12) have recently reported that neither soluble antigen-antibody complexes nor antibody-coated red cells show significant stimulatory or inhibitory effects on the induction of polyclonal antibody synthesis. Thus, a functional role for Fc receptors in the regulation of the immune responses of lymphocytes has not yet been demonstrated.

In the present work it is shown that antigen-antibody complexes immobilized on plastic surfaces profoundly inhibit the response to several mitogens. This inhibition requires the presence of the Fc portion of the antibody molecule.

Materials and Methods

Mice. C57BL/6 mice obtained from Jackson Laboratories, Bar Harbor, Maine, or from the NIH colony were used in all experiments.

Mitogens. Lipopolysaccharide (LPS)¹ was prepared by the method of Westphal et al. (13), from *Escherichia coli* 0111:B4 which was the kind gift of Dr. Abraham Braude, University of California, San Diego, Calif. For many experiments LPS obtained from Difco Laboratories, Detroit, Mich., was used. No differences were noted between the two preparations of LPS. Lipid A was prepared

¹ *Abbreviations used in this paper:* Ag-Ab, antigen-antibody; BSA, bovine serum albumin; BSS, balanced salt solution; Con A, concanavalin A; FITC, fluorescein isothiocyanate; KLH, keyhole limpet hemocyanin; LPS, lipopolysaccharide; MSA, mouse serum albumin; PBS, phosphate-buffered saline; PHA, phytohemagglutinin; TNP, trinitrophenyl.

by the technique of Andersson et al. (14) from both *E. coli* 0111:B4 and *E. coli* 055:B5. Lipid A-bovine serum albumin (BSA) complexes were prepared according to the procedure of Galanos et al. (15). Poly I:C was purchased from Sigma Chemical Co., St. Louis, Mo. Pneumococcal polysaccharide SIII was the kind gift of Ms. Anne Kask, NIAID, NIH, Bethesda, Md. Concanavalin A was purchased from Miles Laboratories, Inc., Miles Research Div., Kankakee, Ill. Purified phytohemagglutinin (PHA) (depleted of hemagglutinating PHA) was obtained from Wellcome Research Laboratories, Beckenham, England.

Preparation of Spleen Cells. Spleens were removed under sterile conditions and teased to single cell suspensions in balanced salt solution (BSS). The cells were filtered through nylon gauze to remove clumps and washed once with BSS. Red cell lysis was effected by resuspension of the pellet into isotonic NH_4Cl (ACK) for 1 min, followed by two more washes with BSS before finally resuspending the cells in culture medium. Viability was assessed by 0.2% trypan blue exclusion.

Preparation of Plates and Culture Conditions. All experiments were done in flat-bottom Microtiter plates (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.). The microtiter wells were coated with antigen-antibody complexes before the addition of cells using a slight modification of the techniques of Henkart and Alexander.² In brief, each well was filled with 20 μl of a 1 mg/ml solution of mouse serum albumin, (MSA), Fraction V, (Sigma Chemical Co.) in phosphate-buffered saline (PBS), pH 7.4. After 20 min at 37°C the plates were inverted, flicked, and washed with a large excess of PBS. 20 μl of a solution of 10 mM trinitrobenzenesulfonic acid in PBS, pH 7.4, was then added to each well and allowed to incubate at 37°C for 15 min. This procedure puts trinitrophenyl (TNP) groups on the MSA which has firmly adsorbed to the plastic wells. The wells were again washed thoroughly with excess PBS and appropriate antibody solutions were added and incubated for 30 min at 37°C to create the immobilized immune complexes. This was followed by a final washing with excess PBS. Measurements in a similar system have shown that at saturation such wells bind about 150 ng of antibody.² Wells were used with or without the final antibody treatment in all experiments. Each well contained 5×10^5 cells in a vol of 200 μl of Eagle's minimal essential medium supplemented with 1 mM glutamine, 100 U/ml penicillin, 10 $\mu\text{g}/\text{ml}$ streptomycin, and 5 $\mu\text{g}/\text{ml}$ gentamicin. No serum supplementation was used at any time. Mitogen was added in a vol of 20 μl or less within a few minutes of addition of the cells. Cultures were incubated in a presence of 7.5% CO_2 at 37°C for 72 h.

Antibody Preparations. Rabbit anti-DNP-BSA serum purchased from Miles Laboratories, Miles Research Div., was used in most experiments. Purified rabbit anti-TNP antibodies were prepared by DNP-lysine Sepharose affinity chromatography using the technique of Robbins et al. (16). Rabbit anti-TNP F(ab')₂ fragments from this antibody were prepared by pepsin digestion and chromatography on Sephadex G-200. The F(ab')₂ fragments showed an identical hemagglutination titer using TNP-red blood cells compared to the intact antibody, but were unable to lyse these cells in the presence of complement. Mouse anti-TNP-keyhole limpet hemocyanin (KLH) was obtained by intraperitoneal immunization of C57BL/6 mice with 100 μg of heavily substituted TNP-KLH in complete Freund's adjuvant, followed by a similar injection 4 wk later in incomplete Freund's adjuvant.

Assay of DNA Synthesis. Cultures were pulsed with 1 μCi of 2 Ci/mmol [³H]thymidine (New England Nuclear, Boston, Mass.) after 48 h. The thymidine was added in a vol of 10 μl in PBS. Cultures were harvested by two techniques. In some experiments DNA was precipitated by the addition of 20 μl of 50% wt/wt TCA to each well. The contents of the wells were mixed, and the precipitates centrifuged and washed two times with cold 5% TCA. The precipitate was dissolved in 50 μl of 1 N NaOH and counted in a Packard Tri-Carb liquid scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.). In other experiments a multiple automated sample harvester (MASH II, Microbiological Associates, Bethesda, Md.) was used. The isotope incorporation data presented are means and standard errors of the means of quadruplicate cultures.

Determination of Fc-Binding Lymphocytes. Fluorescein isothiocyanate (FITC)-conjugated heat-aggregated human Ig was used to determine Fc-positive cells by the procedure of Dickler and Sachs (17). Cells were preincubated for 30 min at 23°C with the test mitogen; FITC-conjugated aggregates were then added without washing and incubated an additional 30 min at 23°C. The cells were then washed, wet mounts prepared, and the slides evaluated by alternate fluorescent and phase microscopy for the percentage of positive lymphocytes.

² Henkart, P. A., and E. Alexander. 1975. Manuscript in preparation.

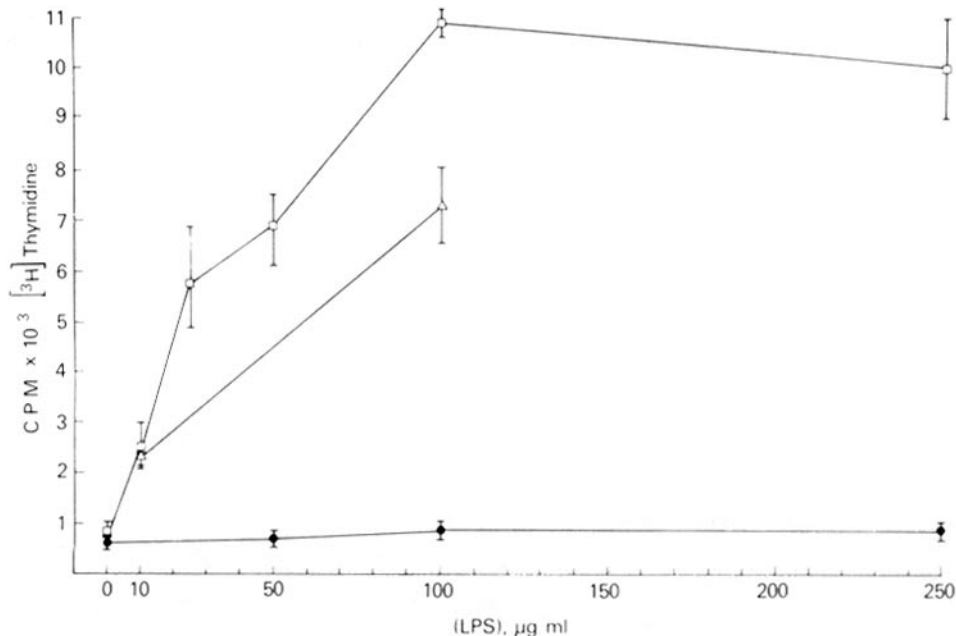


FIG. 1. Stimulation of spleen cells by LPS. In the presence of immobilized complexes (MSA-TNP-IgG), (●); and in the presence of immobilized antigen (MSA-TNP), (□). F(ab')₂ control shown [MSA-TNP-F(ab')₂], (△). Rabbit anti-DNP-BSA (1/10) was used to coat wells in this experiment and in Figs. 3-7. F(ab')₂ was at 270 $\mu\text{g/ml}$ in all experiments.

Results

Inhibition of LPS and Lipid A Mitogenic Response by Immobilized Antigen-Antibody Complexes. Lymphocytes incubated in microtiter wells coated with MSA-TNP responded to LPS with a greater than 10-fold increase in DNA synthesis (Fig. 1). When anti-TNP antibody was bound to the MSA-TNP coating, the capacity of LPS to stimulate DNA synthesis was markedly depressed. Note that antigen-antibody (Ag-Ab) alone did not enhance DNA synthesis. In this experiment rabbit purified anti-TNP antibodies were used; however, in other experiments, hyperimmune mouse anti-TNP antisera gave a similar suppressive effect. It should be emphasized that excess antibody was washed away before addition of cells and medium to the culture wells, so that only antibody complexed to TNP groups remains. If instead of intact anti-TNP, the F(ab')₂ fragments of the purified antibodies were used, inhibition of the LPS response was minimal (Fig. 1). It appears, therefore, that the requirements for suppressing the LPS response are that there be Ag-Ab complexes on the surface of the wells and that the antibody have an intact Fc fragment.

The effect of coating the wells with different concentrations of anti-TNP is shown in Fig. 2. Maximal inhibition occurred at an antibody concentration of 27 $\mu\text{g/ml}$ which is below the level of antibody necessary to saturate the plate.²

Lipid A, the mitogenically active moiety of LPS, has also been shown to be a B-cell mitogen (14). A similar Fc-specific inhibitory effect is observed when spleen cells are cultured with this mitogen in the presence of immobilized

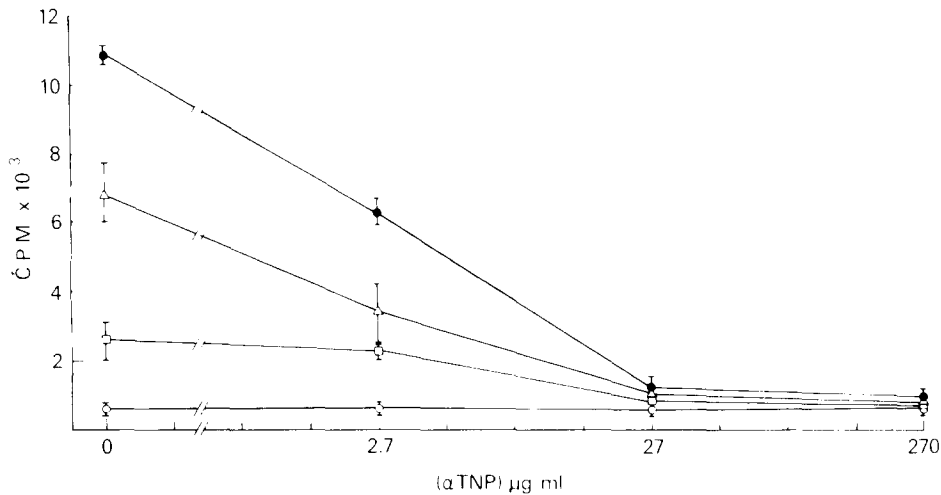


FIG. 2. Antibody titration for inhibition of LPS response of spleen cells. Affinity purified rabbit anti-TNP was used to form immobilized complexes in this experiment. LPS 100 $\mu\text{g/ml}$, (●); 50 $\mu\text{g/ml}$, (Δ); 10 $\mu\text{g/ml}$, (\square); and no LPS (\circ).

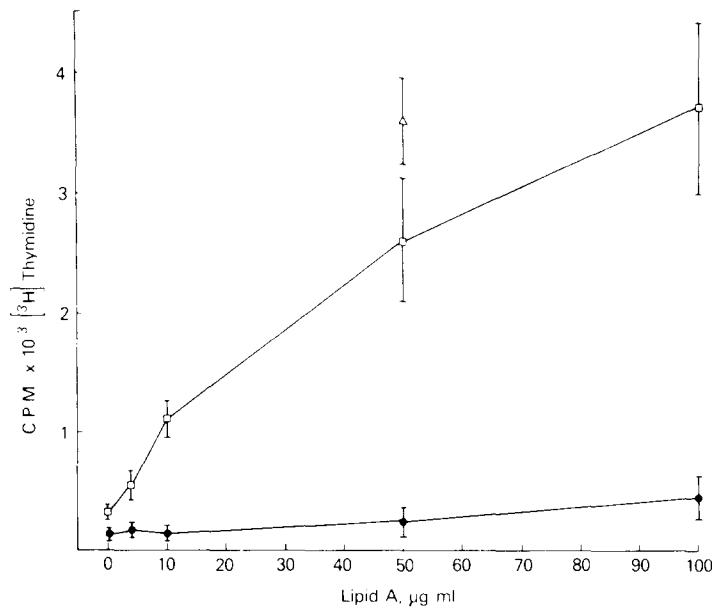


FIG. 3. Stimulation of spleen cells by lipid A. Cells were cultured in the presence of immobilized complexes (MSA-TNP-IgG), (●); and in the presence of immobilized antigen (MSA-TNP), (\square). F(ab')₂ control shown [MSA-TNP-F(ab')₂], (Δ).

complexes (Fig. 3). It should be noted that in this experiment, no inhibition was observed in the presence of the F(ab')₂ complexes.

Since the Fc receptor on mouse B lymphocytes does not bind IgA antibodies (18), the effect of coating the wells with IgA complexes was tested using the TNP-binding IgA mouse myeloma, MOPC 315 (Table I). Only minimal inhibitory

TABLE I
Comparison of MOPC 315 (IgA) and Rabbit IgG for Suppression of LPS Stimulation

Mitogen	MSA-TNP	MSA-TNP-IgA*	MSA-TNP-IgG‡
	<i>cpm</i>	<i>cpm</i>	<i>cpm</i>
None	246 ± 20	ND	36 ± 5
LPS§	13,293 ± 295	8,865 ± 102	39 ± 2

* MOPC 315 used at 100 µg/ml to coat culture wells.

‡ 1/10 dilution of rabbit anti-DNP-BSA used to coat these wells.

§ 100 µg/ml LPS.

TABLE II
Effect of Preincubation of LPS* in Presence of MSA-TNP-IgG

Preincubation wells	[³ H]TdR
	<i>cpm</i>
MSA-TNP	7,762 ± 784
MSA-TNP-IgG	6,357 ± 2,611
MSA-TNP (No LPS)	886 ± 109

* 20 µg of LPS was preincubated in a vol of 100 µl for 24 h, then transferred to new wells coated with MSA-TNP. Cells added in a vol of 100 µl for culture.

activity could be detected with these IgA-TNP complexes supporting the hypothesis that the inhibitory effect was occurring via the Fc receptor.

Interaction of LPS and Immobilized Complexes. One explanation for the inhibitory effect of the immobilized complexes could be that complexes are binding or otherwise inactivating the mitogen. To test this possibility, LPS was preincubated for 24 h in wells coated with antigen alone or with Ag-Ab complexes and then transferred to new wells containing cells. There is no significant difference between the mitogenic activity of LPS preincubated in antigen or Ag-Ab complex-coated wells, indicating that inactivation of the mitogen is not responsible for the inhibition of mitogenesis by immobilized complexes (Table II).

Viability of Cultures. In order to show that the immobilized complexes were not significantly toxic to the lymphocytes, viability and recovery were measured for cultures in the presence of antigen alone and complexes (Table III). No significant difference in viability was observed. These viabilities are in agreement with others (19) for serum-free spleen cell cultures in similar systems.

Effect of Free Antibody and Suspensions of Ag-Ab complexes on the LPS Response. The ability of free IgG in solution and suspensions of Ag-Ab complexes to mediate a similar inhibition of the LPS response was also tested. The antigen used to form the complexes was TNP₃₃MSA, so that the same type of complexes were tested in suspension and immobilized form. Complexes were preformed at equivalence and in slight antigen excess. The results (Table IV) demonstrate that slight inhibition results from the addition of high doses of soluble IgG, but it is evident that the effect of the immobilized complexes is

TABLE III
Viability and Recovery of Cultured Cells

Day	MSA-TNP		MSA-TNP + LPS*		MSA-TNP-IgG + LPS*	
	Viability	Recovery	Viability	Recovery	Viability	Recovery
	%	%	%	%	%	%
0	87	90	93	42	86	60
1	75	80	71	75	75	85
2	64	42	54	70	62	42
3	64	60	60	65	64	40

* LPS at 100 $\mu\text{g/ml}$.

TABLE IV
Effect of Soluble Antibody, Soluble Immune Complexes, and Immobilized Immune Complexes on LPS Mitogenesis

Sample*	[³ H]thymidine
	<i>cpm</i>
Immobilized antigen	17,598 \pm 349
Immobilized complexes	184 \pm 91
Suspensions of complexes [†] (equivalence)	15,579 \pm 241
Soluble complexes [§] (antigen excess)	17,149 \pm 161
Normal rabbit IgG (0.5 $\mu\text{g/ml}$)	16,919 \pm 160
Normal rabbit IgG (5.0 $\mu\text{g/ml}$)	13,644 \pm 600
Normal rabbit IgG (100 $\mu\text{g/ml}$)	13,702 \pm 747
Immobilized complexes, no LPS	42 \pm 6

* 100 $\mu\text{g/ml}$ LPS present in all cultures.

[†] Suspensions of complexes were prepared by adding solutions of TNP₃₃MSA and affinity purified rabbit anti-TNP at a final concentration of 5 $\mu\text{g/ml}$ of antibody and 0.5 $\mu\text{g/ml}$ of antigen. Equivalence was determined by a precipitin curve.

[§] Soluble complexes were added in three times antigen excess above equivalence. Final concentration of 5 $\mu\text{g/ml}$ of antibody was used.

nearly two logs greater, despite the fact that much less Ig is present in the immobilized complexes. In other experiments soluble complexes (formed in antigen excess) were added to cultures over a wide range of concentrations (0.1 μg antibody/well-50 μg antibody/well) without evidence of any inhibition of the LPS-mediated mitogenesis. Thus the inability of suspensions of complexes to inhibit B-cell mitogenesis appears to correlate with their inability to inhibit polyclonal B-cell activation (11).

Inhibition of the Mitogenic Response of Poly I:C and SIII by immobilized Complexes. Two other well-characterized B-cell mitogens, the double-stranded polyribonucleotide poly I:C and Pneumococcal polysaccharide SIII were also tested for their ability to stimulate mitogenesis in the presence of immobilized complexes. Neither of these two substances was as mitogenic as LPS or lipid A. The mitogenic effect of these chemically unrelated B-cell mitogens also showed an Fc-dependent inhibition by the immobilized complexes (Figs. 4 and 5).

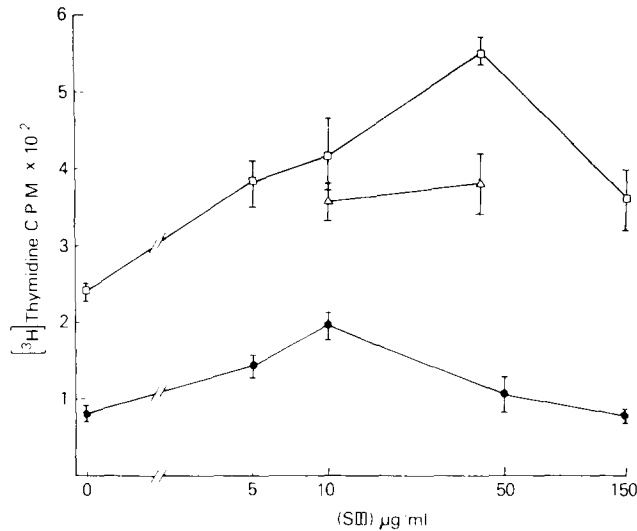


FIG. 4. Stimulation of spleen cells by pneumococcal polysaccharide SIII. Cells were cultured in the presence of immobilized complexes (MSA-TNP-IgG), (●); and in the presence of immobilized antigen (MSA-TNP), (□). F(ab')₂ control shown [MSA-TNP-F(ab')₂], (△).

Effect of Immobilized Complexes on Concanavalin (Con A) and PHA Responses. Soluble Con A and PHA have been shown to stimulate T cells in the mouse. The effect of immobilized complexes on the response to these mitogens is shown in Figs. 6 and 7. While the inhibition of the Con A response was not as striking as that seen for LPS and lipid A, it was highly significant and Fc specific. In contrast, inhibition of PHA mitogenesis was always less than 50%, and was not Fc specific as shown by the comparable inhibition from F(ab')₂-coated wells.

Binding of FITC Heat-Aggregated IgG in the Presence of Mitogens. One mechanism to explain the inhibitory activity of immobilized complexes might involve a competition of mitogen and Ig complexes for a common binding site. This possibility was tested by looking for inhibition of binding of FITC heat-aggregated IgG by the mitogens used in this study (Table V). In no case was it possible to demonstrate any inhibition of binding of aggregates to the Fc receptor by mitogen. Similarly, mitogens tested at 10 times the optimal mitogenic concentration failed to inhibit the binding of aggregated IgG.

Discussion

These experiments demonstrate that immune complexes, immobilized on plastic surfaces, are able to mediate a striking inhibition of B-cell mitogenesis. Inhibition is dependent on an intact Fc portion of the complexed IgG antibody and does not occur with complexed IgA antibodies. These findings suggest that the effect is mediated via the Fc receptors which are known to be present on B lymphocytes and macrophages. These results do not appear to be a direct interaction between LPS and the complexes bound to the plastic surface (Table II), or to the toxicity of immobilized complexes to the cultured lymphocytes

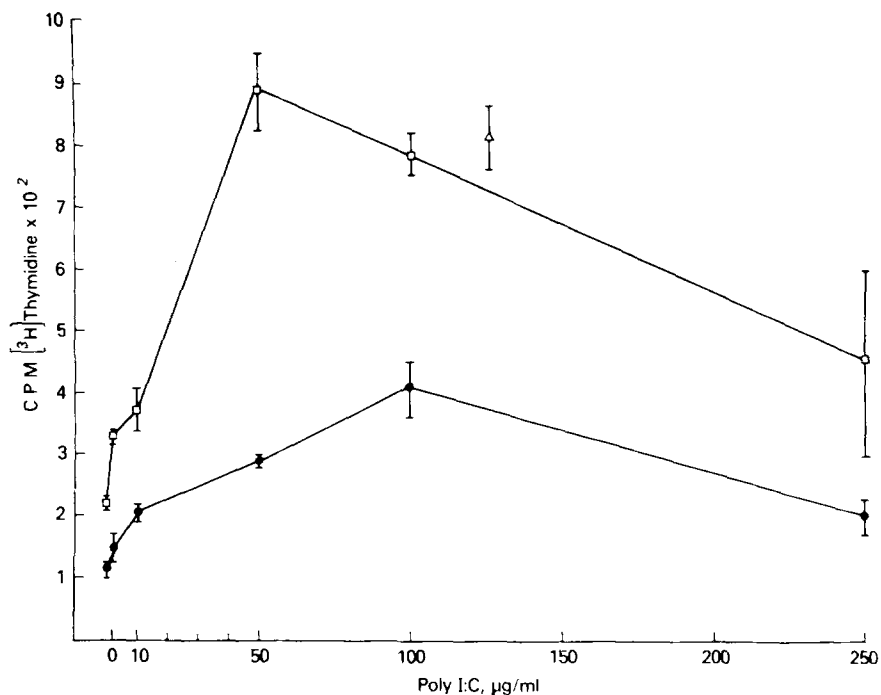


FIG. 5. Stimulation of spleen cells by poly I:C. Cells were cultured in the presence of immobilized complexes (MSA-TNP-IgG), (●); and in the presence of immobilized antigen (MSA-TNP), (□). F(ab')₂ control shown [MSA-TNP-F(ab')₂], (Δ).

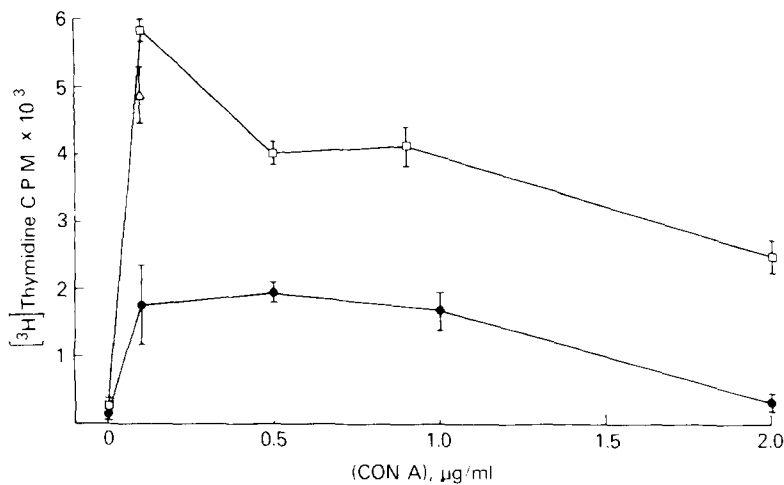


FIG. 6. Stimulation of spleen cells by Con A. Cells were cultured in the presence of immobilized complexes (MSA-TNP-IgG), (●); and in the presence of immobilized antigen (MSA-TNP), (□). F(ab')₂ control shown [MSA-TNP-F(ab')₂], (Δ).

(Table III). The selective killing of a small mitogen-responsive subpopulation has not been ruled out, although this seems unlikely since the vast majority of B lymphocytes proliferate in response to LPS (20).

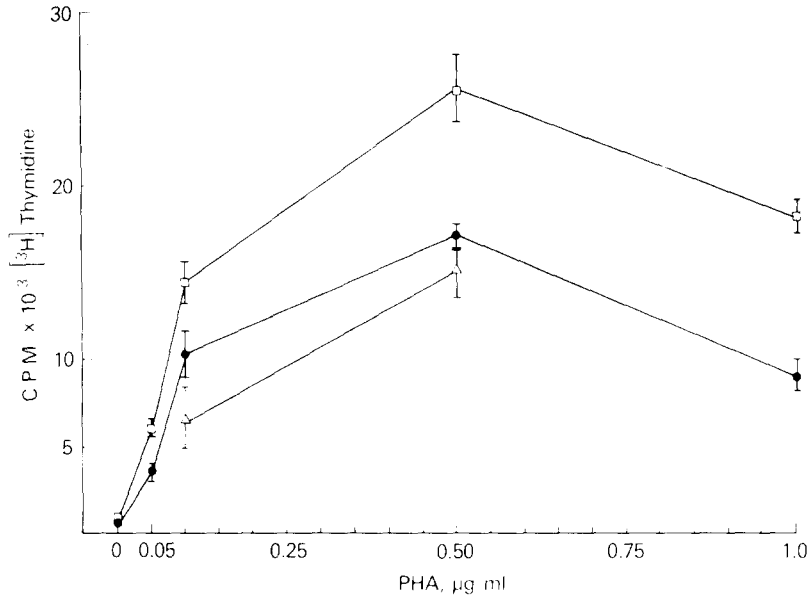


FIG. 7. Stimulation of spleen cells by PHA. Cells were cultured in the presence of immobilized complexes (MSA-TNP-IgG), (●); and in the presence of immobilized antigen (MSA-TNP), (□). F(ab')₂ control shown [MSA-TNP-F(ab')₂], (Δ).

TABLE V
Failure of Mitogens to Inhibit Lymphocyte Binding of Aggregated Ig*

Mitogen	Aggregated Ig binding % positive
Exp. I‡	
Media control	48.0
LPS (100 $\mu\text{g/ml}$)	49.0
Lipid A (100 $\mu\text{g/ml}$)	
Lipid A-BSA (100 $\mu\text{g/ml}$)	46.5
Exp. II‡	50.0
Media control	52.5
Poly I:C (50 $\mu\text{g/ml}$)	50.5
SIII (50 $\mu\text{g/ml}$)	50.5
Con A (0.5 $\mu\text{g/ml}$)	57.0
PHA (0.5 $\mu\text{g/ml}$)	50.5

* 100 cells counted to determine each value.

‡ 44.5–51% (exp. I) and 48.5–57% (exp. II) of the lymphocytes were surface Ig positive and these values were unaffected by the mitogens.

Any explanation of the mechanism of the inhibitory effect of the antigen-antibody-coated plastic must take into account the dramatically different effect produced by immobilized complexes as compared to complexes in suspension. B lymphocytes have previously been shown to respond to stimuli presented on surfaces, while the corresponding stimuli in solution are ineffective. Thus PHA and Con A, typically T-cell mitogens in soluble form, have been shown to

activate B cells when they are cross-linked to surfaces (21, 22). Similarly, hemocyanin, a thymus-dependent antigen in soluble form, becomes T independent when it is attached to Sepharose beads (23).

One mechanism to explain the data is a competitive inhibition for surface binding sites between mitogens and complexed IgG. This may seem unlikely because several chemically unrelated mitogens, which may have separate receptors (20), were inhibited, and we were unable to directly demonstrate any competitive inhibition (Table V). If the mitogens do bind to the Fc receptor, but with much less affinity than heat-aggregated IgG, then competitive inhibition would not have been detected under the conditions used.

A second mechanism to explain the inhibitory effect of immobilized complexes is that Fc receptor binding of a matrix of immobilized complexes (as opposed to binding of suspended complexes) induces an alteration of surface molecules such that normal mitogen receptors become blocked. The Fc receptors may become capped by immobilized complexes more readily than with complexes in suspension. This could be considered a form of noncompetitive inhibition and would not have been detected by our experiments as described in Table V. Unfortunately measurements of the binding of LPS to cells presents many technical difficulties (David C. Morrison, personal communication) and we have not attempted to test this hypothesis directly.

A final mechanism, which is perhaps the most interesting, is one in which binding of immobilized complexes by Fc receptors triggers a central "off" signal to the cell rather than interfering with the binding of the mitogen. This signal could be generated by a capping event or simply because the cell responds more efficiently to the complexes when they are presented as repeating subunits on a fixed matrix. An example of such a signal would be the raising of the intracellular ratio of cAMP to cGMP (24). This mechanism could also explain specific feedback inhibition of antibody synthesis if it is assumed that a specificity component is present (e.g., surface Ig). Thus, surface Ig may concentrate complexes on the appropriate cells via binding to the antigen. The Fc receptors could then bind to the same complexes via the antibody leading to the formation of a matrix which could trigger an off signal. The immobilized complexes on culture dishes may be mimicking this hypothetical *in vivo* mechanism by triggering an off signal for DNA synthesis.

There is evidence that prevention of cell-cell contact will inhibit mitogenesis (25). In our system it might be argued that binding of cells to the complexes could prevent cellular interaction, but with 5×10^5 cells/well, more than 50% of the cells can be observed to be in contact with other cells. It is also clear that the great majority of the cells are not firmly adherent to the substrate, as they may be removed quite easily (Table III).

The role of macrophages in mitogenic responses is poorly defined. Since the macrophage appears to possess Fc receptors (5) and there exists evidence that LPS comes in contact with macrophages first and is subsequently transferred to lymphocytes (26), it is possible that the observed inhibition takes place at the level of macrophage-mitogen or macrophage-lymphocyte interaction.

The Fc receptor has been regarded as a B-lymphocyte marker, and it was not expected that the T-cell mitogen, Con A, would be inhibited by immune com-

plex-coated plastic. This result was puzzling until we learned of recent experiments³ that have defined an Fc receptor-positive subpopulation of T cells which is Con A reactive. The Fc-negative T cells are Con A unreactive and appear to contain the PHA-responsive group. These findings correlate with the present results and suggest that the binding of immobilized complexes to the Fc receptors of Con A-responsive T cells inhibits mitogenesis by a mechanism(s) similar to those outlined above for the B cell.

The recent demonstration of a close association between Fc receptors and Ia antigens on the surface of B lymphocytes (17) suggests that there may be an important role for Fc receptors in the control of the immune response. The exact nature of the relationship between Ia antigens and Fc receptors is still unknown. It is of interest that Niederhuber et al.⁴ have found that anti-Ia antisera show an inhibitory effect on the stimulation of mouse spleen cells by LPS. Since we have shown that immobilized antigen-antibody complexes can exert a similar inhibitory effect, a functional correlation between Ia and Fc receptors may be emerging.

Summary

Mouse spleen cells, cultured on surfaces coated with antigen-antibody complexes, are inhibited from responding to the B-cell mitogens, lipopolysaccharide, lipid A, Pneumococcal polysaccharide SIII, and poly I:C. The response to the T-cell mitogen, concanavalin A, is also substantially inhibited by immobilized antigen-antibody complexes, but specific inhibition of the response to phytohemagglutinin is minimal. Control experiments showed that immobilized complexes prepared from IgG F(ab')₂ fragments and IgA antibodies (both of which fail to bind to Fc receptors when complexed to antigen) did not show significant inhibitory activity when compared with the inhibition observed with complexes prepared from whole IgG. Suspensions of antigen-antibody complexes prepared from the same antigen and intact IgG antibody did not inhibit mitogenesis. None of the mitogens used could be demonstrated to compete with the binding of aggregated immunoglobulin to the B-cell Fc receptor. It appears that the interaction of Fc receptor-bearing lymphocytes and/or macrophages with immobilized complexes prevents lymphocyte activation by mitogens. It is suggested that the mechanism(s) involved may be relevant to antibody feedback control of the humoral immune response.

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