Fc RECEPTOR-MEDIATED INHIBITION OF MURINE B-LYMPHOCYTE ACTIVATION

By JOHN L. RYAN and PIERRE A. HENKART

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

The lymphocyte Fc receptor has been demonstrated on B cells and a subpopulation of T cells in the mouse (1), but the functional role of this receptor is not yet clearly established. Since there is evidence that the ability of immune complexes to inhibit antibody responses is dependent on an intact antibody Fc fragment (2), we have considered the possible role of the Fc receptor in this inhibition. Moller and Coutinho (3) has shown that soluble antigen-antibody complexes do not affect the action of polyclonal B-cell activators. Previous data from this laboratory, however, demonstrated that immobilized antigen-antibody complexes have the ability to inhibit mitogenesis, and that the inhibition is dependent on an intact Fc fragment and is not mediated by soluble complexes (4). In the present report, we have extended these experiments to investigate the mechanism of the inhibition of DNA synthesis in mitogen-stimulated lymphocytes and to show that under certain conditions the in vitro generation of plaque-forming cells (PFC)[1] by LPS may also be inhibited by immobilized complexes.

Materials and Methods

Mice. BALB/c mice, 6–10-wk old, were obtained from the Animal Production Unit, National Institutes of Health. Congenitally athymic *nu/nu* BALB/c mice were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass.

Mitogens. 8 bromo-3′,5′-cyclic guanosine monophosphoric acid (8 BrcGMP) was obtained from Sigma Chemical Co., St. Louis, Mo. Lipopolysaccharide (LPS) was obtained from Difco Laboratories, Detroit, Mich. (*Escherichia coli* 0111:B4 or *E. coli* 055:B5, prepared by the Westphal technique).

Cell Preparation. Mouse spleens were removed aseptically, teased with sterile forceps, and dispersed by passage through a no. 20 hypodermic needle five times. The cells were filtered through sterile nylon gauze to remove clumps, washed once in balanced salt solution (BSS), and suspended in culture medium. Sheep red blood cells (SRBC) were obtained from the National Institutes of Health animal farm, stored in Alsever's solution, and washed in BSS twice before use in cultures.

Culture Conditions. A modified Mishell-Dutton culture system was used. 5×10^5 cells/well were cultured in 5% CO_2 at 37°C for 4 days in 200 μl of medium in flatbottom Microtest II plates, Falcon Plastics, Oxnard, Calif. The microtiter plates were first coated with mouse serum albumin (MSA), modified by trinitrobenzenesulfonic acid to put trinitrophenol (TNP) groups on the protein, and then coated with rabbit anti-dinitrophenol-bovine serum albumin obtained from Miles Laboratories, Kanakee, Ill. Details of this procedure for creating a surface of immobilized

[1] *Abbreviations used in this paper:* 8 BrcGMP, 8 bromo-3′,5′-cyclic guanosine monophosphoric acid; BSS, balanced salt solution; FCS, fetal calf serum; LPS, lipopolysaccharide; MSA, mouse serum albumin; PFC, plaque-forming cells.

complexes have been published (4). No feeding or rocking was used during the culture period. Each sample was set up in a complete row (12 replicates), and all replicates were combined when harvested. Standard Mishell-Dutton medium with 5×10^{-5} M 2-mercaptoethanol supplementation was used. Viability varied between 25 and 50% after 4 days in culture. In many experiments the FCS was omitted or replaced with 0.5% syngeneic normal mouse serum which was prepared fresh each day.

Assay. Anti-SRBC plaques were counted in a modified Jerne plaque assay (5). Mitogen experiments were pulsed with [^{3}H]thymidine and harvested on day 3 as described previously (4).

Antibody Preparations. F(ab')$_2$ fragments from affinity purified rabbit anti-TNP antibodies were prepared with pepsin as before (4). Soluble complexes were prepared by incubating TNP_{33}MSA with rabbit anti-TNP IgG in fourfold antigen excess (determined by a precipitin curve) for 1 h at 37°C.

Results

Kinetics of the Inhibition of LPS Mitogenic Activity by Immobilized Complexes. The activity of mitogens acting on cells bearing the Fc receptor was shown to be profoundly inhibited by immobilized complexes when mitogenesis was assayed on day 3 of the culture period (4). The kinetics of this inhibition of mitogenesis by the B-cell mitogen LPS has been examined (Fig. 1). These results show that the mitogenic activity is inhibited throughout a culture period of 7 days. Thus the previously observed inhibition on day 3 was not due to an alteration in the peak time of response to LPS.

Inhibition of the Mitogenic Effect of 8 BrcGMP. Derivatives of cGMP have been shown to induce DNA synthesis in mouse B lymphocytes (6). Though there is no experimental evidence, one may speculate that this effect may reflect an intracellular increase in cGMP, a proposed intracellular regulator of cell division. Since such a mitogenic mechanism would not obviously involve cell surface receptors, the effect of immobilized complexes on thymidine incorporation induced by 8 BrcGMP was tested. A striking inhibition of the mitogenic effect of this compound was observed over a wide concentration range (Fig. 2).

Inhibition of LPS Mitogenesis in Athymic Mice. One possible interpretation of the inhibition of mitogenesis by immobilized complexes is that an Fc receptor-positive T cell (7) may become activated to become an effective suppressor cell capable of inhibiting mitogenesis in B cells. Since such suppressor T cells have been found lacking in congenitally athymic (*nu/nu*) mice (8), the inhibition of LPS-induced mitogenesis was tested with spleen cells from these mice. The degree of inhibition by immobilized complexes in these cultures was comparable to that achieved using normal spleen cells (Fig. 3) (4) and was entirely dependent on an intact Fc fragment in the antibody molecule. Thus it appears that suppressor T cells are unlikely to play a role in mediating the observed inhibition.

Effect of Immobilized Antigen-Antibody Complexes on the LPS-Induced Polyclonal Antibody Response. LPS has been shown to be a potent polyclonal B-cell activator in that it can induce B cells to differentiate nonspecifically into antibody-secreting cells (9). It was of interest to see if this activation was inhibited by immobilized complexes in a manner similar to that observed for mitogenesis. When the cultures were carried out in the absence of serum, the generation of PFC was inhibited an average of 76% (Table I). Inhibition was observed in every experiment and was highly significant. Immobilized com-

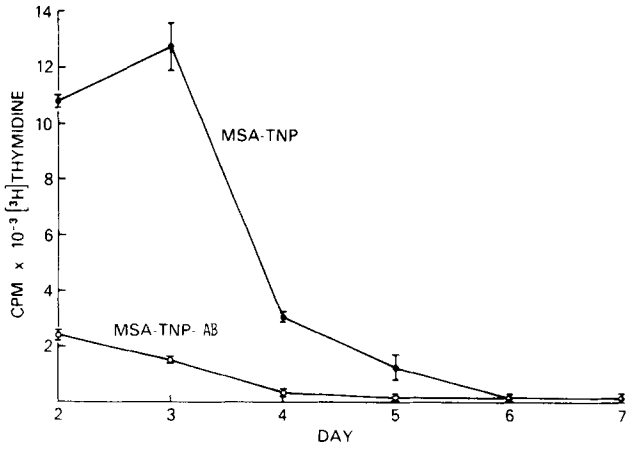

FIG. 1. Kinetics of LPS mitogenesis and its inhibition by immobilized complexes. Identical cultures were set up and harvested on specified days. Each culture was pulsed with 1 μCi [^{3}H]thymidine (2 Ci/mM) 24 h before harvesting. Thymidine incorporation was measured in the presence of immobilized antigen (●), (MSA-TNP), and immobilized antigen-antibody complexes (○), (MSA-TNP-AB). Error bars indicate the standard error of the mean of quadruplicate wells.

plexes made with F(ab')$_2$ antibodies had an insignificant effect (11% inhibition). Soluble complexes, used at a concentration of 5 μg/ml of antibody, had a similar insignificant effect (15% inhibition).

When similar cultures were carried out in medium containing 0.5% syngeneic normal mouse serum, the PFC response and its inhibition (90%) were similar to those observed in serum-free medium (Table I). Heat-inactivated (56°C, 30 min) fresh mouse serum could also be used to support cultures, with similar inhibition observed. This was tested to rule out possible inhibitory effects of complement binding to the C3 receptor after being activated by the immobilized complexes. If medium containing 5% FCS was used, however, the results were markedly different. In this case, the PFC response to LPS was similar to that obtained in serum-free medium and with 0.5% NMS, but no significant inhibition by immobilized complexes was observed.

Since all previous experiments demonstrating the inhibition of mitogenesis had been carried out in the absence of added serum, it was of interest to examine the effect of xenogeneic serum (fetal calf serum, FCS) on this process. Table II shows an experiment in which LPS stimulation was carried out in medium containing 5% FCS. The background level of thymidine incorporation in the absence of added LPS was approximately 10 times that observed in serum-free medium. In the presence of immobilized complexes this background was reduced nearly 50%. The LPS-stimulated incorporation levels were unaffected by immobilized complexes.

In other experiments (data not shown) it was found that a SRBC-induced PFC response could only be obtained in the presence of 5% FCS, but not in 0.5% NMS or serum-free medium. Thus we were unable to effectively test the ability of immobilized complexes to inhibit antigen-induced PFC in medium where inhibition of proliferation could be demonstrated. There was no inhibition of the SRBC-induced response in FCS containing cultures.

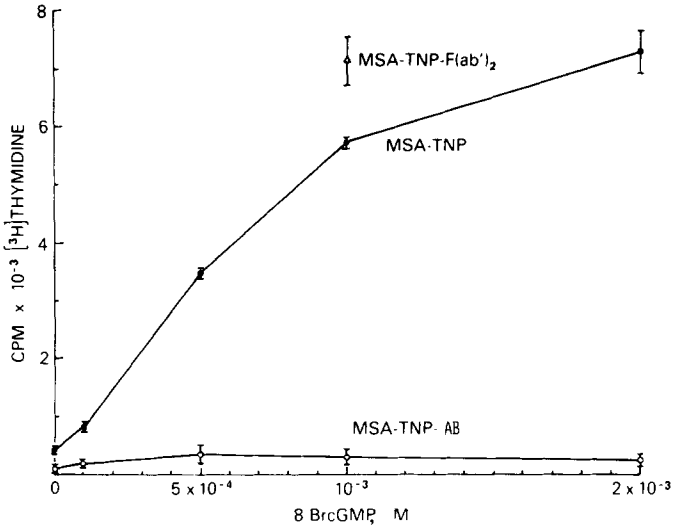

FIG. 2. Stimulation of spleen cells by 8 BrcGMP. Cells were cultured in the presence of immobilized complexes (MSA-TNP-AB), (○); and in the presence of immobilized antigen (MSA-TNP), (●). $F(ab')_2$ control is shown (MSA-TNP-$F(ab')_2$), (△). Cells were pulsed for 24 h with 1 μCi [^{3}H]thymidine (2 Ci/mM) and harvested on day 3.

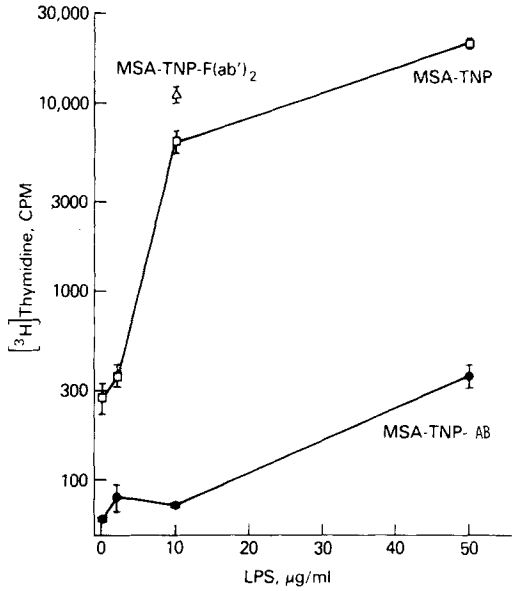

FIG. 3. Stimulation of athymic (*nu/nu*) spleen cells by LPS. Cells were cultured in the presence of immobilized complexes (MSA-TNP-AB), (○), and in the presence of immobilized antigen (MSA-TNP), (●); $F(ab')_2$ control is shown (MSA-TNP-$F(ab')_2$), (△).

The ability of immobilized complexes to inhibit B-cell activation in the presence of FCS was further examined in athymic (*nu/nu*) spleen cell cultures (Table III). The polyclonal response induced by LPS was measured in serum-free medium and in medium containing 5% FCS. In this case immobilized complexes were able to inhibit the LPS response effectively in both serum-free medium and

TABLE I
Inhibition of LPS B-Cell Activation by Immobilized Complexes

Culture conditions	0.1% BSA	0.5% NMS	5% FCS
	*PFC/10⁶**	*PFC/10⁶*	
MSA-TNP‡	0	0	21 ± 10
MSA-TNP-Ab§	0	0	23 ± 6
MSA-TNP‡ + LPS‖	59 ± 14	48 ± 19	49 ± 8
MSA-TNP-Ab§ + LPS‖	14 ± 5	4 ± 2	51 + 10

* Results are given for direct anti-SRBC PFC/10^6 recovered cells on day 4 of culture. All are ± SE for six separate experiments.
‡ Refers to culture wells with immobilized antigen (TNP-modified MSA).
§ Refers to culture wells with immobilized antigen-antibody complexes.
‖ 10 μg/ml LPS/well.

TABLE II
*Lack of Inhibition of LPS Mitogenesis in the Presence of FCS**

Culture conditions	cpm
MSA-TNP‡	15,350 ± 210
MSA-TNP‡ + LPS	29,800 ± 453
MSA-TNP-Ab§	8,714 ± 112
MSA-TNP-Ab§ + LPS	27,420 ± 846

* DNA synthesis measured between 48–72 h. 10 μg/ml LPS added to appropriate wells. cpm are ± SE.
‡ Refers to culture wells with immobilized antigen.
§ Refers to culture wells with immobilized antigen-antibody complexes.

in the presence of 5% FCS. Thus FCS may interfere with the inhibition of normal spleen cells by immobilized complexes, but does not abrogate the inhibition of athymic (*nu/nu*) spleen cell cultures. When 1:1 mixtures of *nu/nu* spleen cells and normal BALB spleen cells were tested, it was found that immobilized complexes did not exert a significant inhibitory effect in the presence of 5% FCS (data not shown).

Discussion

These results demonstrate that the inhibition of LPS-induced mitogenesis by immobilized antigen-antibody complexes is present throughout the culture period and is probably not due to suppressor T cells. Since it has been shown that all B-cell activators tested are inhibited by immobilized complexes (4), one may argue that blocking of surface receptors is not the mechanism of inhibition. The experiments with 8 BrcGMP-induced mitogenesis may further support the interpretation that this Fc receptor-mediated inhibition does not operate by blocking surface receptors for mitogen, but by induction of an intracellular "off" signal. This assumes that exogenously added cGMP derivatives are able to act as intracellular signals for proliferation. This hypothesis, while supported by some data (10), cannot presently be accepted as proven.

In the absence of FCS, immobilized complexes are able to mediate the inhibition of polyclonally stimulated antibody production by normal spleen cell cultures. This inhibition is highly dependent on an intact Fc fragment in the complexes and is not mediated by soluble complexes. This confirms results

TABLE III
Inhibition of the Polyclonal Antibody Response in Athymic (nu/nu) Mice

Culture conditions	5% FCS (4 exp)	0.1% BSA (5 exp)
	*PFC/10⁶**	
MSA-TNP‡	30 ± 11	2 ± 2
MSA-TNP-Ab§	2 ± 1	0
MSA-TNP‡ + LPS‖	87 ± 12	66 ± 14
MSA-TNP-Ab§ + LPS‖	19 ± 11	23 ± 11

* PFC/10^6 recovered cells on day 4 of culture. All results are ± SE.
‡ Refers to culture wells with immobilized antigen.
§ Refers to culture wells with immobilized antigen-antibody complexes.
‖ 10 μg/ml LPS/well.

obtained earlier by other workers (3) and extends our previous work on inhibition of B-cell mitogenesis (4). When nude spleen cells were cultured it was found that they could be equally inhibited in the presence or absence of FCS. This raises many questions concerning the role of FCS in in vitro culture systems. The components of FCS which are supportive are still poorly defined (11). The difference in the ability of immobilized complexes to inhibit PFC in normal and nude spleen cell cultures in the presence of FCS suggests that the serum may be stimulating T cells present in normal spleen populations and absent in athymic spleen populations. It is this T-cell stimulation that may overcome the inhibitory effect of the complexes, since the B cells in both populations are apparently intact (12). In the presence of both FCS and the polyclonal B-cell activator LPS, the positive signals to the B cell may overcome the off signal generated by the immobilized complexes. The lack of inhibition observed when the nude and normal cells were tested as a mixture in the presence of FCS may support this interpretation. Further work with separated cell populations and separated serum components will be necessary to elucidate these phenomena.

The inhibition of the polyclonal activation of spleen cells by immobilized complexes appears to be associated with an inhibition of the proliferative response of the LPS-responsive cell population. This follows from the correlation between inhibition of mitogenesis and inhibition of PFC formation in the serum-free system and the inability of the immobilized complexes to inhibit either DNA synthesis or PFC formation in normal spleen cells in the presence of FCS. None of the experiments reported have dealt with inhibition of antigen-specific responses. It may be more difficult to demonstrate the inhibition of T-dependent responses in this system because of the ability of FCS to block the inhibition. The off signal delivered to T-cell-stimulated B lymphocytes by immobilized antigen-antibody complexes may be inadequate to turn off the B cell. Coordinated binding to both the Fc receptor and surface immunoglobulin might generate an off signal which would be sufficient to turn off the B cells involved (13).

The success that has been achieved in many in vitro systems in inhibiting antibody responses with specific antibody (14) has been suggested to be due to antigen removal. Our data would suggest that binding of complexes to B cells via their Fc receptors is critical for regulation, but that more than simple

binding of complexes is necessary. Perhaps complexes could be formed in vivo on antigen-carrying cells such as macrophages and interact with B-cell Fc receptors and surface immunoglobulin simultaneously. Clonal proliferation is an essential characteristic of immune responses and a feedback inhibition of the proliferation of a specific clone is a desirable mechanism for control.

Summary

Immobilized antigen-antibody complexes are able to inhibit the mitogenic response of murine spleen cells to the B-cell mitogen 8-bromo-3′,5′-cyclic guanosine monophosphoric acid. This inhibition is dependent on intact Fc fragments in the immobilized complexes. Soluble complexes do not mediate this inhibition. When lipopolysaccharide (LPS) activation of B cells was studied, it was found that the mitogenic response was inhibited at all times tested between 2 and 7 days of culture. Also, the LPS-induced mitogenesis of nude spleen cells was inhibited by immobilized complexes, indicating that suppressor T cells probably play no significant role in the inhibition. Immobilized complexes inhibit polyclonal antibody responses in a serum-free system and in the presence of normal mouse serum, but are unable to inhibit in the presence of fetal calf serum (FCS). If *nu/nu* spleen cells are used, however, the FCS does not block the ability of the complexes to inhibit the polyclonal response. It is suggested that antigen-antibody complexes under appropriate conditions may bind to B lymphocytes via their Fc receptors and trigger a central "off" signal which blocks proliferation and consequently antibody production.

The authors acknowledge the helpful comments of Dr. Howard Dickler, Dr. Richard Hodes, and Dr. William Terry. The meticulous animal care delivered by Mr. Walter Lyles is also greatly appreciated, as is the secretarial help of Ms. Judy Steckel.

Received for publication 10 May 1976.

References

1. Anderson, C. L., and H. M. Grey. 1974. Receptors for aggregated IgG on mouse lymphocytes. Their presence on thymocytes, thymus-derived, and bone marrow-derived lymphocytes. *J. Exp. Med.* **139**:1175.
2. Sinclair, N. R. StC. 1969. Regulation of the immune response. I. Reduction in ability of specific antibody to inhibit long lasting IgG immunological priming after removal of the Fc fragment. *J. Exp. Med.* **129**:1183.
3. Moller, G., and A. Coutinho. 1975. Role of C'3 and Fc receptors in B-lymphocyte activation. *J. Exp. Med.* **141**:647.
4. Ryan, J. L., R. D. Arbeit, H. B. Dickler, and P. A. Henkart. 1975. Inhibition of lymphocyte mitogenesis by immobilized antigen-antibody complexes. *J. Exp. Med.* **142**:814.
5. Mishell, R. I., and R. W. Dutton. 1967. Immunization of dissociated spleen cell cultures from normal mice. *J. Exp. Med.* **126**:423.
6. Weinstein, Y., S. Segal, and K. L. Melmon. 1975. Specific mitogenic activity of 8 Br-Guanosine 3′,5′-monophosphate (Br-cyclic GMP) on B lymphocytes. *J. Immunol.* **115**:112.
7. Stout, R. D., and L. D. Herzenberg. 1975. The Fc receptor on thymus-derived lymphocytes. II. Mitogen responsiveness of T lymphocytes bearing the Fc receptor. *J. Exp. Med.* **142**:1041.

8. Dutton, R. W. 1973. Inhibitory and stimulatory effects of concanavalin A on the response of mouse spleen cell suspensions to antigen. *J. Exp. Med.* **138**:1496.
9. Andersson, J., F. Melchers, C. Galanos, and O. Luderitz. 1972. The mitogenic effect of lipopolysaccharide on bone marrow-derived mouse lymphocytes. Lipid A as the mitogenic part of the molecule. *J. Exp. Med.* **137**:943.
10. Watson, J. 1975. The influence of intracellular levels of cyclic nucleotides on cell proliferation and the induction of antibody synthesis. *J. Exp. Med* **141**:97.
11. Shiigi, S., and R. I. Mishell. 1975. Sera and the in vitro induction of immune responses. I. Bacterial contamination and the generation of good fetal bovine sera. *J. Immunol.* **115**:741.
12. Kindred, B. 1971. Antibody response in genetically thymus-less nude mice injected with normal thymus cells. *J. Immunol.* **107**:1291.
13. Sidman, C. L. 1976. Inactivation of B lymphocytes by interaction with ligands. *Fed. Proc. Abst.* **35**:820.
14. Kappler, J. W., A. vander Hoven, U. Dharmarajan, and M. Hoffman. 1973. Regulation of the immune response. IV. Antibody-mediated suppression of the immune response to haptens and heterologous erythrocyte antigens in vitro. *J. Immunol.* **111**:1228.