PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE **174**, 229–238 (1983)

Antigen–Antibody Complex Binding and Cell Interaction in Stimulating Normal Rabbit Lymphocytes (41730)

LEE S. F. SODERBERG

Department of Microbiology and Immunology, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205

Abstract. Complexes of antigen with specific antibody have been shown to enhance or suppress the specific antibody response *in vivo. In vitro,* antigen–antibody (Ag–Ab) complexes prepared in a slight antigen excess with rabbit antibody induced proliferation of unprimed rabbit lymphocytes. The Ag–Ab stimulated cells from a number of different normal lymphoid organs, including peripheral blood, bone marrow, spleen, and lymph node, but not thymus. Cells exposed to Ag–Ab for 1 hr and washed, bound Ag–Ab through Fc and complement receptors (CR), but were not induced to proliferate unless additional Ag–Ab was added. Specific antigen, which was otherwise unstimulatory, interacted with Ag–Ab-coated cells to activate them, probably through the cross-linking of membrane-bound ligands. Proliferation stimulated by Ag–Ab involved the interaction of three bone marrow cell subpopulations; a macrophage-enriched, a B-cell-enriched, and an $mIgM^-$ cell-enriched population. The separated subpopulations were poorly responsive to Ag–Ab stimulation, even though Ag–Ab bound to cells in each of the populations. Low levels of responsiveness to Ag–Ab also resulted when any two of the three subpopulations were combined. Only when all three subpopulations were mixed, was stimulation equivalent to the levels of stimulation reached by unseparated cells.

Regulation of antibody synthesis through circulating specific antibody complexed with residual or secondary antigen has been recognized for some time (1, 2). Antibody administered at about the same time as, or complexed to, antigen has been shown to inhibit (2, 3) or augment (4–6) specific antibody synthesis. The ratio of antigen to antibody in complex has been shown to be critical to whether the antibody response is enhanced or inhibited, (4, 7, 8) as has the immunoglobulin class of the antibody in complex (9). Both primary (10) and secondary (11) antibody responses have been reported to be regulated by specific antibody.

While the studies of stimulatory effects of Ag–Ab on antibody formation have been done *in vivo,* reports of *in vitro* proliferative responses to Ag–Ab have also appeared. Moller (12) and Uhr and associates (13) found that Ag–Ab stimulated human peripheral blood leukocytes to proliferate, but disagreed on whether stimulation was dependent on complement (13) or not (12). Our investigations have shown that lymphocytes from unprimed rabbits were stimulated by Ag–Ab and that the response was complement-dependent (8). Both Fc receptors (FcR) (14, 15) and complement receptors (CR) (16) have been proposed as lymphocyte triggering sites. Purified murine Fc fragments acting through FcR were found to be mitogenic and enhance specific antibody responses (15). Complement and CR apparently do not have roles in Fc fragment-mediated lymphocyte activation, but they might function in activation by intact Ag–Ab (5, 8).

In the regulation of secondary antibody responses, Ag–Ab was found to stimulate primed T helper cells more than primed B cells (17). Interaction of Ag–Ab with T cells might be through antigen receptors or, with some T cells, FcR (18). B cells have been demonstrated to bind Ag–Ab preferentially through CR (19), but it is not clear whether binding alone can lead to stimulation.

In the present paper we further investigated the role of Ag–Ab in the *in vitro* stimulation of unprimed lymphocytes. A variety of Ag–Ab and different lymphoid cell populations were tested. Cell binding of Ag–Ab, membrane-bound ligand cross-linking and different cell types were examined for their roles in Ag–Ab-induced stimulation.

Materials and Methods. *Animals.* Unimmunized, 4–5 lb rabbits (New Zealand white,

0037-9727/83 $1.50

Laboratory Animal Medicine, UAMS, Little Rock, Ark.) were used as the source of normal lymphoid cells. Such rabbits had no detectable circulating antibodies to the antigens used and *in vitro* exposure of their lymphocytes to the antigens did not result in proliferation above background. Female mice (BALB/c, 19–25 g), were obtained from Jackson Laboratories (Bar Harbor, Me.).

Cell culture. Peripheral blood leukocytes (PBL), used in most experiments, were prepared according to the method of Boyum (20). Briefly, freshly drawn rabbit blood was heparinized (Schwarz/ Mann, Orangeburg, N.Y.) to a concentration of 16 units/ml and diluted in 2 vol of RPMI 1640. The diluted blood was layered over Ficoll (6.35%)–Hypaque (10%) and centrifuged at 400*g* for 40 min. The cells at the interface, which were predominantly mononuclear cells, were collected and washed three times in RPMI 1640. Cells were maintained in 96-well culture plates at 2×10^5 cells per well in RPMI 1640 supplemented with 2.5% fresh, autologous serum, 10 m*M* Hepes, and 2 m*M* glutamine unless otherwise indicated. Cultures were incubated at 37°C in 5% CO_2.

Cells were also prepared from spleen, thymus, femoral bone marrow, and popliteal lymph nodes. The organs were excised, teased, and cell clumps were allowed to settle out for 5 min on ice. Single cell supensions so obtained were washed three times in RPMI 1640 and cultured at various cell concentrations in microwell plates.

Lymphocyte separation. Unprimed rabbit bone marrow cells were separated into subpopulations by adherence to plastic or nylon wool. Cells which could not be readily dislodged from the plastic surfaces of microwell culture plates after 1 hr incubation at 37°C were 60% phagocytic for zymosan particles. Such cells were predominantly mononuclear and were designated the macrophage-enriched population. Cells were separated by adherence to nylon wool using the method of Handwerger and Schwartz (21). Briefly, bone marrow cells were incubated 45 min at 37°C in a sterilized column containing nylon wool. Cells, which could be eluted from the column with RPMI 1640, were 83% nonmembrane immunoglobulin M-bearing ($mIgM^-$) as determined by staining with fluorescent goat anti-rabbit IgM. Cells, which could be forcibly extruded from the columns by compressing the nylon wool, were 61% $mIgM^+$ cells. Such cell populations were cultured alone, in combination at equal proportions, or added to culture wells containing plastic adherent macrophages to final concentrations of 2×10^5 lymphocytes per well.

Immune complex preparation. Specific antiserum to diphtheria toxoid (Connaught Laboratories, Toronto, Ontario) was prepared in New Zealand white rabbits, by footpad injection of 150 Lf toxoid (1 Lf = 2.5 μg toxoid protein) in Freund's complete adjuvant (Difco Laboratories, Detroit, Mich.). Sera containing more than 10 units/ml antitoxin as determined by passive hemagglutination were collected 4 to 8 weeks later. Booster injections of 150 Lf toxoid into the hind footpads maintained high titers for many months.

Specific antibody was precipitated by the appropriate toxoid at the concentration empirically found to be most stimulatory to PBL. For diphtheria toxoid–antitoxin, which was used in most experiments, a ratio of 0.83 μg diphtheria toxoid protein/μg antitoxin, as determined by the method of Stavitsky (22) was found to be most stimulatory. Such an antigen–antibody mixture produced a molar ratio of two antibodies per antigen molecule and was slightly into antigen excess. Other ratios of antibody to antigen gave no stimulation or much lower stimulation. The precipitate was washed three times in saline and dissociated in 0.2 *M* glycine, pH 2.8, before filter sterilization. After 24 hr of cell culture, 20 μl of dilutions of the dissociated Ag–Ab solution was added to triplicate test cultures and 20 μl acid-glycine was added to control cultures. The most stimulatory concentration of diphtheria toxoid–antitoxin was 1.3 μg/ml. Addition of the complex in dissociated form was deemed the best way to dispense Ag–Ab accurately. The Hepes-buffering system and elevated CO_2 atmosphere used in culture, maintained the pH at neutrality after the addition of acid-glycine. Neither the acid-glycine nor the dissociated form of the Ag–Ab affected stimulation, since the Ag–Ab precipitate suspended in saline was equally as stimulatory as the dissociated Ag–Ab mixture. The proliferative

response was measured by the incorporation of [*methyl*-^{3}H]thymidine (Schwarz/Mann) 0.2 μCi/ml, over the final 18 hr of culture.

Tetanus toxoid (Connaught Labs, Toronto, Ontario) keyhole limpet hemocyanin (KLH) (Pacific Biomarine Labs, Inc., Venice, Calif.) dinitrophenylated-KLH (DNP-KLH), and DNP-bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, Mo.) Ag–Ab were prepared in a manner similar to the preparation described above for diphtheria toxoid Ag–Ab.

Ag–Ab binding cells. To demonstrate the binding of Ag–Ab, immune complexes were prepared as described above using rhodamine-labeled antitoxin. Antitoxin was labeled with rhodamine isothiocyanate by the method of Coons and Kaplan (23). Cells were stained with fluorescein-labeled goat anti-rabbit IgM and then incubated for 5 min with rhodamine-labeled Ag–Ab. Stained and double-stained cells were enumerated using a Zeiss fluorescence microscope.

Rossette-forming cells (RFC). To demonstrate FcγR, washed ox erythrocytes (E) were coated (EA) with a nonagglutinating dose of rabbit IgG anti-E, washed, and mixed with lymphocytes at a ratio of 20 EA per lymphocyte (24). The cell mixture was pelleted at 400*g* for 10 min, incubated for 1 hr on ice, gently resuspended, and stained with a drop of euchrysine. Lymphocytes binding three or more EA were scored as RFC. To demonstrate CR, EA were prepared using rabbit IgM anti-E (Pel-Freez, Rogers, Ark.), and were then incubated with 1/10 diluted fresh mouse serum for 1 hr at 37°C before rosetting. The CR rosetting procedures were similar to those used for FcγR rosettes.

Results. *Stimulation of normal rabbit lymphoid cells with Ag–Ab.* Normal rabbit lymphocytes were stimulated to proliferate by Ag–Ab prepared with various antigens (Table I). The levels of tritiated thymidine incorporation reported, represent the proliferation produced by the optimal antigen to antibody ratio and the optimal concentration of each Ag–Ab from a series of concentrations. Complexes prepared from diphtheria toxoid and DNP-BSA were most stimulatory, but all Ag–Ab tested substantially increased proliferation. Cultures treated with equivalent concentrations of antigen alone or antibody alone induced only background levels of [^{3}H]thymidine incorporation.

TABLE I. STIMULATION OF UNPRIMED LYMPHOCYTES BY VARIOUS Ag–Ab COMPLEXES

Ag used in Ag–Ab[a]	Control[b]	Ag–Ab[c]	e/c[d]
Diphtheria toxoid	857 ± 146	4669 ± 592	5.45
Tetanus toxoid	400 ± 35	1355 ± 73	3.39
KLH	595 ± 230	2193 ± 132	3.69
DNP-KLH	785 ± 160	3017 ± 402	3.84
DNP-BSA	785 ± 160	4956 ± 1196	6.31

[a] KLH, keyhole limpet hemocyanin; DNP-KLH, dinitrophenyl-KLH; DNP-BSA, DNP-bovine serum albumin.

[b] Unprimed rabbit peripheral blood lymphocytes were cultured for 7 days in RPMI 1640. Results are reported as counts per minute (cpm) ± standard error of triplicate cultures labeled with [^{3}H]thymidine for the final 18 hr of culture.

[c] Optimally stimulatory dose of Ag–Ab of the designated specificity reported as cpm ± standard error of triplicate cultures.

[d] Stimulation index: (experimental (cpm))/(control (cpm)).

Proliferation was highly dependent upon the ratio of antigen to antibody as shown in Table II. The optimal ratio of 2.3 μg diphtheria toxoid protein and 2.8 μg specific antibody protein was slightly into antigen excess, as determined by quantitative precipitation. Proliferation of cells cultured with antibody alone

TABLE II. STIMULATION OF UNPRIMED LYMPHOCYTES BY Ag–Ab AT VARIOUS RATIOS

Antigen (μg/ml)[a]	Antibody (μg/ml)[b]	cpm[c]
0	0	1532 ± 214
0	5.6	2060 ± 137
2.3	0	1353 ± 49
2.3	0.7	2210 ± 119
2.3	1.4	3123 ± 156
2.3	2.8	7082 ± 307
2.3	5.6	5503 ± 278
2.3	11.2	1316 ± 76

[a] Diphtheria toxoid was added to cultures at 24 hr.

[b] Hyperimmune rabbit antiserum containing the indicated amounts of specific anti-diphtheria toxoid antibody protein was added to cultures at 24 hr.

[c] Normal rabbit peripheral blood lymphocytes were maintained in RPMI 1640 with normal rabbit serum, added to adjust all cultures to 6.6% serum. Results were reported as counts per minute ± standard error of quadruplicate cultures labeled with [*methyl*-^{3}H]thymidine over the final 18 hr of 5-day cultures.

TABLE III. STIMULATION WITH Ag–Ab OF UNPRIMED LYMPHOCYTES FROM VARIOUS SOURCES

	Optimal No. cells/culture[a]	Control[b]	Ag–Ab[c]	e/c[d]
Rabbit				
Peripheral blood	2×10^5	265 ± 30	2612 ± 310	9.86
Bone marrow	3×10^5	1086 ± 59	9042 ± 981	8.33
Spleen	8×10^5	1036 ± 66	2566 ± 105	2.48
Lymph node	4×10^5	366 ± 3	1484 ± 311	4.05
Thymus	2×10^5	109 ± 43	146 ± 52	1.34
Mouse (BALB/cJ)				
Spleen	4×10^5	762 ± 3	1729 ± 300	2.27
Bone marrow	2×10^5	1620 ± 200	3480 ± 260	2.15
Thymus	3×10^5	64 ± 25	102 ± 17	1.59

[a] Single cell suspensions were maintained for 7 days in RPMI 1640 and labeled with [^{3}H]thymidine for the last 18 hr of culture.
[b] cpm ± standard error of triplicate cultures.
[c] Diphtheria toxoid plus specific rabbit antibody.
[d] Stimulation index: (experimental (cpm))/(control (cpm)).

or with antigen alone were not significantly (t test) different from that of control cultures.

Cell suspensions from various lymphoid organs obtained from normal rabbits or mice were stimulated to proliferate by Ag–Ab (Table III). Rabbit PBL and bone marrow cells showed particularly high levels of proliferation after stimulation with Ag–Ab. Spleen cell and lymph node cell populations also contained cells that proliferated in response to Ag–Ab. Mouse spleen and bone marrow cells were stimulated only marginally by Ag–Ab and, therefore, only rabbit cells were used in subsequent experiments. Thymus cells from both rabbits and mice were unresponsive to Ag–Ab stimulation.

Binding of Ag–Ab to lymphocytes. To examine the relationship of Ag–Ab binding to lymphocyte stimulation, rabbit PBL were incubated for 1 or 24 hr with Ag–Ab, washed free of unbound complexes, and then exposed again to Ag–Ab. As shown in Table IV, cells preincubated with Ag–Ab for 1 hr without further treatment showed only a slight increase (1.85-fold) in the level of proliferation. When cells were preincubated with Ag–Ab for 24 hr and washed, stimulation was greater than 5-fold. The level of proliferation of cells pretreated with Ag–Ab for 24 hr was not different from the levels reached when cells were stimulated with Ag–Ab without washing away unbound Ag–Ab. This suggested that the cells were irreversibly stimulated by 24 hr.

Even though the 1 hr preincubation with Ag–Ab did not result in very much stimulation, it did affect the responsiveness of the cells. The addition of Ag–Ab, immediately or 24 or 48 hr after the 1 hr pretreatment with Ag–Ab, resulted in higher levels of stimulation than achieved by cells that were not preincubated with Ag–Ab. Specific antigen, which was not stimulatory to untreated cells, was stimulatory (4.5-fold) to cells pretreated for 1 hr with Ag–Ab, suggesting an interaction with membrane-bound Ag–Ab. Heterologous antigen, tetanus toxoid, did not stimulate cells pretreated for 1 hr with the Ag–Ab prepared with diphtheria toxoid. The stimulatory activity of the specific antigen declined dramatically when its addition was delayed 24 or 48 hr after the 1-hr pretreatment.

Cells, which were preincubated with Ag–Ab for 24 hr, washed, and then stimulated with an additional dose of Ag–Ab or specific antigen, showed substantially higher levels of stimulation (8-fold) than cells that were not petreated (5-fold). All cultures received an optimally stimulatory dose of Ag–Ab and higher doses of Ag–Ab have been shown to give lower, not higher, responses (8).

Membrane receptors, which have been implicated in lymphocyte triggering (14–16),

TABLE IV. RESPONSES OF CELLS PREINCUBATED WITH Ag–Ab TO Ag–Ab OR SPECIFIC ANTIGEN

	Time of preincubation[a]		
	None	1 hr	24 hr
No second additives	857 ± 146	1585 ± 141 (1.85)	4862 ± 501 (5.67)
Ag–Ab added at[b]			
1 hr	3838 ± 239 (4.48)	4613 ± 91 (5.38)	—
24 hr	4669 ± 592 (5.45)	5563 ± 282 (6.49)	7497 ± 446 (8.75)
48 hr	2509 ± 268 (2.93)	8803 ± 1408 (10.27)	—
Specific Ag added at[c]			
1 hr	749 ± 72 (0.92)	3873 ± 669 (4.52)	—
24 hr	492 ± 70 (0.49)	2007 ± 423 (2.34)	7132 ± 610 (8.32)
48 hr	851 ± 148 (0.99)	1268 ± 493 (1.45)	—
Heterologous Ag added at[d]			
1 hr	556 ± 8 (0.65)	831 ± 59 (0.97)	—

[a] Unprimed rabbit PBL were exposed to 1.3 μg/ml diphtheria toxoid–antitoxin (Ag–Ab) for 1 or 24 hr. Unbound Ag–Ab was removed by washing. The levels of incorporation of [^{3}H]thymidine over the final 18 hr of 7 days culture are reported as the mean cpm ± standard error of triplicate cultures. Numbers in parentheses indicate stimulation index: experimental cpm/control cpm.

[b] Diphtheria toxoid–antitoxin (Ag–Ab) (1.3 μg/ml) was added to cultures at 1, 24, or 48 hr.

[c] Diphtheria toxoid (specific Ag) (0.6 μg/ml) was added to cultures at 1, 24, or 48 hr.

[d] Tetanus toxoid (heterologous Ag) (0.6 μg/ml) was added to cultures at 1 hr.

were assayed by rosetting. Untreated rabbit PBL were found to have an average of 14% FcγR$^+$ cells and 12% CR$^+$ cells (Table V). Treatment of the cells with Ag–Ab for 1 hr followed by washing, totally blocked rosetting for both FcγR and CR. With bone marrow cells, which were normally 39% FcγR$^+$ and 25% CR$^+$, rosettes for FcγR and CR were likewise completely blocked by Ag–Ab treatment. When PBL were maintained in culture for 7 days, the levels of FcγR$^+$ cells dropped, while the levels of CR$^+$ cells remained unchanged. Cells, which were stimulated with Ag–Ab, cultured for 7 days and washed, were enriched in both FcγR$^+$ and CR$^+$ cells. CR$^+$ cells comprised one-third of all cultured PBL after stimulation with Ag–Ab.

In contrast to PBL, untreated bone marrow cells lost most of their CR rosetting activity in culture without a similar drop in the proportion of FcγR$^+$ cells. This difference between PBL and bone marrow cells cannot be explained at present. Cultures of bone marrow cells stimulated with Ag–Ab contained only one-third the number of FcγR$^+$ cells in untreated cultures, but the number of CR$^+$ cells was increased in stimulated cultures. Because of variation in rosetting levels from experiment to experiment, the means reported obscured the actual CR increases, which in five experiments averaged 9.4-fold over the number of CR$^+$ cells in control cultures.

Three bone marrow cell populations contribute to Ag–Ab stimulation. To try to better

TABLE V. CELL SURFACE RECEPTORS BEFORE AND AFTER 7 DAYS IN CULTURE

	Before culture[a]	After culture[b]	Percentage RFC	
			Untreated	Ag–Ab treated
PBL				
FcγR	+		14.4 ± 4.2	0
		+	6.6 ± 1.0	17.1 ± 2.3
CR	+		11.9 ± 1.5	0
		+	11.6 ± 2.5	32.0 ± 7.2
Bone marrow				
FcγR	+		38.9 ± 6.6	0
		+	35.6 ± 6.1	13.3 ± 2.7
CR	+		25.5 ± 4.3	0
		+	4.2 ± 2.5	16.7 ± 3.4

[a] Single cell suspensions, untreated or exposed to 1.3 μg/ml for 60 min and washed, were used for rosetting assays for Fcγ and complement receptors.

[b] Cells, untreated or stimulated, with 1.3 μg/ml Ag–Ab, were maintained for 7 days in RPMI 1640 with 2.5% autologous serum, washed, and then used for rosetting assays for Fcγ and complement receptors.

characterize the cell populations involved in Ag–Ab-mediated stimulation, rabbit bone marrow cells were separated into subpopulations on the basis of adherence to plastic or nylon wool. Plastic-adherent cells were enriched (60%) in mononuclear phagocytes (macrophages). Virtually all of the macrophages bound rhodamine-labeled Ag–Ab. Lymphocytes were separated by nylon wool into nonadherent cells, which were 83% $mIgM^-$, and adherent cells which could be dislodged from the nylon wool, that were enriched (61%) in $mIgM^+$ (B) cells. All of the $mIgM^+$ cells and 44% of the $mIgM^-$ cells bound rhodamine-labeled Ag–Ab. However, as shown in Fig. 1, the separated $mIgM^-$ and B-cell populations responded to Ag–Ab with only slightly enhanced proliferation. The responses of each of the separated cell populations to Ag–Ab were substantially lower than the responses of cultures of unseparated cells. Proliferative responses to Ag–Ab, stimulated in cultures where the three separated populations were combined, were large and were not different from those of the unseparated cells. All cultures were maintained at 2×10^5 lymphocytes per culture. While a synergistic response was observed in cultures containing cells from each of the three cell populations, combinations of only two of the cell populations did not increase responses (Fig. 2). The addition of either the $mIgM^-$ or the B-cell-enriched population to the macrophage-enriched cells did not increase the proliferative responses to Ag–Ab. Mixing the $mIgM^-$ and the B-cell populations in the absence of macrophages also did not increase the level of proliferation.

Discussion. The present study describes the binding of Ag–Ab to normal rabbit lymphocytes and macrophages and interactions leading to increased lymphocyte proliferation. It was shown that Ag–Ab could bind to lymphocytes without activating them to proliferate. Cells treated for 1 hr with Ag–Ab and then washed free of unbound Ag–Ab were not induced to proliferate to a very great extent. Dickler *et al.* (14) reported that when murine B cells were coated with Ag–Ab prepared in antigen excess, capping of FcγR was poor unless they incubated the Ag–Ab-coated cells a second time with the specific antibody, suggesting ligand cross-linking. The stimulatory activity of purified Fc fragments (15) would, on the other hand, suggest that monovalent ligands could activate lymphocytes, but the mechanism of such activation may be different from the activation of rabbit cells by Ag–Ab, which required complement (8). In addition to Fc fragments, it has been reported that aggregated γ-globulin could be stimulatory (25) or inhibitory (26) to mouse cells. Heat-aggre-

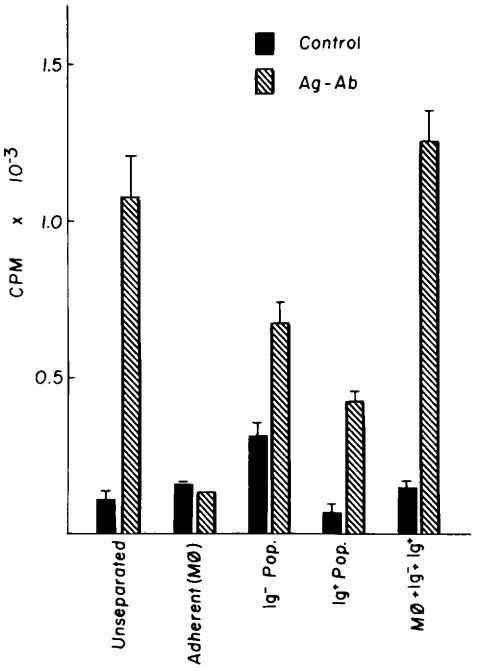

FIG. 1. Stimulation of subpopulations of rabbit bone marrow cells with Ag–Ab. Normal rabbit bone marrow cells were separated into a plastic adherent, macrophage-enriched (MØ) population; a nylon wool nonadherent, $mIgM^-$ cell-enriched population; and a nylon wool dislodgable, $mIgM^+$ cell-enriched population. All cell populations were maintained at 2×10^5 cells per well. Triplicate cultures were stimulated with 1.3 μg/ml diphtheria toxoid–antitoxin (Ag–Ab). Proliferation was measured by uptake of [*methyl*-^{3}H]thymidine over the final 18 hr of 7-day cultures. Vertical bars represent standard error.

gated rabbit γ-globulin in a wide range of doses was not stimulatory to rabbit cells (8).

Lymphocyte activation stimulated by Ag–Ab required an interaction beyond simple binding to receptors. The cells, which were treated for 1 hr with Ag–Ab and not activated, could be induced to proliferate by treatment with the specific antigen. Since the antigen was otherwise not stimulatory, it must have interacted with membrane-bound Ag–Ab to induce the response. Cross-linking FcR and antigen receptors has been shown to produce stimulatory and inhibitory signals (9, 27). Cross-linking of those receptors would make stimulation dependent upon to the availability of antigen and the Fc portion of antibody. A dependence upon the ratio of antigen to antibody was shown for the induction of proliferation (Table II) and for antibody synthesis (4, 7, 11) by Ag–Ab.

When the cells treated with Ag–Ab for 1 hr were washed and immediately exposed to a second dose of Ag–Ab they were induced to proliferate. When the second dose of Ag–Ab was delayed for 48 hr, the level of stimulation was approximately doubled. Similar high levels of stimulation occurred when cells were treated with Ag–Ab for 24 hr, washed, and then exposed to Ag–Ab or specific antigen. Such high levels of stimulation suggested that a change in cell responsiveness was induced after the first exposure to Ag–Ab, which could occur in the absence of proliferation. Several lines of evidence have suggested that stimulatory signals leading to lymphocyte proliferation could be distinguished from stimu-

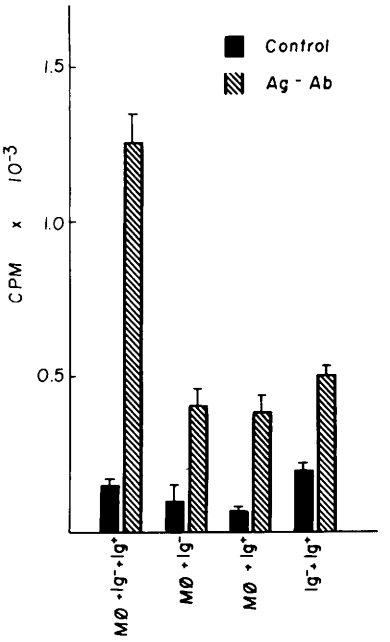

FIG. 2. Stimulation of combinations of bone marrow cell subpopulations with Ag–Ab. Combinations of subpopulations of normal rabbit bone marrow cells were stimulated with 1.3 μg/ml diphtheria toxoid–antitoxin (Ag–Ab). Cells were separated into macrophage-enriched (MØ), $mIgM^-$ cell-enriched, and $mIgM^+$ cell-enriched populations. Proliferation was measured by the uptake of [*methyl*-^{3}H]thymidine over the final 18 hr of 7-day cultures. Vertical bars indicate the standard error of triplicate cultures.

latory signals leading to cell maturation (9, 28). The higher than expected responses may, alternatively, represent the stimulation of an increased number of cells resulting from the initial treatment with Ag–Ab. An increase in the number of cells over the first 72 hr of culture, however, was not detected.

Stimulation of *in vitro* proliferation of unprimed lymphocytes by Ag–Ab was dependent upon three separable cell populations. One of these was enriched in macrophages, which have been shown to participate in most types of lymphocyte activation (29). The other two cell populations were enriched on nylon wool for B cells and $mIgM^-$ cells. Nylon wool was reported to deplete 99% of primed B cells (30) from a nonadherent rabbit spleen cell population without affecting the T-cell responses to concanavalin A (31). The B-cell population obtained in that nylon wool separation was, however, functionally contaminated with primed T cells. Since proliferation of rabbit bone marrow cells was synergistically increased only when all three cell populations were combined, functional separation was more complete than indicated by the presence or absence of mIgM. In the rabbit system, mIg has been regularly detected on cells other than B cells (32). The mouse system was not useful for the present studies because of the low levels of proliferation generated.

The requirement for three types of cells for Ag–Ab-induced proliferation of rabbit bone marrow cells correlated with studies using mature cells from other species. Thoman *et al.* (15) reported that using mice, Fc fragments could act as polyclonal B-cell activators, if macrophages and T cells were also present. The intact Ag–Ab might affect cells differently than Fc fragments, since the Fc portion of antibody has also been reported to block lymphocyte stimulation (26, 33) and complement was required for Ag–Ab stimulation of rabbit cells (8). Morgan and Weigle (34) recently reported that immune complexes, in doses 250-fold higher than those reported to be optimal here, stimulated proliferation and polyclonal antibody responses in normal murine spleen cells. These antibody responses required macrophages, B cells, and T cells, but T cells contributed little to the proliferative response. This suggested that the nylon wool nonadherent rabbit bone marrow cells, which contributed to proliferation in the present report, might have been cells other than T cells. Antigen-induced blastogenesis of human T cells required two other cell types; monocytes and CR^-, FcR^+ null (L) cells (35). While T cells may be present in rabbit bone marrow (24), the increase in CR^+ cells after Ag–Ab stimulation suggested that neither T cells nor L cells were the principal target of the proliferative signal.

Antigen–antibody complexes in stimulatory doses bound to both FcγR and CR on macrophages, B cells, and some $mIgM^-$ cells. Both FcR (14, 15) and CR (16) have been postulated to be critical initiators of lymphocyte activation. Interactions with FcR may enhance or suppress lymphocyte responses depending on the Ig class involved (9, 26). In the rabbit most FcR^+ lymphocytes are reported to be B cells (24) and B-cell precursors (36). B cells are reported to acquire CR as they mature (37), suggesting that the increase in CR^+ cells observed after Ag–Ab stimulation correlated with cell maturation. Since CR^+ B cells were found to be much more responsive to anti-Ig stimulation than CR^- cells, (38) an increase in the number of CR^+ cells could explain the higher responsiveness of Ag–Ab-coated cells which were stimulated again after 48 hr in culture.

The unresponsiveness of thymocytes to Ag–Ab could be explained by the absence of one or more of the three cell types involved. Rabbit thymus has been reported to have 0.3% cells bearing IgM or IgA, while 10% of rabbit bone marrow lymphocytes, which were highly responsive to Ag–Ab, carried thymic antigens (24). The relative absence of FcγR reported on rabbit thymocytes (24) may also be correlated with unresponsiveness to Ag–Ab.

The author gratefully acknowledges the very capable assistance of Holly R. Steinkamp and Lawrence M. Kerr. This work was supported by U.S.P.H.S. Grant AI 16685.

1. Weigle WO. Cyclical production of antibody as a regulatory mechanism in the immune response. Advan Immunol **21**:87–111, 1975.
2. Uhr JW, Moller G. Regulatory effect of antibody on the immune response. Advan Immunol **8**:81–127, 1968.

3. Zubler RH, Benacerraf B, Germain RN. Feedback suppression of the immune response *in vitro*. II. IgV-restricted antibody-dependent suppression. J Exp Med **151**:681–694, 1980.
4. Heyman B, Andrighetto G, Wigzell H. Antigen-dependent IgM-mediated enhancement of the sheep erythrocyte response in mice. Evidence for induction of B cells with specificities other than that of the injected antibodies. J Exp Med **155**:994–1009, 1982.
5. Klaus GGB. The generation of memory cells. II. Generation of B memory cells with preformed antigen–antibody complexes. Immunology **34**:643–652, 1978.
6. Terres G, Habicht GS, Stoner RD. Carrier-specific enhancement of the immune response using antigen–antibody complexes. J Immunol **112**:804–811, 1974.
7. Tite JP, Morrison CA, Taylor RB. Immunoregulatory effects of covalent antigen–antibody complexes. III. Enhancement of suppression depending on the time of administration of complex relative to a T-independent antigen. Immunology **42**:355–362, 1981.
8. Soderberg LSF, Coons AH. Complement-dependent stimulation of normal lymphocytes by immune complexes. J Immunol **120**:806–811, 1978.
9. Taylor RB. Regulation of antibody responses by antibody towards the immunogen. Immunol Today **3**:47–51, 1982.
10. Hoffman MK. Antigen-specific induction and regulation of antibody synthesis in cultures of human peripheral blood mononuclear cells. Proc Natl Acad Sci USA **77**:1139–1143, 1980.
11. Brooks KH, Feldbush TL. Generation of antibody-mediated regulation during *in vitro* clonal expansion of memory B lymphocytes. J Immunol **127**:963–967, 1981.
12. Moller G. Induction of DNA synthesis in normal mouse lymphocyte culture by antigen–antibody complexes. Clin Exp Immunol **4**:65–82, 1969.
13. Bloch-Shtacher N, Hirschorn K, Uhr JW. The response of lymphocytes from non-immune humans to antigen–antibody complexes. Clin Exp Immunol **3**:889–899, 1968.
14. Dickler HB, Kubicek MT, Finkelman FD. Interactions between lymphocyte membrane molecules. II. Characterization of an interaction between B lymphocyte surface IgD and Fc IgG receptors that differs from the surface IgM-Fc IgG receptor interaction. J Immunol **128**:1271–1277, 1982.
15. Thoman ML, Morgan EL, Weigle WO. Polyclonal activation of murine B lymphocytes by Fc fragments II. Replacement of T cells by a soluble helper T cell-replacing factor. J Immunol **125**:1630–1633, 1980.
16. Sitia R, Rabellino EM, Sorkell M, Hammerling V. A spatial association between membrane IgD and the receptor for C3b (CR_1) at the cell surface of murine B lymphocytes. J Immunol **126**:107–112, 1981.
17. Kaattari S, Scibienski RJ, Benhamini E. The immunoregulatory role of antigen–antibody complexes. I. Assessment of B and T-cell responses. Immunology **40**:9–16, 1980.
18. Janeway CA Jr, Murgita RA, Weinbaum FI, Asofsky R, Wigzell H. Evidence for an immunoglobulin-dependent antigen-specific helper T cell. Proc Natl Acad Sci USA **74**:4582, 1977.
19. Miller GW, Nussenzweig V. Complement as a regulator of interactions between immune complexes and cell membranes. J Immunol **113**:464–469, 1974.
20. Boyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest **21**(Suppl 97): 77–89, 1968.
21. Handwerger BS, Schwartz RH. Separation of murine lymphoid cells using nylon wool columns. Recovery of the B cell-enriched population. Transplantation **18**:544–548, 1974.
22. Stavitsky AB. Micromethods for the study of proteins and antibodies. II. Specific applications of hemagglutination and hemagglutination-inhibition reactions with tannic acid and protein-treated red blood cells. J Immunol **72**:368–375, 1954.
23. Coons AH, Kaplan MH. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. J Exp Med **91**:1–13, 1950.
24. Bast BJEG, Manten-Slingerland R, Roholl P, Vangraft M, Ballieux RE. Fc receptors on rabbit lymphocytes. Identification and organ distribution of rosette-forming cells—cocapping with surface immunoglobulin. Eur J Immunol **10**:198–202, 1980.
25. Morgan EL, Weigle WO. Aggregated human-globulin-induced proliferation and polyclonal activation of murine B lymphocytes. J Immunol **125**:226–231, 1980.
26. Saito-Taki T, Nakano M. Suppression of lipopolysaccharide-induced polyclonal B cell activation of murine spleen with heat-aggregated murine immunoglobulin G. J Immunol **130**:2022–2026, 1983.
27. Sidman CL, Unanue ER. Requirements for mitogenic stimulation of murine B cells by soluble anti-IgM antibodies. J Immunol **122**:406–413, 1979.
28. Pure E, Isakson PC, Takatsu K, Hamaoka T, Swain SL, Dutton RW, Dennert G, Uhr JW, Vitetta ES. Induction of B cell differentiation by T cell factors. I. Stimulation of IgM secretion by products of a T cell hybridoma and a T cell line. J Immunol **127**:1953–1958, 1981.
29. Soderberg LSF, Solotorovsky M. Host-parasite interactions with macrophages in culture. In: Gadebusch HH, ed. Phagocytes and Cellular Immunity. CRC Press, 1979.
30. Redelman D, Scott CB, Sheppard HW Jr, Sell S. *In vitro* studies of the rabbit immune system. II. Functional characterization of rabbit T and B populations separated by adherence to nylon wool or lysis with anti-thymocyte serum and complement. Cell Immunol **24**:11–23, 1976.

31. Sheppard HW Jr, Redelman D, Sell S. *In vitro* studies of the rabbit immune system. IV. Differential mitogen responses of isolated T and B cells. Cell Immunol **24**:34–44, 1976.
32. Raffel C, Sell S. Individual rabbit peripheral blood lymphocytes reactive to anti-immunoglobulin and phytohemagglutinin or concanavalin A identified by immuno-electronmicroscopic demonstration of endocytosis. J Immunol **127**:2530–2535, 1981.
33. Oberbarnscheidt J, Kolsch E. Direct blockade of antigen-reactive B lymphocytes by immune complexes. An "off" signal for precursors of IgM-producing cells provided by the linkage of antigen and Fc-receptors. Immunology **35**:151–157, 1978.
34. Morgan EL, Weigle WO. Polyclonal activation of murine B lymphocytes by immune complexes. J Immunol **130**:1066–1070, 1983.
35. Carvalho EM, Horwitz DA. Characterization of a non-T, non-B human blood lymphocyte that mediated the enhancing effects of immune complexes on lymphocyte blastogenesis. J Immunol **124**:1656–1661, 1980.
36. McElroy PJ, Wilcox N, Cotty D. Early precursors of lymphocytes—B. 1. Rabbit–mouse species differences in the physical properties and surface phenotype of pre-B cells and in the maturation sequence of early B-cells. Eur J Immunol **11**:76–85, 1981.
37. Feldbush TL. Separation of memory cell subpopulations by complement receptors—*In vivo* analysis. Eur J Immunol **10**:443–448, 1980.
38. Nariuchi H, Kakiuchi T. B cells with or without C3 receptor: Murine B cell subsets highly and lowly responsive to anti-Ig stimulation. Cell Immunol **56**:184–192, 1980.

Received April 8, 1983. P.S.E.B.M. 1983, Vol. 174.