THE INFLUENCE OF ANTIBODIES ON IMMUNOLOGIC RESPONSES

I. The Effect on the Response to Particulate Antigen in the Rabbit*

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(Received for publication 2 March 1967)

A limited amount of information concerning the influence of antibodies on immunologic responses has been available for some time, but data recently accumulated have implied an even more profound influence than previously suspected. In particular, the interference of passively acquired maternal antibody on active immunization in the newborn has long been known and the ability of antibody to inhibit antibody responses has also been demonstrated in experimental animals (1). Immunologic complexes in ratios of antibody excess have been shown to be less antigenic than antigen alone (2, 3), and the administration of antibody before, with, or after antigen has been reported to lead to the development of immunologic tolerance to the antigen used (4, 5). On the other hand, the combination of antibody with antigen, particularly in ratios of antigen excess, can markedly enhance the process of antibody formation (6, 7). Moreover, circulating antibody has been reported to be of special importance in facilitating the response of newborn piglets to antigenic stimulation (8, 9).

Although the ability of antibody both to enhance and to suppress immunologic responses is apparent, the mechanism of these effects is not. However, some information about the ability of antibodies to suppress immunologic responses is available and the amount of antigen used, the ratio between antigen and antibody, and antibody avidity have all been implicated as being of importance. In addition, the particular effectiveness of 7S antibody in suppressing antibody formation has been the subject of a number of recent reports, and it has been suggested that antibodies in general (2, 5), and 7S antibody in particular (10–12), may have a fundamental role in the regulation of immunologic responsiveness. Sahiar and Schwartz demonstrated specifically that the administration of 7S antibody to rabbits manufacturing 19S antibody could cause the essentially immediate cessation of 19S antibody production (10). Other investigators have reported that 7S antibody could inhibit the formation of both 19S and 7S antibody, but that 19S antibody was either unable to do so at all or had a rela-

* This work was supported by United States Public Health Service Grants No. AI 06305, No. 06787, and No. AI 4152. It was presented in part at the April 1966 meeting of the Federation of American Societies for Experimental Biology, Atlantic City, N. J.

‡ Recipient of United States Public Health Service Career Research Development Award (National Institute of Allergy and Infectious Diseases).

tively small effect in this regard (11–13).[1] In contrast, the report of Rowley and Fitch has suggested that both 19S and 7S antibody can effectively suppress antibody production in rats (5). Similar information about the importance of immunoglobulin type on the ability of antibody to enhance antibody formation has not been available.

Much additional information is needed before a clear interpretation of the role of antibodies in immunologic responses is possible. To define further the nature of the influence of antibodies on immunologic responses as well as to provide information about the mechanism of these effects, experiments were undertaken focusing on the relationship of antigen-antibody ratio, the effect of immunoglobulin type, and the specificity of the antibodies employed. Since handling of antigenic material appears to differ, at least initially, for particulate and soluble antigens, experiments were designed using prototypes of both forms of antigens. Experiments performed with particulate antigens are the subject of this report.

Materials and Methods

Animals.—Albino rabbits weighing 2–3 kg were used in all experiments. Animals of the same sex were used in a given experiment; in most instances, female animals were used. Animals were purchased from the B. & B. Rabbitry, Fort Lupton, Colo.

Antigens.—Sheep red blood cells (SRBC) conjugated with dinitrobenzene (DNB) were used as antigens. DNB-conjugated sheep red blood cells (SRBC-DNB) were prepared on the day of experiment as follows: 0.1 ml of 1-fluoro-2, 4-dinitrobenzene (Eastman Organic Chemicals, Rochester, N. Y.) was dissolved in 50 ml of 0.005 M sodium carbonate–normal saline buffer (pH 9.4) containing 1% dextrose, using a magnetic stirrer. 4.5 ml of this preparation were added to 0.5 ml of packed SRBCs (obtained in Alsever's solution from Colorado Serum Co., Denver, Colo. and freshly washed that day) and incubated at room temperature for 30 min by rotation at moderate speed using a Multipurpose rotator (Scientific Industries, Springfield, Mass.). The conjugated cells were sedimented, the supernatant removed, and the cells washed at least three times with normal saline and finally standardized to a 1% suspension in normal saline using a Klett-Summerson photoelectric colorimeter according to the method for SRBC standardization described by Taliaferro (14). When less concentrated cell suspensions were used, dilutions were made from suspensions originally standardized to 1%. Although this method regularly resulted in the production of DNB-conjugated SRBCs, there appeared to be some variation in the degree of consistency of DNB conjugation between antigens prepared on different days. The variation in anti-DNB responses between experiments undoubtedly reflects this fact to some extent. DNB–bovine serum albumin (BSA-DNB), used for secondary injections in some experiments, was prepared according to the method of Eisen et al. (35). The preparation used had an average of 16 DNB groups per BSA molecule.

Antisera.—Sheep red cell antisera were prepared by injecting female albino rabbits with boiled sheep red blood cell stromata (37) or intact sheep red blood cells. Blood was collected by cardiac puncture 5 days after the last injection. Antiserum-I was obtained from animals injected three times intravenously with sheep red blood stromata at 2 wk intervals. 7S-II antiserum was collected from animals injected two or three times a week for 5 wk with 1 ml of 10% SRBCs. 19S-II rabbit antiserum was prepared by Colorado Serum Co. and purchased from

[1] Jerne, N., A. Nordin, C. Henry, H. Funi, and A. Koros. 1965. The agar plate technique. The target of the antigen and clonal selection. Personal communication.

them in 50% glycerin. This preparation was dialyzed against large volumes of normal saline to remove the glycerin prior to fractionation (see below). All preparations were inactivated at 56°C for 30 min.

Fractionation and Characterization of Antisera.—Sephadex G-200 (Pharmacia, Uppsala, Sweden) gel filtration was used to separate antisera into "19S" and "7S" antibody fractions as described by Onoue et al. (15). 19S and 7S antibody fractions were presumed to correspond

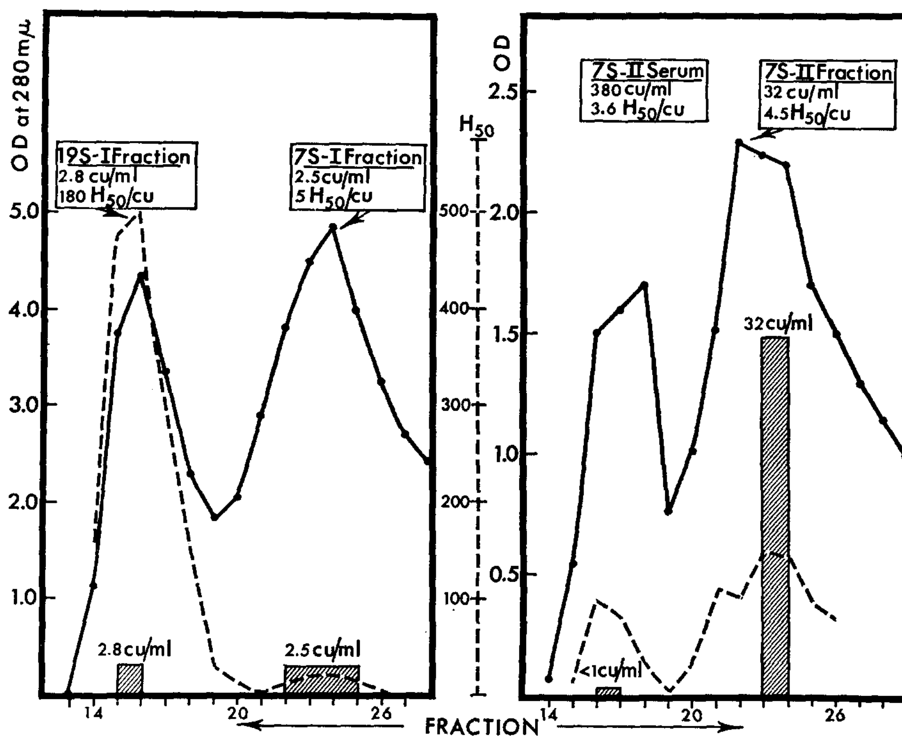

FIG. 1. Fractionation, on Sephadex G-200 columns, of two rabbit antisera. These fraction were used as source of SRBC antibody in some of the following experiments. The optical density is given by the solid line and H_{50} units by broken line (cu, combining unit).

to γM- and γG-antibody, respectively, and these terms have been used in this context within this paper. A 19 × 600 mm column was used and the eluted fractions were collected in the cold in 2.5–3.0 ml aliquots. Antisera to be fractionated were first precipitated in a final concentration of 50% ammonium sulfate, pH 7.0, centrifuged, and the precipitate dialyzed against 0.02 M tris (hydroxymethyl) aminomethane phosphate-buffered saline, pH 8.0. This same buffer was used for equilibrating the columns and eluting proteins. The protein concentration of eluted fractions was determined by measurement of optical density at 280 mμ, using a Beckman DU model spectrophotometer. Appropriate fractions were pooled and designated as 19S or 7S fractions as indicated in Figs. 1 and 2. Numerals I and II were used in conjunction with 19S or 7S to identify a given antiserum or pooled antiserum fraction (see Figs. 1 and 2). Antibody activity of each antiserum preparation was characterized in terms of hemolysin activity and combining activity as described below.

Assay for Combining Activity.—Combining activity was determined by the method of Talmage et al. (16) and depends upon the ability of antiserum to inhibit the uptake of a designated amount of radioactively labeled antibody by a standard amount of SRBCs. A combining unit (cu) in these experiments is expressed as the reciprocal of that dilution of antiserum required to inhibit the uptake of 50% of radioactively labeled SRBC antibody. SRBC antibody was labeled with ^{125}I (Nuclear Consultants Corp., St. Louis, Mo.) by the

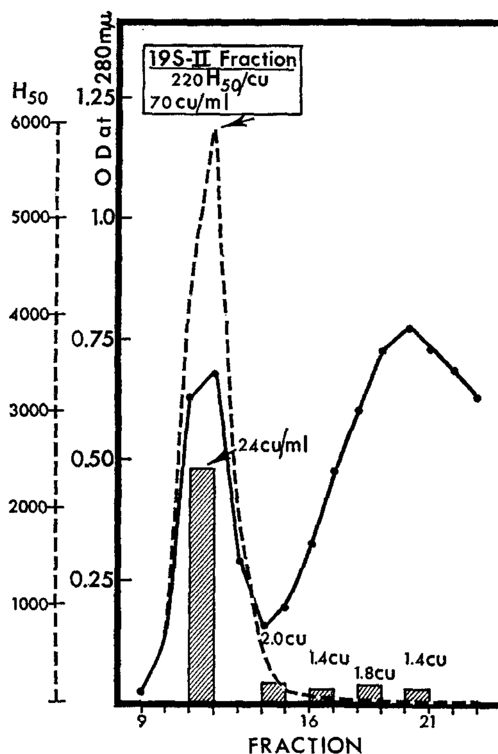

FIG. 2. Fractionation, on a Sephadex G-200 column, of a rabbit antiserum used as the source of the 19S-II fraction used in some of the following experiments. The optical density is given by the solid line and H_{50} units by broken line.

method of McConahey and Dixon (17). The procedure and preparation of reagents are otherwise as described by Talmage et al. (16). During these experiments, two differently labeled antibody preparations were used in assaying combining activity. Consequently, the combining activities of antisera used in early experiments are not necessarily comparable to those used in later experiments.

Assay for Hemolysin Activity.—The hemolysin assay as described by Taliaferro was used (14). Titers were expressed in 50% hemolytic units (H_{50}). 6 units of guinea pig complement (Texas Biologicals, Fort Worth, Tex.) were used for all titrations except when antibody levels were extremely low, in which case 12 units of complement were used. All sera were inactivated at 56°C for 30 min prior to titration.

Assay for Dinitrobenzene Antibody.—The ammonium sulfate precipitation method of Farr (20) was used to detect antidinitrobenzene antibody. Fluorodinitrobenzene (Eastman Organic

Chemicals) was conjugated to rabbit serum albumin (Cohn fraction V, Pentex, Inc., Kankakee, Ill.) according to the method of Eisen (36). Dinitrobenzene-conjugated rabbit serum albumin (RSA-DNB) had an average ratio of 4.6 DNB groups per RSA molecule as determined by the spectrophotometric method described by Eisen (18). RSA-DNB was radioiodinated with ^{125}I Nuclear Consultants Corp.) by the method of Weigle and Dixon (19). Titers were expressed as the reciprocal of that dilution of antiserum required to precipitate 33% of 0.01 μg N ^{125}I-RSA-DNB. When antisera from animals which had received BSA-DNB were assayed, 10 μg N BSA was added to each aliquot of test antiserum prior to incubation with labeled antigen. This was done to eliminate any possible reaction between anti-BSA

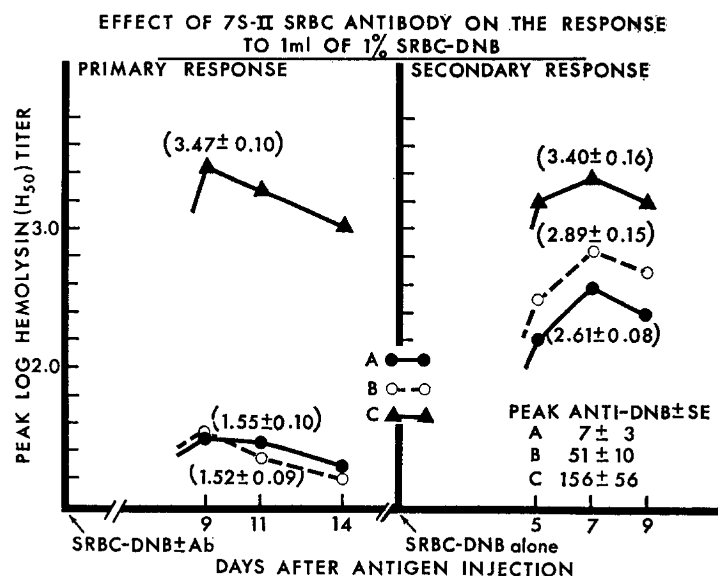

FIG. 3. Each animal in A received 760 cu (2760 H_{50}) 7S-II antibody (whole antiserum, see Fig. 1) with antigen. Each animal in B received 760 cu (2760 H_{50}) 7S-II antibody (whole antiserum) 3 hr after antigen. Each animal in C received antigen alone. Peak log hemolysin titers ± SE are given in parentheses. P values (Student's t test):

	Primary hemolysin	Secondary hemolysin
A vs C	0.001	0.001
B vs C	0.001	0.025

antibody and labeled antigen. Anti-DNB antibody usually could not be detected after one injection of antigen, and titers reported refer only to responses obtained after secondary injection.

Reduction of Antibody by Mercaptans.—Reduction of antibody was performed at 37°C for 30 min using a final concentration of 0.1 M 2-mercaptoethanol (2-ME) (Matheson, Coleman, Bell, Cincinnati, Ohio.) Reduced preparations were then dialyzed against successive changes of Veronal-buffered saline, pH 7.3, (VBS) over a 2-day period at 10°C, or alkylated by dialysis against a large volume of 0.02 M iodoacetamide (Nutritional Biochemical Corp., Cleveland, Ohio) in VBS, with final dialysis against VBS alone. Antibody activity remaining after reduction with 2-ME was designated as mercaptoethanol-resistant (MER) antibody.

Experimental Design and Procedure.—Animals were divided into groups of 5 to 8, similar in weight variation. All injections were given intravenously into the marginal ear vein. When animals were injected with antibody at the same time as antigen, the antigen and antibody were first incubated at room temperature for at least 30 min; the entire preincubated mixture was used for injection. Combining activity was used as the basis for equilibrating the amounts of 19S and 7S antibody used in a given experiment. In the first experiments, "antibody excess" was determined by absorption of various amounts of antiserum preparation with that amount of antigen used for immunization; it refers to an amount of antiserum preparation in which at least 20% of the hemolysin activity remain unabsorbed after incubation with antigen.

TABLE I

Effect of 2-ME Treatment on 19S and 7S* Hemolysin Activity*

Fraction	Hemolysin activity (H_{50})/ml		Per cent residual activity
	Control	2-ME-treated	
19S	6.7	<0.12	<1.8
	69.0	3.2	5.6
	339.0	5.0	1.5
	600.0	<5.0	<0.9
	36,000.0	100.0	0.3
7S	17.7	13.2	75
	17.4	13.8	79
	13.2	10.5	79
	19.0	16.6	87
	17.4	14.8	85
	29.0	19.5	67
19S	4.4	0	0
7S	2.3	1.7	72
19S + 7S	5.3 (6.7)‡	1.7 (1.7)‡	—

* Refers to fractions obtained by Sephadex G-200 gel filtration, and used in original, concentrated, or diluted form.

‡ Predicted values.

"Antigen excess" refers to 1/50th of that amount of antiserum designated as "antibody excess". All antigen-antibody preparations used were examined microscopically for hemagglutination; with the exception of one experiment (see Fig. 3), profound hemagglutination was always found even in preparations designated as "antibody excess". All animals received the same volume of inoculum. Those animals injected with antigen alone received antigen with a specifically absorbed serum fraction from an unimmunized animal, otherwise comparable to the fraction used in the antibody-treated animals (first three experiments) or in normal saline (all other experiments). Secondary injections always consisted of antigen alone and, unless otherwise specified, were given 3 wk after primary injections. Animals were bled from the marginal ear vein after each injection, from 7 to 14 days after primary injections and 5 to 9 days after secondary injections. The blood was allowed to clot at room temperature for at least 2 hr, and the serum separated from the clot and inactivated at 56°C for 30 min before being assayed for antibody activity.

RESULTS

Separation of 19S and 7S Hemolysin Activity by Mercaptoethanol Treatment.— The inordinate sensitivity of 19S compared with 7S antibody to the action of sulfhydryl-reducing agents has been utilized as a means of distinguishing antibody activity of one type from the other. Although this differential susceptibility has been applied most successfully to agglutinating antibody systems, it was thought that it might be of some use in the hemolysin system as well. Table I lists the results of an experiment designed to test its applicability to the hemolysin system. Since sulfhydryl agents interfere with complement fixation and complement-dependent hemolysis, the sulfhydryl agent used, mercaptoethanol, was removed by dialysis after its incubation with the antibody preparation. (see Materials and Methods). As indicated in Table I, this method

TABLE II

Effect of 19S-I SRBC Antibody on the Response to 1 ml of 1% SRBC-DNB*

Experimental group	Mean log peak H_{50} ± SE			Anti-DNB titer peak ± SE (secondary)
	Primary	Secondary	MER antibody (secondary)	
A (Ab excess)	3.02 ± 0.13	3.43 ± 0.28	1.66 ± 0.16	78 ± 48
B (Ag excess)	3.56 ± 0.14	3.52 ± 0.11	2.12 ± 0.15	78 ± 41
C (control)	3.59 ± 0.09	3.62 ± 0.19	1.90 ± 0.19	27 ± 12

P values (Student's *t* test):

A vs C	<0.005	NS	NS	NS
A vs B	<0.01	NS	<0.1	NS
B vs C	NS	NS	NS	NS

* Prepared from antiserum I by the Sephadex fractionation (see Fig. 1).

NS, not significant. (For statistical analysis, anti-DNB titers were converted to logarithms in order to equalize variances.)

usually resulted in the inactivation of at least 98% of the hemolysin activity in 19S fractions. On the other hand, hemolysin activity in 7S antibody fractions was reduced by only 15–25%. Contamination of 7S fractions with 19S antibody, known to be an especially efficient hemolysin, probably accounted in large part for the loss of activity in 7S fractions since some trailing of 19S globulin in these fractions commonly occurs with the method of fractionation employed. This procedure, performed on a mixture of a known amount of 19S and 7S hemolysin, resulted in the recovery of that amount of activity originally attributed to 7S antibody.

On the basis of these findings, resistance to mercaptoethanol treatment was considered a useful means of estimating 7S hemolysin activity, and it was em-

ployed for this purpose in some of the experiments that follow. Since the degree to which the procedure used specifically affected 7S hemolytic antibody was not rigorously defined, figures obtained by this procedure were used for comparative purposes only. Mercaptoethanol-resistant (MER) hemolysin titers of the individual sera in the experiments that follow were reproducible and independent of the total hemolysin activity of an individual antiserum. Treatment of numerous primary sera from these experiments resulted regularly in more than 99% reduction of hemolysin activity, whereas secondary response antisera usually exhibited significant MER hemolysin activity. Since production of MER hemolytic antibody was found to be negligible in primary responses, only assays performed on secondary response sera are reported.

The Effect of Different Amounts of 19S or 7S Anti-SRBC on the Response to 1% SRBC-DNB.—In order to determine the importance of antibody concen-

TABLE III

Effect of 7S-I SRBC Antibody on the Response to 1 ml of 1% SRBC-DNB*

Experimental group	Mean log peak H_{50} ± SE			Anti-DNB titer peak ± SE (secondary)
	Primary	Secondary	MER antibody (secondary)	
A (Ab excess)	3.12 ± 0.12	3.17 ± 0.12	1.34 ± 0.29	20 ± 11
B (Ag excess)	3.40 ± 0.12	3.13 ± 0.12	1.49 ± 0.25	19 ± 7
C (control)	3.42 ± 0.15	3.37 ± 0.09	1.57 ± 0.33	10 ± 3

* Prepared from antiserum I by Sephadex fractionation (see Fig. 1).

P values (Student's *t* test): A vs B, A vs C, B vs C: No values less than 0.05. (For statistical analysis, anti-DNB titers were converted to logarithms.)

Each animal in A received 5.7 cu (25 H_{50}) antibody with antigen. Each animal in B received 0.29 cu (1.25 H_{50}) antibody with antigen. Each animal in C received antigen alone.

tration on the influence exerted on antibody formation, two different amounts of antibodies were used. As a starting point, 19S antibody was used with antigen in amounts thought to represent relative antibody or relative antigen excess based upon absorption of varying amounts of 19S antibody by the dose of antigen used for immunization. A 1% red cell suspension was selected as the antigen dose, since this amount of antigen had previously been found to elicit a maximal hemolysin response. Despite the fact that more than 20% of the antibody used in the antibody excess group could not be absorbed by the red cell inoculum, no antibody prozone effect was observed; that is, profound hemagglutination occurred in both the "antibody excess" and "antigen excess" preparations.

The results of the first experiment with 19S antibody are listed in Table II. As can be seen, 19S antibody in amounts designated as antibody excess reduced the primary hemolysin response to one third that of control animals, whereas antibody in amounts designated as antigen excess failed to affect this

response. Secondary hemolysin responses equalized in all groups, but (7S) MER responses of animals which had received antibody excess with the first antigen injection were somewhat lower than those of the other two groups. Anti-DNB antibody titers, on the other hand, were elevated in both groups of animals which received 19S SRBC antibody. Despite the threefold elevation of mean titers, however, the variation in individual responses was too great to bear out these differences statistically. Two animals, not listed in Table II, that received antibody excess without antigen had negligible hemolysin titers at the time primary responses were being assayed in other experimental groups.

Table III lists the results of a similar experiment using 7S antibody in amounts equivalent in combining activity to those used in the previous ex-

TABLE IV

Effect of Coprecipitation with Anti-Rabbit γG on 19S-I Antibody Activity

Sample	Hemolysin titer log H_{50}	Per cent inhibition of ^{125}I-Anti-SRBC uptake		Per cent precipitation by BSA-^{131}I (Farr test)	
Control	2.41	23	15	40	83
Coprecipitated*	2.37	23	15	0	25

* Aliquots of presumed 19S or 7S antibody were added to a mixture of goat anti-γG (100 μg abN) and rabbit Cohn fraction II (14.5 μg N), incubated for 60 hr at 4°C, centrifuged, and the supernatant assayed for appropriate antibody activity. Controls received identical treatment except that normal saline was substituted for anti-γG.

Two differently diluted aliquots of 19S-I anti-SRBC fractions or 7S (Sephadex fraction) anti-BSA from hyperimmunized rabbits were used. Titrations were performed in triplicate.

periment. As in the previous experiment, 7S antibody excess appeared to suppress the primary response to sheep red blood cell antigen to approximately one-third of control animals, an effect which also tended to reflect itself in the secondary 7S hemolysin response. These differences, however, were not statistically significant. Anti-DNB responses were again higher in both antibody-treated groups, but these differences were not significant.

Since 19S antibody has been reported to be ineffective in suppressing antibody responses, especially in comparison with 7S antibody, it was important to rule out the possibility that the suppression attributed to 19S antibody in the 19S-I fraction used might have been due to the contamination of this fraction with specific 7S (γG-1) antibody. Using immunodiffusion techniques and passive cutaneous anaphylaxis (PCA) reactions in guinea pigs, it was possible to establish that less than 1% but more than 0.1% of the total protein in the 19S fraction had the antigenic characteristics of γG-globulin. In order to determine whether a significant amount of anti-SRBC antibody was present in this small amount of γG-globulin, the effect of specific coprecipitation of γG-globulin on the anti-SRBC activity of this fraction was tested. As can be seen

in Table IV, neither the combining nor hemolysin activities of the 19S fraction were significantly altered, despite the fact that this procedure effectively coprecipitated 7S anti-BSA antibody added to the 19S fraction.

The Effect of 19S and 7S Antibody Excess on the Response to 0.1% SRBC-DNB.—Results recorded in Tables II and III suggested that sufficient amounts of both 19S and 7S antibody could suppress the response to homologous antigens and that their effectiveness in doing so was somewhat similar if employed in amounts comparable in combining capacity. The degree of suppression produced in these experiments, however, was relatively small. An attempt was made, therefore, to substantiate these findings further by producing greater inhibition of the antibody response using 19S and 7S antibody. Because of the

TABLE V

Effect of 19S-I and 7S-I SRBC Antibody on the Response to 1 ml of 0.1% SRBC-DNB*

Experimental	Mean log peak $H_{50} \pm$ SE			Animals with anti-DNB antibody (secondary)
	Primary	Secondary	MER antibody (secondary)	
A	2.82 ± 0.22	3.04 ± 0.10	1.24 ± 0.14	4/5
B	2.62 ± 0.23	3.15 ± 0.43	1.41 ± 0.10	2/5
C	2.12 ± 0.30	2.31 ± 0.25	0.40 ± 0.32	0/5

P values (Student's *t* test):

A vs C	<0.005	<0.05	<0.001	
B vs C	<0.05	<0.10	<0.001	

* Prepared from antiserum I (see Fig. 1).

Each animal in A received 5.7 cu of 19S-I antibody with antigen. Each animal in B received 5.7 cu of 7S-I antibody with antigen. Each animal in C received antigen alone.

limited amount of 19S and 7S material then available, the amount of antibody relative to antigen was increased by decreasing the antigen dose by 10 but using the amount of antibody employed previously in antibody excess groups. Despite the tenfold increase in antibody-antigen ratio, remarkable hemagglutination still occurred in all antigen-antibody preparations. As Table V indicates, rather than suppressing the response to sheep red blood cell antigens, the administration of either 19S or 7S antibody significantly enhanced both primary and secondary hemolysin responses over the response to antigen alone. It should also be noted that hemolysin responses elicited by the 0.1% antigen dose were far below those induced by 1% antigen. Anti-DNB responses using this reduced antigen dose were also much lower and in fact could not be titered accurately by the method used in previous experiments. Consequently, anti-DNB responses are reported instead in terms of animals responding with

detectable amounts of antibody. As can be seen, 6 to 10 animals which received 19S or 7S antibody with antigen made detectable DNB antibody whereas none of the 5 control animals did.

Results of the foregoing experiments seemed to indicate the following: (*a*) The ability of antibodies to enhance or suppress antibody formation is not confined to a particular immunoglobulin type. (*b*) These effects are influenced by the amount of antigen as well as the relative amount of antibody used. (*c*) The specificity of enhancement and suppression differ; suppression occurred only to those determinants to which administered antibody was specifically directed, whereas enhancement could occur to heterologous as well as homologous determinants. (*d*) These opposing effects of antibody could occur concomitantly since the response to some antigenic determinants (SRBC) could be inhibited at the same time that the response to others (DNB) was enhanced. The seemingly paradoxical nature of some of these findings could be reconciled by the following explanation, adopted at that point as a working hypothesis: Enhancement or suppression of immunologic responses by antibodies represents a different balance of at least two competing factors, specific neutralization of appropriate determinants resulting in a decrease of total effective concentration of these determinants and a nonspecific increase in the availability of antigen to immunologically competent cells. A number of experiments were performed in an attempt to separate the enhancing from suppressive influence of antibody on a given immunologic response as well as to further document the findings already reported.

The Effect of SRBC Antibody Given with or after 1 ml of 1% SRBC-DNB on the Antibody Response.—Particulate antigen is known to be almost cleared from the circulation within a few hours (20). If antibody were to enhance antibody formation by some effect on the distribution of antigen, the administration of antibody at a time when much of the antigen has already been cleared (and presumably distributed) should markedly diminish its enhancing influence on antibody formation. Antibody given hours or days after antigen, on the other hand, has been shown to effectively suppress the antibody response. Thus, antibody administered a suitable period after antigen might be expected to lose its ability to enhance yet retain its ability to suppress antibody formation.

It was apparent that the amounts of antibody used in the first experiments, although designated as antibody excess, represented antigen excess instead since (*a*) a significant amount of antibody formation against SRBC antigens was induced despite the presence of this amount of antibody and (*b*) no antibody prozone effect occurred in vitro, rather, profound hemagglutination was consistently induced by antibody. (The inability of the antigen dose used in the first experiments to absorb 20% or more of the antibody used in the antibody excess groups undoubtedly reflects the presence in these preparations of significant amounts of low avidity hemolysin). Consequently, in order to use

larger amounts of antibody, antisera with high combining activity were prepared prior to further experimentation.

Fig. 3 illustrates the results of an experiment somewhat similar to the first experiments, but using much larger amounts of 7S antibody obtained from hyperimmunized rabbits. Antibody was injected with antigen (group A) or 3 hr after antigen (group B). Primary hemolysin responses of all animals injected with antibody either with or after antigen were markedly suppressed.

TABLE VI

Effect of 7S-II SRBC Antibody on Absorption of DNB Antibody by SRBC-DNB

Cells used for absorption	Per cent RSA-DNB precipitated by anti-DNB serum (Farr test method)		
	NRS*	7S-II serum*	7S-II serum 1:10*
	%	%	%
None	27‡ (25, 27, 29)	—	—
SRBC	25 (19, 29, 30)	34 (27, 37)	29 (19, 34, 36)
SRBC-DNB	10 (10, 10, 12)	22 (19, 22, 27)	19 (19, 19, 20)

* Refers to serum with which cells were incubated prior to absorption of anti-DNB.

‡ Average of duplicate or triplicate titrations. Individual titrations are given in parentheses.

2 ml of appropriate serum were added to the sediment of 1 ml of 1% cells, incubated at 37°C for ½ hr, then overnight at 4°C. Suspensions were centrifuged and the supernatants carefully removed. 2 ml aliquots of an anti-DNB serum (diluted 1:2000 in BBS) were mixed with the appropriate sedimented cell preparations and incubated at 37°C for ½ hr and then on a rotating drum at 10°C for 3 days. They were then centrifuged, the supernatant removed and assayed for remaining anti-DNB activity by the Farr test method using 0.01 μg N RSA-DNB. (BBS, borate buffer solution).

Secondary hemolysin responses in these groups were also significantly lower than control animals and were, in fact, much lower than the primary responses of animals injected with antigen alone. Differences in the amount of passively administered antibody remaining prior to secondary injection probably existed, and they may have exerted a direct influence on the second injection of antigen. The amounts of antibody present just prior to secondary injections were not specifically measured, however. In contrast to the anticipated effect on the formation of DNB antibody, anti-DNB responses of animals treated with SRBC antibody were significantly lower than those of the group which received antigen alone.

On the basis of the observation that, for the first time in these experiments, appreciable hemagglutination had not occurred in the antigen-antibody preparations used for immunization, two explanations for the unexpected results were considered to be likely possibilities. First, the amount of antibody used, although specific for sheep red blood cell antigens, may have been sufficient to coat the red cells to such an extent that a steric hindrance effect against DNB determinants was achieved; second, that agglutination or aggregation of sheep red blood cells facilitated their antigenicity even in control animals known to have "natural" SRBC antibody, and prevention of such aggregation therefore abolished this facilitory effect. To test the plausibility of the first

TABLE VII

Effect of 7S-II SRBC Antibody with, or Three hr after, 1 ml of 1% SRBC-DNB on the Antibody Response*

Experimental group	Mean log peak H_{50} ± SE	Anti-DNB titer peak ± SE
	Primary	Secondary
A	1.68 ± 0.09	236 ± 73
B	1.58 ± 0.20	374 ± 229
C	3.31 ± 0.11	63 ± 12

P values (Student's *t* test):

A vs C	<0.001	—‡
B vs C	<0.001	—‡

* 7S-II whole antiserum was used (see Fig. 1).

‡ Different variances precluded comparative analysis by Student's *t* test.

Each animal in A received 155 cu 7S-II antibody with antigen. Each animal in B received 155 cu 7S-II antibody 3 hr after antigen. Each animal in C received antigen alone.

explanation, the ability of 7S-II SRBC antibody to inhibit the absorption of anti-DNB antibody by SRBC-DNB was examined. The results listed in Table VI indicate that the amount of SRBC antibody used in the previous experiment was sufficient to interfere significantly with DNB determinants on the SRBC-DNB antigen. Such interference, therefore, could have accounted at least in part for the diminished anti-DNB responses observed in the antibody-treated groups. Since the two explanations offered are not mutually exclusive, they both may have been factors in the results obtained.

Table VII lists the results of a similar experiment using a smaller amount of 7S-II antibody than that used in the previous experiment. This amount of antibody induced considerable hemagglutination of antigen-antibody preparations, but was still successful in profoundly inhibiting primary hemolysin re-

sponses. Since it was recognized that marked differences in SRBC antibody titers among the experimental groups prior to secondary stimulation could complicate the interpretation of secondary responses in this experiment and may have contributed to the differences in the previous experiment, secondary injections were made substituting 5 mg BSA-DNB/kg for SRBC-DNB antigen. Anti-DNB antibody responses in animals that had originally received antigen-antibody preparations were enhanced and were not appreciably different from those of animals that had received antibody 3 hr after antigen.

TABLE VIII

Effect of 7S-II SRBC Antibody With, or One Day After, 1 ml of 1% SRBC-DNB on the Antibody Response

Experimental group	Mean log peak H_{50} ± titer	Anti-DNB titers
	Primary	Secondary
A	1.00 ± 0.10	53 ± 29
B	1.10 ± 0.17	50 ± 28
C	2.93 ± 0.18	16 ± 4

P values (Student's *t* test):

A vs C	<0.001	<0.1
B vs C	<0.001	<0.1

Anti-DNB titers were converted to logarithms for statistical comparisons, in order to equalize the variances. Animals in A received 155 cu 7S-II whole antiserum with antigen (see Fig. 1). Animals in B received 155 cu 7S-II whole antiserum one day after antigen (see Fig. 1). Animals in C received antigen alone.

In the experiment on Table VIII, antibody injected with, or 1 day after, antigen served again to suppress SRBC antibody formation and increase anti-DNB responses. Secondary injections were given 2 wk after primary injections and consisted of 5 mg/kg BSA-DNB. Anti-DNB responses of animals that received antibody 1 day after antigen were essentially the same as the responses of animals given antibody and antigen together.

Effect of 7S-II Compared with 19S-II SRBC Antibody and the Response to 1% SRBC-DNB.—In contrast to some previous reports, experiments listed earlier in this paper indicated that both 19S and 7S antibodies could inhibit antibody responses effectively. However, in agreement with previous investigations, the results of an experiment listed in Table IX suggest that there may indeed be some difference in the relative efficiency of these two types of immunoglobulins in suppressing antibody formation. Although 100 combining

units of 19S antibody effectively inhibited the primary hemolysin response, the suppression produced by 20 combining units of 7S antibody was significantly greater; furthermore, suppression of responses of two rabbits given 200 combining units of 19S antibody was no greater than that of animals treated with 20 units of 7S antibody.

Because 19S-II antiserum contained significant amounts of 7S SRBC antibody (see Fig. 2), it was important to be certain that the 19S-II fraction was not contaminated by sufficient 7S antibody to account for the results obtained.

TABLE IX

Effect of Different Amounts of 19S and 7S SRBC Antibody on the Response to 1 ml of 1% SRBC-DNB

Experimental group	Mean log peak $H_{50} \pm$ SE
	Primary
A	2.90 ± 0.08
B	2.41 ± 0.20
C	3.47 ± 0.16
D	2.52 ± 0.24

P values (Student's *t* test):

A vs C	<0.005
B vs C	<0.005
A vs B	<0.05

Each animal in A received 100 cu of 19S-III antibody fraction with antigen (see Fig. 2). Each animal in B received 20 cu of 7S-II antibody fraction with antigen (see Fig. 1). Each animal in C received antigen alone. Each animal in D (2 animals) received 200 cu of 19S-III antibody fraction (see Fig. 2).

Most of the hemolytic and combining activities of this fraction could be specifically precipitated by anti-rabbit γM-antibody which did not appreciably affect the activity of 7S BSA antibody added to the preparation prior to precipitation. Furthermore, sulfhydryl reduction coupled with alkylation resulted in the complete elimination of combining activity in the 19S-II fraction, whereas this same procedure left the combining activity of a 7S fraction uneffected.

DISCUSSION

The results of the experiments reported here indicate that the ability to suppress antibody formation is not a property exclusive to a particular immunoglobulin type. 19S and 7S antibody, at least, were both shown to be capable of suppressing the formation of antibody to significant degrees. A number of

previous reports have indicated that 7S but not 19S antibody is an effective inhibitor of antibody production (11, 12).[1] Differences in results may relate partly to differences in methods used to assay the activity of 7S and 19S antibody administered. In the present experiments, the amounts of 19S and 7S antibody used were based solely on relative combining capacity, whereas methods of antibody measurement used in some of the experiments alluded to are dependent on biologic activities other than combining activities. It is well known that these biologic activities may vary significantly between different types of immunoglobulins. For instance, the agglutinating activity of 19S antibody may be over 1000-fold greater than that of 7S antibody, mole for mole (21), and the ratio of hemolytic to combining activity has been shown (22) (and further documented in this report) to be up to 100 times higher for 19S than for 7S antibody. The apparent discrepancy between this report and others probably reflects the fact that suppression of antibody formation is more a function of combining activity than of other biologic properties related to antibody molecules. The amounts of 19S antibody used in the present experiments, therefore, may have been much more comparable in this regard. Factors such as specificity and avidity which relate to the "specific" portion of the antibody molecule have previously been shown to be of critical importance to the suppressive effects exerted by antibodies (2); the "nonspecific" portion (Fc fragment of γG-globulin), to which many differences in biologic properties have been attributed, is not essential for this effect (23). The inhibitory effect of antibody was shown to be specific at the antigenic determinant level in these experiments.

The ability of antibody to enhance antibody formation was also shown to be independent of immunoglobulin type. However, in contradistinction to the suppressive phenomenon, the enhancement of antibody formation appeared to be nonspecific. Although differences in the formation of DNB antibody between animals given SRBC antibody and those given antigen alone were not always significant in any given experiment, in all but one experiment, regardless of the response to red cell antigens, anti-DNB titers were greater in the antibody-treated groups. Furthermore, if suboptimal amounts of antigen were used, passively administered SRBC antibody was able to increase both anti-SRBC and anti-DNB responses. Thus, appropriate amounts of antibody could decrease the response to large doses of antigen but increase the response to small antigen doses. Moreover, significantly smaller amounts of antibody were capable of inducing enhancement than were required to suppress antibody formation. There have been previous indications of a similar effect using red cell antigens in rabbits (24), humans (25), and mice (12). In an experiment in which the amount of antibody used was sufficiently large to produce a prozone effect with antigen in vitro, anti-SRBC formation was negligible. The use of this amount of antibody, however, with a combining capacity many times in

excess of that used in the first experiments, suppressed rather than enhanced anti-DNB formation. That the use of this excessive amount of SRBC antibody may have inhibited the response to DNB determinants by an effect of steric hindrance is indicated by the reduced capacity of SRBC-DNB antigen preincubated with the amount of SRBC antibody used to absorb DNB antibody. The suppressive effect of such high doses of antibody may also be related to the prozone effect induced since aggregation of antigen frequently enhances antibody formation and inhibition of aggregation would serve to reduce this effect. The amount of "natural antibody" known to be present in these rabbits prior to immunization was probably sufficient to affect some agglutination of the antigen administered, thus acting perhaps to increase the response to antigen even in control groups.

Thus, the presence of antibody on antigen, depending on the particular circumstances, seemed to have some facilitatory value for certain determinants even though it could also be shown to be inhibitory in nature for others. These paradoxical effects appeared to occur concomitantly, for anti-SRBC responses could be inhibited at the same time that anti-DNB responses were enhanced. These observations taken together suggested that enhancement or suppression of immunologic responses by antibodies represents a different balance of at least two competing factors operating concurrently: specific neutralization of appropriate determinants thus decreasing total effective concentration of these determinants available to stimulate the formation of antibodies, and a nonspecific increase in the availability of antigen to immunologically competent cells. Some separation of these competing effects was attempted by administering antibody 3 hr or 1 day after antigen. There was no appreciable difference, however, in the responses of animals that received antibody with or after antigen.

It has been suggested that antibody plays an important role in regulating immunologic responses by some specific feedback mechanism (2, 5) and, further, that γG-antibody has a particular role in this regard (10–12). The assignment of a special role to this immunoglobulin has been based largely on the efficiency of γG-antibody in inhibiting antibody formation, and the relative ineffectiveness of γM-antibody in doing so. The demonstration that effective inhibition of antibody formation is not peculiar to the 7S γG-immunoglobulin by no means renders this suggestion invalid. In fact, the results of the experiment listed in Table IX are consistent with previous reports (11–13)[1] which indicated that significant differences between the inhibitory activity of 19S and 7S antibody do exist, since 20 combining units of 7S antibody more effectively inhibited antibody formation than did 100 combining units of 19S antibody. The faster catabolic rate of 19S compared with 7S antibody may be responsible for the difference in effectiveness observed (26). Different catabolic rates have also been implicated as a possible cause of the reduced effectiveness of pepsin-

digested antibody compared with the same amount of undigested antibody in suppressing antibody formation (23). However, it should also be pointed out that even though an attempt was made in the present experiments to approximate the amounts of 19S and 7S antibody used, as closely as possible by using relative combining activity rather than other secondary immunologic tests, this method of antibody measurement is based upon the ability of antibody to compete with a known quantity of radioactively labeled antibody in a limited environment and cannot be construed to be an accurate reflection of avidity for antigen. Since it has been shown that avidity is one of the decisive factors in the suppressive effects of antibody on antibody formation, critical differences between the 19S and 7S preparations may well have existed in this respect. There has been some suggestion that 7S antibody obtained early in the course of immunization is less immunosuppressive than is antibody obtained later after initial immunization (11, 27), and that it is reportedly easier to inhibit primary responses than secondary responses with antibody (5, 30). The average avidity of antibodies found late in the course of immunization or after secondary stimulation is also known to be significantly greater than that of antibody present early (28, 36). It is extremely tempting to relate these observations, especially since the latter has been shown to be true for 7S globulin specifically (29). In addition, the fact that immunization usually leads to the prolonged production of γG-antibodies out of proportion to those of other immunoglobulin classes would suggest, on the basis of these considerations alone, that γG-antibody may have an especially important role in regulating the overproduction of antibody.

Although the exact mechanism by which antibody exerts its inhibitory effects is not clear, it has been suggested that antibody acts by tying-up antigen and, in essence, simply reducing the amount of free antigen available to stimulate antibody formation (2, 23). This effect is apparently not mediated by preventing access of antigen to antibody-forming cells since, when the response to some determinants was suppressed, the response to others on the same antigen was enhanced. Furthermore, it has been shown that antibody may allow sensitization to occur to the antigen at the same time it inhibits the production of antibody (30), and that antibody is capable of inhibiting antibody production after antigen has been largely cleared from the circulation (11). These observations suggest that neutralization of antigen represents the effect at a cellular level of competition for antigen between antibody and specific receptors somehow involved in the antibody-forming process. The nature of these specific receptors has been the subject of much speculation; reasons for implicating preformed strategically located antibody have been described elsewhere (31). Whatever the exact nature of these receptors are, however, it is apparent that if such competition were indeed to occur the comparative affini-

ties of antibody and receptor site for antigen would be of strategic importance, and variations in receptor site affinity as well as antibody affinity would have considerable bearing on the outcome of competition for antigen. Receptor sites of low affinity would, of course, be at a disadvantage to those of high affinity. Assuming a relation between receptor site affinity and the affinity of antibodies formed, it would follow that the inhibitory effect of antibody passively administered or actively formed would be to some degree selective, differentially affecting the production of antibodies of different avidities. This may be a partial explanation for the observation previously noted, that the average avidity of antibody increases during the course of immunization.

Much consideration has been given here and elsewhere to the possible role of antibody in regulating its own overproduction. It may also be suggested that antibody plays a further role in regulating immunologic responses by facilitating antibody formation. Whether or not the presence of small amounts of antibody is critical to immunologic responsiveness (32, 34) is controversial, but the fact that antibody can enhance the response to antigen under experimental conditions, not entirely dissimilar to those which occur naturally, certainly raises the possibility that it may have an effect under ordinary circumstances. Some facilitatory effect may well occur whenever antibody to antigen is present. However, since enhancement and suppression seem to be effects exerted simultaneously by antibodies, the formation and presence of increasing amounts of antibody probably acts as an increasingly effective counterbalance to enhancement, serving more and more to diminish its expression. The clearest expression of enhancement, in fact, probably occurs early in the course of immunization. For reasons given earlier, large amounts of antibody and antibody of high avidity may be especially inhibitory; for similar reasons, small amounts of antibody and antibody of low avidity may be of particular value in enhancing immunologic responses. Both 19S and 7S antibody appear able to enhance antibody formation in relatively small amounts. Experiments reported here do not provide information concerning the comparative efficiency of these immunoglobulins in enhancing antibody formation. However, whereas γG-antibody may have a suppressive role out of proportion to that of other immunoglobulins, it might be suggested that γM-antibody may have an especially significant role in enhancing immunologic responses by virtue of its "natural" presence in small amounts to a large variety of antigens. Currently available information does not permit a definitive interpretation of the mechanism of enhancement, and the presumption that it relates to an increase in the availability of antigen to immunologically competent cells was mentioned only as one possible explanation for this phenomenon. Whether this crude interpretation reflects an effect on the fixation of antigen to membranes and processes of reticular cells as suggested by Mitchell and Nossal (33), or

whether it represents a previously hypothesized effect of aggregation of antibody on antibody formation (31), or whether enhancement is instead dependent upon some other unexplained mechanism cannot be ascertained at present.

SUMMARY

The influence of antibody on antibody formation to particulate antigen was examined in the rabbit with special reference to the importance of immunoglobulin type, the amount and relative proportion of antigen and antibody involved, and the specificity of this influence. 19S as well as 7S antibody was shown to be an effective inhibitor of antibody formation, although there was some evidence that 7S antibody was the more efficient of the two in doing so. The inhibitory effect of antibody was found to be specific for homologous antigenic determinants. Both 19S and 7S antibody were also able to enhance antibody formation. In contrast to the suppressive phenomenon, however, enhancement appeared to be nonspecific since antibody reactive with homologous (sheep red blood cell) determinants could enhance the response not only to homologous determinants but to heterologous (dinitrobenzene) determinants conjugated to the red blood cells as well. Smaller amounts of antibody were needed to enhance than to suppress antibody formation, and suppression and enhancement depended to some extent on the amount of antigen as well as to the amount of antibody used. The enhancing and suppressing influence of antibody on antibody formation appeared to be exerted concomitantly, for the response to some antigenic determinants was sometimes suppressed at the same time that the response to others was enhanced. It is suggested that enhancement or suppression of immunologic responses by antibody represents a different balance of at least two competing factors operating together: specific neutralization of appropriate determinants thus decreasing the total effective concentration of these determinants available to stimulate the formation of antibodies, and a nonspecific increase in the availability of antigen to immunologically competent cells.

It is a pleasure to acknowledge the invaluable technical assistance of Mrs. Christine Terry in the performance of these experiments. I wish to express my appreciation to Dr. David W. Talmage, Professor of Medicine and Microbiology, University of Colorado School of Medicine, for many stimulating discussions during the course of these experiments, as well as for critically reviewing this manuscript. I am also indebted to Dr. Kimishige Ishizaka, Childrens Asthma Research Institute, Denver, Colo., for generously providing specific anti-rabbit γM- and γG-antisera.

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