IMMUNE RESPONSES IN VITRO

II. Suppression of the Immune Response in Vitro by Specific Antibody

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In vivo, antibody administered at the proper time in relation to the antigenic stimulus specifically suppresses the immune response to that antigen (see discussion in ref. 1). This suppression is probably the result of antibody combining with antigenic determinants, neutralizing the antigen and thus preventing it from stimulating potential antibody-forming cells (1–3).

The precise step(s) at which antibody acts in the sequence of events culminating in an immune response can be more effectively studied in tissue culture systems. Spleen cell suspension cultures stimulated with heterologous erythrocytes develop plaque-forming cell responses similar to those in mouse spleens in vivo (4, 5). However, in vitro, cellular and chemical constituents and their interactions can be regulated. Cell populations can be separated, and events such as interactions of macrophages with antigen and lymphoid cells can be isolated and controlled (6, 7). Changes in physical characteristics of cells (8) and the onset of cell division (9) after stimulation with antigen can be detected. In such a system the mechanism of antibody suppression of specific antibody synthesis can be precisely analyzed.

The data to be presented indicate that antibody acts specifically during the macrophage-dependent step of the primary type of response by neutralizing the antigenic stimulus.

Materials and Methods

Animals.—8–10 month old C57BL/6N male mice and 6–8 month old New Zealand white male rabbits, from the National Institutes of Health Rodent and Rabbit Production Section, were maintained on water and laboratory chow ad libitum.

Antigens.—Erythrocytes from one sheep (SRBC), one burro (BRBC), and one pig, maintained at the National Institutes of Health Ungulate Division, were collected weekly under sterile conditions and washed by centrifugation three times with 40 volumes of sterile Hanks' balanced salt solution (H-BSS). Erythrocytes for immunization of animals to produce antisera and for use in the hemolytic plaque assay were resuspended to 10×10^8 erythrocytes/ml; erythrocytes for addition to cultures were resuspended to 2×10^8 erythrocytes/ml.

Antibody.—36 mice were injected intraperitoneally with 5×10^8 SRBC every 14 days for

a total of four injections. One third of the mice were bled from the retro-orbital plexus each day from the 5th to 13th days after the second, third, and fourth injections. Another group of mice was bled from days 5 to 10 after the third intraperitoneal injection of 5×10^8 pig erythrocytes. Blood was collected by cardiac puncture from one rabbit 7 days after the third weekly intravenous injection of 10×10^8 SRBC. Normal blood was collected from unimmunized mice and an unimmunized rabbit. Within 3 hr after collection, serum was separated at 4°C and sterilized by passage through a prewashed 0.22 μ Millipore filter (Millipore Filter Corp., Bedford, Mass.). An aliquot was cultured to ensure sterility; the remainder was frozen at −20°C. After the final bleeding, each type of serum was thawed and pooled; an aliquot was removed for antibody titration, and the remainder was divided into 0.05 ml portions and frozen.

Hemagglutination titers were from 40,000 to 80,000 against the immunizing erythrocyte and less than 10 against the nonimmunizing erythrocytes. Normal serum had no detectable titer against any of the erythrocytes tested. All antibody activity, within the limits of the system, was resistant to treatment with 0.1 M 2-mercaptoethanol for 30 min.

Preparation of Antigen-Antibody Complexes and Elution of Antibody from Complexes.—SRBC–anti-SRBC complexes were prepared by sedimenting 4×10^8 SRBC in sterile plastic tubes (Falcon Plastics, Los Angeles, Calif., No. 2001) by centrifugation. The supernatant fluid was removed, and the erythrocytes were resuspended in 2 ml of tertiary response C57BL/6N anti-SRBC antibody diluted 1:100 (A), 1:1000 (B), 1:10,000 (C), and 1:100,000 (D) in H-BSS. After 24 hr at 4°C with periodic agitation, the erythrocytes were sedimented by centrifugation, and the separated supernatant fluids were labeled supernatant fluid A, B, C, or D. The erythrocytes were washed three times by centrifugation in large volumes of H-BSS and resuspended in 2 ml H-BSS; 1 ml of the suspension was transferred to another plastic tube labeled complex A, B, C, or D, and the remaining 1 ml was heated at 56°C for 30 min with periodic agitation. The erythrocytes were sedimented, and the separated supernatant fluids were labeled eluate A, B, C, or D. The erythrocytes were resuspended in 1 ml H-BSS and labeled postelution SRBC A, B, C, or D. An erythrocyte suspension containing 2×10^8 erythrocytes/ml and 2 ml of a 10^{-2} dilution of the antibody were also heated at 56°C for 30 min to control for the effects of heating.

Approximately 10^7 SRBC were added to cultures as complexes or postelution SRBC. The complexes were used to determine the effect on the plaque-forming cell (PFC) response of complexing SRBC with antibody at various dilutions; the postelution SRBC provided an estimate of suppressive activity remaining complexed to SRBC after heat elution of antibody. The supernatant fluids were used to estimate the suppressive activity not bound to the SRBC during the preparation of the complexes; the eluates were used to estimate the amount of suppressive antibody eluted from complexes with heat.

Preparation of Spleen Cell and Separated Cell Population Cultures.—Tissue culture reagents were obtained from Microbiological Associates, Bethesda, Md.; fetal bovine serum, added to the completely supplemented Eagle's minimal essential medium, monolayer type (S-MEM) (5, 10), at a final concentration of 10%, was obtained from Reheis Chemical Company, Division of Armour Pharmaceutical Company, Chicago, Ill., lot 162.

Spleen cell suspensions containing $1.0–1.5 \times 10^7$ cells/ml in S-MEM were prepared as previously described (4, 5) and were incubated for 4 days with 10^7 erythrocytes in plastic tissue culture grade Petri dishes (35 × 10 mm) (Falcon Plastics, Los Angeles, Calif., No. 3005) in a water-saturated atmosphere of 83% nitrogen, 7% oxygen, and 10% carbon dioxide in a Lucite box on a slowly rocking platform (Bellco, Vineland, N.J.). Each experimental group had four to six dishes, which were supplemented daily with 0.05 ml fetal bovine serum and 0.05 ml of a nutritional mixture (5).

In some experiments "macrophages" were separated from "lymphoid cells" by incubating the spleen cell suspensions without antigen and allowing the macrophages to attach to the plastic culture dishes (5, 6). Dishes containing approximately 1×10^6 macrophages were incubated with 10^7 erythrocytes for 30–45 min, after which free erythrocytes were removed; the culture was then reconstituted with 1×10^7 lymphoid cells. This limited exposure to antigen stimulated PFC responses equivalent to responses in cultures where erythrocytes were present

TABLE I

Suppression of the Primary Immune Response in Vitro: Effect of Antibody Concentration

Culture variables: 1.5×10^7 spleen cells incubated with		PFC /10^6 recovered cells after 4 days in culture*			
		C57BL/6N anti- SRBC:			Rabbit anti-SRBC: tertiary response
10^7 SRBC	0.05 ml antibody at dilution of	secondary response	tertiary response	quaternary response	
+	No antibody (control response)	553	726	866	676
+	10^{-1}	70	80	83	53
+	10^{-2}	80	84	141	41
+	10^{-3}	192	276	304	194
+	10^{-4}	375	450	333	618
+	10^{-5}	506	670	333	811
+	10^{-6}	578	702	493	793
+	10^{-7}	569	779	833	700
+	10^{-1} normal mouse serum	322	347	359	300‡
+	10^{-2} normal mouse serum	588	780	821	674‡
−	10^{-2} antibody	69	97	106	73
−	No antibody§	73	105	100	52

* Data are from a representative, reproducible experiment.

‡ Same dilution of normal rabbit serum substituted for normal mouse serum.

§ The PFC response in these cultures indicates the stimulation of PFC against SRBC by fetal bovine serum in the culture medium.

for 4 days. Neither separated macrophages nor lymphoid cells alone incubated with 10^7 erythrocytes for 4 days developed a significant PFC response (5, 6).

Hemolytic Plaque Assay.—The number of direct PFC, cells presumably releasing γM antibody, in each experimental group was determined by a modification of the Jerne hemolytic plaque technique (11) using agarose and glass microscope slides (12) and were expressed as PFC/10^6 recovered cells. Only experiments in which comparable cell recoveries were obtained in the experimental groups were reported.

RESULTS

Effect of Antibody Concentration and Specificity of Suppression.—Spleen cells were incubated with or without 10^7 SRBC and 0.05 ml of 10-fold dilutions of homologous or heterologous anti-SRBC serum, normal mouse serum, or rabbit serum as indicated in Table I. Homologous and heterologous antibody at dilutions of 10^{-1} and 10^{-2} caused marked suppression of the PFC response. However,

a 10^{-1} dilution of normal serum also suppressed the response, apparently nonspecifically, to about the same extent as the 10^{-4} dilution of the antisera; a 10^{-2} dilution of normal serum had no suppressive effect. At dilutions greater than 10^{-2}, as the amount of antibody added to the cultures was reduced, the degree of suppression of the response decreased.

Generally, the PFC response was more effectively suppressed at higher antibody dilutions by homologous antibody than by heterologous antibody with the same hemagglutinin titers. Also, homologous quaternary response antibody was more effective at higher dilutions than either homologous secondary or tertiary response antibody; e.g. compare the 10^{-5} and 10^{-6} antibody dilutions in Table I.

TABLE II

Suppression of the Primary Immune Response in Vitro: Specificity of Suppression

Culture variables: 1.5×10^7 spleen cells incubated with		PFC/10^6 recovered cells after 4 days in culture		
10^7 erythrocytes	0.05 ml of antibody at 10^{-2} dilution	Expt 1	Expt 2	Expt 3
SRBC	No antibody (control response)	961	676	670
SRBC	C57BL/6N anti-SRBC, tertiary response	122	140	137
SRBC	C57BL/6N anti-pig erythrocyte, tertiary response	907	648	860
SRBC and BRBC	No antibody (control responses in doubly immunized cultures)	967 680*	926 434*	872 1010*
SRBC and BRBC	C57BL/6N anti-SRBC, tertiary response	137 695*	155 458*	167 960*

* PFC to burro erythrocytes.

The small PFC response which developed in cultures to which no SRBC had been added, presumably the result of stimulation by the fetal bovine serum (4), was not abolished by the 10^{-2} dilution of the antisera tested. The suppression of the PFC response therefore was not due to neutralization by the antibody of a necessary constituent of the fetal bovine serum.

The PFC response against SRBC was not suppressed by homologous tertiary response, anti-pig erythrocyte antibody at a dilution of 10^{-2} (Table II). Cultures of spleen cells stimulated with 10^7 SRBC and 10^7 BRBC developed a primary type of response to each erythrocyte. Anti-SRBC antibody suppressed only the response to sheep erythrocytes in these doubly stimulated cultures, demonstrating further the specificity of the suppression (Table II).

Effect of Complexing Antibody with Antigen.—The ability of the supernatant fluids, eluates, postelution SRBC, and complexes to suppress the primary type of response in vitro was tested by incubating these preparations with 1.5×10^7 spleen cells and 10^7 SRBC where necessary. The ability of erythrocytes to

stimulate and of antibody to suppress the primary type of response in vitro was not diminished by heating at 56°C for 30 min (Table III).

Sufficient antibody combined with SRBC in complexes prepared at antibody dilutions of 10^{-2} and 10^{-3} to suppress the response as effectively as 0.05 ml of the same dilution of free antibody (Table III). When higher dilutions of antibody were used to prepare the complexes, the degree of suppression was less with the

TABLE III

Suppression of the Primary Immune Response in Vitro: Effect of Complexing Antibody with Antigen and Antibody Eluted from Antigen-Antibody Complexes

Culture variables: 1.5×10^7 spleen cells incubated with	PFC/10^6 recovered cells after 4 days in culture		
	Expt 1	Expt 2	Expt 3
10^7 SRBC (control response)	967	655	953
10^7 SRBC heated at 56°C for 30 min	915	824	965
10^7 SRBC + 0.05 ml 10^{-2} Ab*	83	172	145
10^7 SRBC + 0.05 ml 10^{-2} Ab heated at 56°C for 30 min	110	230	157
(10^7 SRBC–10^{-2} Ab)‡ complex A	125	175	100
(10^7 SRBC–10^{-3} Ab) complex B	367	161	394
(10^7 SRBC–10^{-4} Ab) complex C	1110	1150	955
(10^7 SRBC–10^{-5} Ab) complex D	890	681	900
10^7 SRBC + 0.05 ml of supernatant fluid A	304	450	400
10^7 SRBC + 0.05 ml of supernatant fluid B	520	526	712
10^7 SRBC + 0.05 ml of supernatant fluid C	1030	664	939
10^7 SRBC + 0.05 ml of eluate A	161	155	192
10^7 SRBC + 0.05 ml of eluate B	869	845	1090
10^7 SRBC + 0.05 ml of eluate C	955	870	1090
10^7 SRBC + 0.05 ml of eluate D	895	645	941
10^7 SRBC postelution A	240	346	530
10^7 SRBC postelution B	978	675	958
10^7 SRBC postelution C	975	687	952

* C57BL/6N tertiary response anti-SRBC antibody.

‡ For preparation of complexes, see the text.

complexes than when the free antibody and SRBC were added separately. Complexes prepared at an antibody dilution of 10^{-4} usually stimulated responses greater than those in cultures to which only SRBC were added, whereas addition of 0.05 ml of free antibody at a dilution of 10^{-4} caused 50% suppression of the response (not shown in Table III).

However, all the suppressive antibody was not bound to the SRBC during the preparation of the complexes. Supernatant fluids from complexes prepared at antibody dilutions of 10^{-2} and 10^{-3} retained suppressive activity approximating that of 10^{-3} and 10^{-4} dilutions, respectively, of the original antibody (Table III). The fact that suppressive activity was removed from antibody preparations by

mixing SRBC and anti-SRBC antibody further demonstrated the specificity of the suppression of the PFC response by antibody. Sufficient antibody was eluted from complexes prepared at an antibody dilution of 10^{-2} to suppress the response almost as effectively as 0.05 ml of a 10^{-2} dilution of free antibody or the whole complex from which the eluate was prepared. However, little if any suppressive antibody could be eluted from complexes prepared with higher dilutions of antibody.

TABLE IV

Suppression of the Primary Immune Response in Vitro: Effect of Increased Antigen Dose

Culture variables: 1.5×10^7 spleen cells incubated with		PFC/10^6 recovered cells after 4 days in culture with and without antibody‡	
SRBC dose ($\times 10^7$)	Antibody*	10^{-2} antibody, 0.05 ml	10^{-3} antibody, 0.05 ml
1	−	946	1172
1	+	202	238
2	−	948	1243
2	+	163	277
4	−	784	1178
4	+	112	248
8	−	913	1447
8	+	123	361
16	−	818	1232
16	+	136	991
50	−	873	1400
50	+	161	1112
100	−	827	1175
100	+	486	1306

* C57BL/6N tertiary response anti-SRBC antibody.

‡ PFC responses shown are from a representative, reproducible experiment.

Only postelution SRBC from the complex prepared at a 10^{-2} dilution of antibody retained sufficient antibody to exhibit any suppression of the PFC response.

Less antibody could be eluted from complexes prepared at an antibody dilution of 10^{-2} at 37°C than at 56°C. The suppressive activity in normal mouse serum could not be absorbed by SRBC. Suppressive activity was not removed from any dilution of anti-SRBC serum by burro erythrocytes.

Effect of Increased Antigen Dose.—One mechanism of action of suppressive antibody suggested by the preceding experiments is neutralization of the antigenic stimulus. The suppressive effect decreased as less antibody was added to the system (Table I). If the proposed mechanism is correct, the suppressive effect of a constant amount of antibody should be overcome by increasing the antigen dose so that the antibody no longer neutralizes the antigenic stimulus.

Spleen cells were incubated with doses of SRBC from 1×10^7 to 100×10^7/culture. One half of the cultures at each SRBC dose were control responses for that SRBC dose; 0.05 ml of a 10^{-2} or 10^{-3} dilution of tertiary response C57BL/6N anti-SRBC antibody was added to the remaining cultures.

The PFC responses to the increasing SRBC doses were quite constant within an experiment (Table IV). Even at a dose of 100×10^7 SRBC, the 10^{-2} dilution

TABLE V

Suppression of the Primary Immune Response in Vitro: Effect of Adding Antibody at Intervals after Initiation of Culture

Culture variables: 1.5×10^7 spleen cells incubated with 10^7 SRBC and 0.05 ml of 10^{-2} dilution of antibody		PFC/10^6 recovered cells after 4 days in culture		
Antibody type*	Hours after culture initiated when antibody added	Expt 1	Expt 2	Expt 3‡
No antibody	Control response	880	778	826
Secondary	0 (control suppression)	134	89	117
Quaternary	0 (control suppression)	167	82	87
Secondary	12	166	131	204
Quaternary	12	137	133	122
Secondary	24	343	295	356
Quaternary	24	166	124	107
Secondary	48	910	708	910
Quaternary	48	883	717	700
Secondary	72	873	883	933
Quaternary	72	881	891	900

* C57BL/6N anti-SRBC antibody, secondary or quaternary response.

‡ In this experiment, macrophages and lymphoid cells were separated from the spleen cell suspension as described in the text. The macrophages were incubated with 10^7 sheep erythrocytes for 30 min; excess erythrocytes were removed, and 1×10^7 lymphoid cells were added to each macrophage dish.

of antibody suppressed the PFC response 40%. This dilution of antibody neutralized the stimulus of 50×10^7 SRBC; the degree of suppression at this SRBC dose was comparable to that at all lower SRBC doses. At an antibody dilution of 10^{-3}, suppression of the response was partially overcome by a SRBC dose of 16×10^7; suppression was completely overcome at higher doses of SRBC.

Effect of Adding Antibody at Intervals after Initiation of Cultures.—Secondary or quaternary response C57BL/6N anti-SRBC antibody was added to spleen cell cultures at intervals after stimulation with SRBC. Antibody added 12 hr after the initiation of the cultures was almost as effective as antibody added at zero time (Table V). The PFC response was suppressed at least 50% by either antiserum added as long as 24 hr after initiation of the cultures. Either anti-

body added 48 hr or later after the initiation of the cultures had little suppressive effect.

The quaternary response antibody clearly had a greater suppressive effect than secondary response antibody when added 24 hr after the initiation of the culture. When antibody was added at 6 hr intervals between 24 and 48 hr, the degree of suppression gradually decreased from near the control suppression value at 24 hr to almost no suppression at 48 hr.

Cultures in which only separated macrophages were exposed to the SRBC in the absence of lymphoid cells had responses comparable to those cultures in which the SRBC were present for 4 days (Table V). The experiments reported in Tables I, II, III, and V were repeated using this "limited antigen exposure system"; the results were comparable. In other experiments, antibody was added at the initiation of the cultures and was partially removed after intervals of incubation by washing the cells twice by centrifugation in large volumes of H-BSS and resuspending them in S-MEM at 10^7 cells/ml. Some suppression resulted from exposure of the spleen cells to antibody for as little as 30 min. But exposure for 4–6 hr was required before the degree of suppression equaled that in cultures where antibody was present for 4 days. Since not all the antibody could be removed from the system by the washing procedure, conclusions regarding the minimum time of exposure of the spleen cells to antibody to produce maximum suppression must be guarded.

Effect of Exposing Separated Cell Populations to Antibody.—In addition to neutralizing the antigenic stimulus, antibody might also act directly on either the phagocytic or responding lymphoid cells to suppress the immune response. Separated lymphoid cells were incubated in S-MEM with or without 0.05 ml of a 10^{-2} dilution of antibody for 24 hr. Separated macrophages were treated in one of four ways. (*a*) Macrophages were incubated with 10^7 SRBC for 30 min; the excess SRBC were removed, and the macrophages were washed with H-BSS and incubated for the next 24 hr in 1 ml of S-MEM (A, B, and G in Table VI). (*b*) After incubation for 24 hr in S-MEM, 10^7 SRBC were incubated with the macrophages for 30 min, after which the excess SRBC were removed (C, D, and H). (*c*) Macrophages were incubated with 10^7 SRBC for 30 min; the excess SRBC were removed, and the macrophages were washed and incubated for the next 24 hr in S-MEM containing 0.05 ml of a 10^{-2} dilution of antibody (E). (*d*) Macrophages were incubated for 24 hr in S-MEM containing 0.05 ml of a 10^{-2} dilution of antibody; medium was removed, and the macrophages were washed and then incubated in S-MEM with 10^7 SRBC for 30 min before removal of the excess SRBC (F).

At the end of the 24 hr treatment period, all separated macrophages and lymphoid cells were gently washed with H-BSS and the cultures were reconstituted by adding 10^7 lymphoid cells to macrophages according to the

protocol in Table VI. The PFC response was determined 4 days after the cultures were reconstituted.

Culture A demonstrated that macrophages exposed to SRBC for 30 min 24 hr before reconstitution with lymphoid cells stimulated a PFC response that was

TABLE VI

Suppression of the Primary Immune Response in Vitro: Effect of Exposing Separated Cell Populations to Antibody

Culture variables	PFC/10^6 recovered cells after 4 days in culture		
	Expt 1	Expt 2	Expt 3
A. MR* + SRBC‡ for 30 min, wash, add new medium, incubate for 24 hr; add LC§	727	656	549
B. MR + SRBC for 30 min, wash, add new medium, incubate for 24 hr; add LC and Ab‖	254	175	56
C. After incubation for 24 hr; MR + SRBC for 30 min, wash, and add LC	942	323	440
D. After incubation for 24 hr; MR + SRBC for 30 min, wash, and add LC and Ab	300	134	103
E. MR + SRBC for 30 min, wash, add new medium with Ab, incubate for 24 hr; wash and add LC	290	172	89
F. Incubate MR + Ab for 24 hr; wash, add new medium with SRBC for 30 min; wash, and add LC	163	134	80
G. MR + SRBC for 30 min, wash, add new medium, incubate for 24 hr; add LC incubated with Ab for 24 hr¶	974	743	526
H. After incubation for 24 hr; MR + SRBC for 30 min, wash, and add LC incubated with Ab for 24 hr	821	456	1,022
I. LC + SRBC at 24 hr	5	0	5

* MR = macrophage-rich population derived from spleen cell suspension, each dish containing approximately 10^6 cells.

‡ SRBC = 10^7 SRBC.

§ LC = lymphoid cell-rich population derived from spleen cell suspension, 1×10^7 cells added to cultures.

‖ Ab = C57BL/6N anti-SRBC tertiary response antibody, 0.05 ml of a 10^{-2} dilution added per 1 ml of culture medium.

¶ After incubation of lymphoid cells with antibody for 24 hr, lymphoid cells were washed three times by centrifugation with H-BSS.

suppressed by free antibody added when the cultures were reconstituted (culture B, Table VI). Culture C demonstrated that after 24 hr in vitro, macrophages after exposure to SRBC for 30 min stimulated a PFC response which could be suppressed by antibody (culture D).

Addition of antibody to the S-MEM in which macrophages were incubated for 24 hr after a 30 min exposure to SRBC (culture E) suppressed the PFC response in the reconstituted culture even though the antibody had been added

after the interaction of the SRBC and macrophages and most antibody had subsequently been removed from the system by washing. The PFC response was also suppressed in cultures where the macrophages had been incubated with antibody for 24 hr and washed before exposure to SRBC (culture F).

Incubation of the lymphoid cells with antibody for 24 hr did not impair their ability to respond to the stimulus provided by macrophages exposed to SRBC for 30 min (cultures G and H). Similar results were obtained in cultures C, D, F, and H, where the cell populations were treated as indicated, but when the

TABLE VII

Suppression of the Primary Immune Response in Vitro: Effect of Antibody Eluted from Separated Cell Populations

Culture variables*	PFC/10^6 recovered cells after 4 days in culture		
	Expt 1	Expt 2	Expt 3
Spleen cells + SRBC + 0.05 ml of macrophage eluate	732	691	1115
Spleen cells + SRBC + 0.05 ml of lymphoid cell eluate	700	650	1154
Spleen cells + SRBC + 0.05 ml of antibody-treated macrophage eluate	367	399	444
Spleen cells + SRBC + 0.05 ml of antibody-treated lymphoid cell eluate	687	660	1158

* 10^6 macrophages and 10^7 lymphoid cells were incubated in medium with or without antibody at a concentration of 10^{-2}. After 24 hr, the supernatant fluids were separated and discarded; the cells were washed and new medium added. The cells were heated at 56°C for 1 hr, and the supernatant fluid (eluate) was separated.

cultures were reconstituted SRBC were added and were present throughout the 4 days of incubation. The PFC response in experiments in which the separated cell populations were treated with normal mouse serum at a dilution of 10^{-2} instead of antibody were not suppressed.

Separated macrophages and lymphoid cells were incubated in S-MEM with or without antibody at a concentration of 10^{-2}. After 24 hr the supernatant fluids were discarded, and the cells were washed three times. Macrophages were resuspended to 10^6 cells/ml, and lymphoid cells to 10^7 cells/ml, in S-MEM and heated at 56°C for 1 hr; the supernatant fluids were separated and added to cultures of spleen cells and SRBC as indicated in Table VII. Addition of eluates from macrophages and lymphoid cells not incubated with antibody provided controls. The experiments clearly demonstrated that suppressive antibody could be eluted by heat only from macrophages. Furthermore, the suppressive activity eluted from the macrophages was removed from the eluate by absorption with SRBC. The supernatant fluid from the third washing of either the macrophages or lymphoid cells contained no suppressive activity.

DISCUSSION

Hyperimmune anti-SRBC antibody suppressed the primary type of PFC response against SRBC by mouse spleen cells in vitro. The specificity of the suppression was established by three types of experiments.

1. Absorption with SRBC removed suppressive activity from the anti-SRBC serum. Supernatant fluid from antigen-antibody complexes prepared at an antibody dilution of 10^{-2} had less suppressive activity than an equal volume of a 10^{-2} dilution of antibody. Repeated absorption of the supernatant fluid from this complex further reduced its suppressive activity.[1] Furthermore, specific suppressive activity could be eluted from washed antigen-antibody complexes with heat. Burro erythrocytes did not remove the suppressive activity from anti-SRBC serum.

2. The primary type of response to burro erythrocytes was not suppressed by anti-SRBC antibody, whereas a concomitant response to SRBC in doubly immunized cultures was suppressed. In vivo, the specificity of suppression has been demonstrated in similar experiments, using two types of erythrocytes (13) or two haptenic determinants on the same carrier protein, in which suppression of the response to one hapten had no effect on the response to the other (14). Studies with a hapten-protein conjugate demonstrated that antihapten antibody suppressed only the antihapten response (15).

3. The response to SRBC was not suppressed by anti-pig erythrocyte antibody or normal mouse serum (at dilutions greater than 10^{-2}). The nonspecific suppression of high concentrations of normal mouse serum was demonstrated. Studies with specific antibody should utilize dilutions at which this nonspecific suppression is nonexistent.

The data strongly indicate that antibody specifically suppressed the response by combining with antigen. The fact that decreasing the amount of antibody reduced the degree of suppression did not show that antibody neutralized antigen, since less antibody would be less suppressive if it acted directly on antibody-producing cells. However, increasing the antigen dose in the presence of a constant amount of antibody overcame the suppression, indicating there was not enough antibody to neutralize all the antigen, and demonstrating most dramatically that the spleen cells were still able to respond to antigen.

When antigen was complexed with antibody, antigenic sites were covered and the response was suppressed (complex B in Table III). However, when antibody was eluted and the antigenic sites were uncovered, the erythrocytes then stimulated a response (postelution SRBC from complex B, Table III).

Other investigators (1, 16) have shown that antigen-antibody complexes prepared at certain ratios of reactants stimulated increased responses; in the

[1] Pierce, C. W. Unpublished observations.

present experiments, complexes prepared at an antibody dilution of 10^{-4} always stimulated a response greater than the control.

The antisera used contained mostly, if not all, "7S" antibodies by the criterion of resistance to 2-mercaptoethanol. Sera were not fractionated, thus avoiding introduction of endotoxins which suppress the response in vitro nonspecifically.[1] Studies in which antibody molecules have been digested into Fc, F(ab'), and $F(ab')_2$ pieces indicate that the F(ab') and $F(ab')_2$ pieces mediate suppression by virtue of their antibody-combining site (1, 2, 17).

Antibodies produced after the fourth immunization were more effective at higher dilutions than antibodies produced after the second and third immunizations. In vivo, late antiserum suppressed the responses more effectively than antibody formed early in the response (1, 3, 13, 18, 19). Later in the course of immunization, the antibody formed generally has a higher binding affinity (20); the higher the binding affinity of antibody for an antigenic determinant, the greater its ability to suppress the antibody response to that antigen (19). Potential antibody-forming cells are thought to have receptor sites, representing samples of specific immunoglobulin products, which bind antigen in a manner that initiates the immune response (3, 21–25). If an antibody has a binding affinity for an antigen greater than the cell receptor site, the antibody would effectively compete with the receptor, and capture and neutralize the antigenic stimulus: the result would be a suppressed response (1, 21).

The interval after initiation of the cultures during which the antibody can be added with complete suppression of the response suggests that up to 24–36 hr the immune apparatus is still antigen-dependent. This 24–36 hr interval appears to be critical; during this interval cells which will become PFC are not dividing (9), and the in vitro PFC response appears dependent on cell clusters and macrophages[2] and is highly susceptible to suppressive effects of antibody.

The response in cultures in which only lymphoid cells were exposed to antibody was not suppressed; thus, antibody did not suppress the response by a direct effect on these cells. In contrast, the responses in cultures where only the macrophages were exposed to antibody, either before or after incubation with SRBC, were suppressed. Antibody does not incapacitate all macrophages, since a response to burro erythrocytes was not suppressed in doubly immunized cultures to which anti-SRBC serum was added. When macrophages were incubated with SRBC before exposure to antibody, antibody could neutralize the "antigen product" of the macrophage. Antibody would have had to remain on the cell surface or within the cell to function in this manner, since non–cell-bound antibody was removed from the system by washing. Similarly, when macrophages were exposed to antibody before incubation with SRBC, the effective

[2] Pierce, C. W., and B. Benacerraf. Independence of "activated" lymphoid cells in the immune response in vitro. In preparation.

antibody would have had to be in or on the cell. The macrophages bind or contain considerable suppressive antibody, as shown by the suppressive activity that was eluted from these cells by the inefficient method of heating. The formation of SRBC-antibody complexes and neutralization of the antigenic stimulus at the cell surface of the macrophage can be envisioned; however, more efficient utilization of the antibody would be combination with the antigen product of the macrophage and neutralization of the antigenic stimulus at this step. By acting in this manner, antibody can combine with antigen, whether the macrophages are exposed to antibody before or after exposure to antigen.

These experiments show that suppressive antibody does not act directly on the lymphoid cell population of the spleen, but most probably is sequestered by macrophages and neutralizes the antigenic stimulus by combining with the antigen product of macrophages within or on the surface of the macrophage. These experiments also clarify how the response of normal spleen cells, after exposure to anti-SRBC antibody in donor animals or in vitro, can be suppressed when stimulated with SRBC in X-irradiated recipients (26).

SUMMARY

The effects of hyperimmune anti–sheep erythrocyte (SRBC) antibody on the plaque-forming cell (PFC) response to SRBC by mouse spleen cells in vitro were studied. Anti-SRBC antibody specifically suppressed the PFC response against SRBC. The degree of suppression was directly related to the amount of antibody added and was overcome by large amounts of antigen. Suppressive activity was absorbed from the sera by SRBC and could be partially eluted from the antigen by heat. The PFC response in cultures stimulated with antigen-antibody complexes prepared with high concentrations of antibody were suppressed; however, some complexes prepared at lower antibody concentrations stimulated greater responses than SRBC alone.

Antibodies collected after four immunizations had greater suppressive ability than those collected after two immunizations. The degree of suppression was as great whether antibody was added at the initiation of the cultures or 24 hr later, suggesting that during the first 24 hr the culture system was antigen-dependent.

Incubation of separated lymphoid cells with antibody did not impair their ability to develop a PFC response in vitro. However, if macrophages were incubated with antibody either before or after incubation with SRBC, the subsequent PFC response by lymphoid cells was suppressed.

The data are consistent with the conclusion that antibody suppresses the PFC response in vitro by neutralizing the antigenic stimulus at the macrophage-dependent phase of the response.

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