ANTIBODY FORMATION*

I. The Suppression of Antibody Formation by Passively Administered Antibody

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In 1909, Theobald Smith (1) showed that injection of partially neutralized diphtheria toxin-antitoxin mixtures induced antitoxin formation in experimental animals, but that immunity did not result from mixtures containing a sufficient excess of antitoxin. These observations were subsequently confirmed (2–6) and have been extended to the study of additional antigens, such as tetanus toxoid (7–10), sheep red blood cells (11), poliomyelitis virus (12, 13), and bacteriophage (14, 15).

In this paper, several factors affecting the capacity of passively administered antibody to suppress antibody production have been examined. It has been shown that the degree of antibody suppression is largely determined by the intensity of the antigenic stimulation and the dissociation of the specific complex *in vivo*. It has also been found that antibody injected several days after immunization can prevent a primary antibody response in guinea pigs. It is suggested, therefore, that serum antibody may play a role in the regulation of antibody formation.

Materials and Methods

Antigens.—Three different diphtheria toxoids (To) were used in this study. Two of these, KP59A (50 Lf/ml. and 1730 Lf/mg. N) and PT 55 (1400 Lf/ml., 66 per cent specifically precipitable) were obtained from the Massachusetts Department of Health. The third, 42929–225, containing 820 Lf/ml. and 1790 Lf/mg. N, was obtained from Lederle Laboratories, Pearl River, New York. All the toxoid preparations contained 1:10,000 merthiolate as a preservative. Diphtheria toxins Nos. 8 and 5 were prepared from a culture filtrate of the PW8 strain grown on Mueller and Miller's medium (16), and partially purified by ammonium sulfate fractionation and dialysis. They contained 282 Lf/ml. and 3 Lf/ml., respectively.

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The other antigens employed were ovalbumin (Ea, 2 and 5 times recrystallized) from the Worthington Biochemical Corporation, Harrison, New Jersey, crystalline bovine serum albumin (BSA), bovine gamma globulin fraction II (BGG), and rabbit gamma globulin fraction II (RGG), all from Armour Inc., Kankakee, Illinois, digested horse gamma 1 globulin (HGG) prepared by the method of Glaubiger (17), and guinea pig gamma globulin (GPGG) purified by starch block electrophoresis after its preliminary precipitation from guinea pig serum with 34 per cent saturated ammonium sulphate.

Antisera.—Horse diphtheria antitoxic globulin containing approximately 2000 units/ml. was obtained from Lederle (Lot No. 1520–5319). Rabbit, guinea pig, and rat diphtheria antitoxins were prepared by injecting groups of animals (at least 4 rabbits, 12 guinea pigs, or 12 rats) with To PT 55 and/or To 42929–225. The initial injections contained complete Freund's adjuvant and were distributed into numerous subcutaneous and intramuscular sites. The final injection 1 to 2 weeks prior to exsanguination usually contained aluminum phosphate gel and was sometimes injected intravenously. Rabbits received 3 injections containing a total of 3 to 10 mg. of To over a 6 to 8 week period before exsanguination, guinea pigs 2 to 4 injections containing 1 to 4 mg. To for 4 to 7 weeks, and rats 3 injections containing 1 to 4 mg. To for 7 weeks. In this way, three rabbit antitoxins, RI (65 units/ml.), RII (85 units/ml.), and RIII (90 units/ml.), three guinea pig antitoxins, GPI (70 units/ml.), GPII (100 units/ml.), GPIII (30 units/ml.), and a rat antitoxin (90 units/ml.) were obtained. Rabbit anti-egg albumin (2.80 mg. antibody protein/ml.), rabbit antibovine gamma globulin (3.21 mg. antibody protein/ml.), and guinea pig anti-egg albumin (1.25 mg. antibody protein/ml.) were prepared using an immunization schedule similar to that employed for the diphtheria antitoxins. All sera were stored without preservatives at −20°C. until just before use. The rabbit and guinea pig antitoxins were characterized by: (*a*) toxin neutralization with toxin No. 5 tested in the skin of rabbits as described by Fraser (18); (*b*) toxin neutralization test with toxin No. 8 in rabbit skin (the antitoxin content was considered to be equal to the number of Lf of toxin necessary to give minimal erythema with "1 unit" of antitoxin, as determined by *a*); (*c*) precipitation with To KP59A in very slight antitoxin excess confirmed by determination of the biological activity of the supernatant (the amount of antitoxin in the serum was considered to be equal to the number of Lf of To precipitated); (*d*) avidity by the method of Glenny using peptone water as the diluent (19); (*e*) agar diffusion studies including immunoelectrophoresis (20) and double Preer agar diffusion (21) utilizing as antigen To PT 55 (containing at least 1 mg. of impurities/ml.); (*f*) quantitative precipitation with determination of antitoxin nitrogen content in the case of those antitoxins showing single lines of precipitation by *e* (both guinea pig antitoxins I and II appeared to give a single line of precipitation whereas guinea pig antitoxin III and the rabbit and rat antitoxins revealed multiple lines of precipitation); (*g*) optimal proportions flocculation with To PT 55. Of the 6 antitoxic sera studied, the antitoxin content of 4 did not vary appreciably with the type of test employed In contrast, guinea pig antitoxin II and rabbit antitoxin II had 100 and 85 units of antitoxin/ml. by each test, respectively, except by the neutralization test of Fraser which revealed only 30 units/ml. in each serum. This marked discrepancy was not due to: (*a*) inherent differences between *in vitro* and *in vivo* tests (22), since a second *in vivo* test (referred to under *b*) did not yield these low results; (*b*) poor avidity, since both of these antitoxins had avidities of 2 or under which were not significantly different from the other 4 antitoxin sera. The cause of the poor neutralizing efficiency of these 2 antitoxic sera in the Fraser test is not yet known.

The antibody contents of the rabbit anti–egg albumin, rabbit anti-bovine gamma globulin, and guinea pig anti–egg albumin sera were determined by quantitative precipitation according to the method of Gitlin (23).

Specific Precipitates.—Specific precipitation was carried out at an antigen concentration of

25 to 50 μg. protein/ml. Unless otherwise stated, toxoid-antitoxin precipitation was carried out in 2½ times antitoxin excess and precipitation of other protein antigens in 4 times antibody excess.

After incubation at 37°C. for 30 minutes and at 4°C. for 24 hours, the precipitates (containing 200 to 500 μg. antigen) were washed 3 times, usually with 5 ml. of chilled physiological saline. The precipitates were resuspended by vigorous shaking between dropwise addition of saline which resulted in a macroscopically homogeneous suspension. The suspension was usually emulsified with an equal volume of complete Freund's adjuvant containing 8.5 parts bayol F, 1.5 parts arlacel A, and 2 mg./ml. of lyophilized *Mycobacterium butyricum*, giving a final concentration of 6 μg./ml. of antigen. Emulsification, which was accomplished by mixing in a 20 ml. syringe, occurred within several minutes in the case of precipitates but usually took longer if soluble antigens were used. The stability of the emulsion was tested by allowing one drop to fall 2 cm. above water. If the drop of emulsion remained spherical for 30 seconds the emulsification was considered satisfactory. In one experiment, precipitates were also absorbed on aluminum phosphate gel as described by Holt (24).

Immunization.—Hartley albino guinea pigs weighing 350 to 400 gm., white rabbits of 1 to 2 kg., and 150 gm. albino rats were employed. Unless otherwise stated, rabbits received a dose of 9 μg. of antigen ("free", or as a specific precipitate) in 1.5 ml. of complete Freund's adjuvant; guinea pigs and rats received 3 μg. of antigen in 0.5 ml. of complete Freund's adjuvant. Rabbits were usually immunized intramuscularly into a front and rear leg, guinea pigs intramuscularly into a rear leg, and rats intraperitoneally.

Antibody Determinations.—Serum was obtained from guinea pigs and rats by bleeding from the retro-orbital space and from rabbits by bleeding from the marginal ear vein or heart. Tests for serum antibody included quantitative precipitation, immunoelectrophoresis, double Preer agar diffusion, toxin neutralization test of Fraser, signs of systemic anaphylaxis, passive cutaneous anaphylaxis (PCA), and active cutaneous anaphylaxis (ACA) (25). In order to demonstrate signs of systemic anaphylaxis in guinea pigs, 3 mg. of antigen in 0.5 ml. saline were injected into a vein in the hind foot. PCA was performed by injecting 0.1 ml. serum intradermally into 350 gm. albino guinea pigs followed 5 hours later by 3 mg. of antigen in 0.75 ml. of 0.5 per cent Evans blue dye (EBD) intravenously. ACA involved the injection of 20 μg. antigen intradermally followed immediately by 0.2 ml. of 2 per cent EBD intravenously.

RESULTS

Ratio of Antibody to Antigen in the Precipitate.—It was previously shown that injection into guinea pigs of 3 μg. of diphtheria toxoid–rabbit antitoxin precipitate (To-RGG) prepared in antitoxin excess does not stimulate antitoxin formation for many weeks (26). To determine the number of sites on a To molecule which must be bound with antitoxin in order to prevent antitoxin formation, specific precipitates were prepared which contained the same amount of To but varying amounts of rabbit antitoxin I.

Groups of 8 guinea pigs were immunized with 3 μg. of either "free" To, or specific precipitates prepared at equivalence (average molecular composition, one To to 3 antibody, To–RGG_3) (27) or in 2½ times antitoxin excess (average molecular composition To–RGG_5). In this and in all subsequent experiments, the immunizing antigens were injected intramuscularly in 0.5 ml. of complete adjuvant, unless otherwise stated. The animals were bled at 2, 3, and 4 weeks, the serum obtained from individual animals in each group was pooled, and the antitoxin content was then determined.

Fig. 1 shows that To-RGG_3 was as effective as To-RGG_5 in suppressing detectable antitoxin formation for 4 weeks.

The suppression of antitoxin formation could have been due to antigenic competition between the To and the RGG components of the complex. In order to examine this possibility, specific precipitates of varying composition were prepared with homologous[1] antibodies. Thus, To–guinea pig antitoxin I precipitates (To-GPGG) were injected into groups of 8 guinea pigs each,

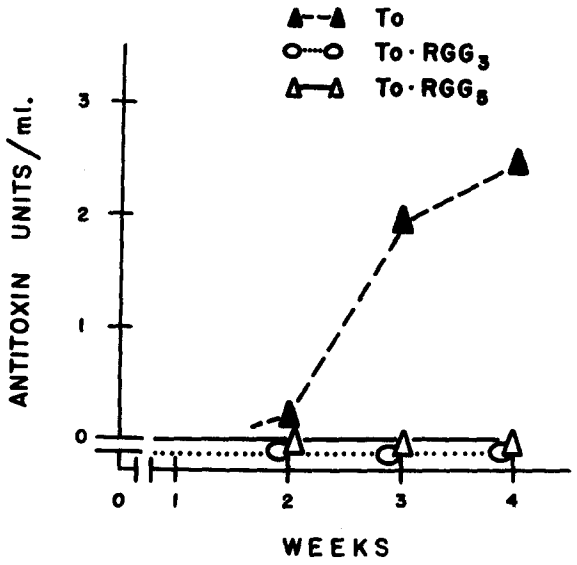

FIG. 1. Suppression of antitoxin formation in guinea pigs by rabbit antitoxin. Groups of 8 animals were immunized with 3 μg. of either "free" To, or with specific precipitates whose average molecular composition was To-RGG_3 or To-RGG_5 (see text).

and bovine serum albumin (BSA)–RGG precipitates into groups of 8 rabbits each.

The results of these experiments (Figs. 2 and 3) indicate that homologous antibody can also inhibit antibody formation in both species. This finding indicates that the suppressive effect of antibody in specific precipitates is not due predominantly to antigenic competition. These experiments also confirm the previous observation that inhibition is as effective with antigen-$antibody_3$ as with antigen-$antibody_5$. Lescowitz (28) also found no essential differences in anti-BSA formation in rabbits injected with BSA precipitates formed at equivalence or far in antibody excess provided that the precipitates were

[1] Refers to antibody obtained from the same species that is to be immunized. Heterologous refers to antibody from a different species than the one to be immunized.

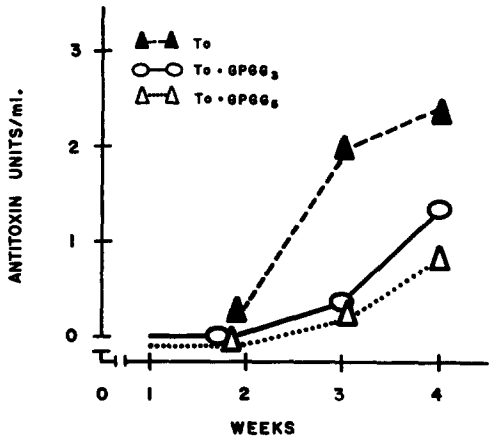

FIG. 2. Suppression of antitoxin formation in guinea pigs by guinea pig antitoxin. Groups of 8 animals were immunized with 3 μg. of either "free" To, or with specific precipitates whose average molecular composition was To-GPGG$_3$ or To-GPGG$_5$.

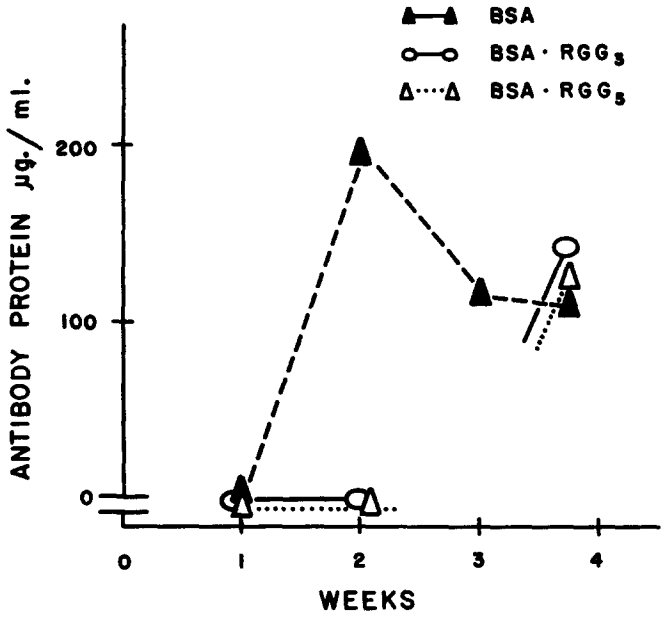

FIG. 3. Suppression of antibody formation to bovine serum albumin (BSA) in rabbits by rabbit anti-BSA. Groups of 8 animals were immunized with 9 μg. of either "free" BSA, or with specific precipitates whose average molecular composition was BSA-RGG$_3$ or BSA-RGG$_5$.

administered in complete adjuvant. When analogous precipitates were injected in saline, however, only those formed far in antibody excess suppressed anti-BSA formation. The cause of this discrepancy is not known.

These experiments also show that rabbit antitoxin is more effective than guinea pig antitoxin in suppressing antitoxin formation in the guinea pig. The possibility, therefore, that the antigenicity of the antibody gamma globulin is an additional factor determining the extent of suppression of antibody formation cannot be excluded by this experiment.

Nature of Antigen.—The duration of antibody suppression against 4 different antigens injected as specific precipitates was studied. The antigenicity of the

TABLE I

Nature of the Antigen

Antigen*	Antibody detectable by				
	Passive cutaneous anaphylaxis			Systemic anaphylaxis‡	
	2 wks.	3 wks.	6 wks.	3 wks.	6 wks.
Toxoid	0/10	0/10	0/6	0/4	0/6
Bovine serum albumin	0/10	0/10	0/6	0/4	8/10
Bovine gamma globulin	0/10	2/10	1/6	3/5	6/8
Egg albumin	0/10	5/10	5/7	4/4	6/6

* Groups of 15 guinea pigs were immunized with antigen-RGG precipitate formed in 4 times antibody excess.

‡ Challenged intravenously with 3 mg. of specific antigen.

proteins varied from that shown by BSA, which is incapable without adjuvants of stimulating a detectable antibody response in guinea pigs (29, 30), to that shown by Ea which is highly antigenic in this species.

Groups of 10 to 15 guinea pigs were immunized with 3 μg. of protein antigen previously precipitated with its corresponding rabbit antibody in 4 times antibody excess. The majority of animals were bled at intervals and their sera examined for antibody by PCA. The remaining animals were injected intravenously with 3 mg. antigen at 4 or 6 weeks to test for signs of systemic anaphylaxis.

Table I shows that antibody production was suppressed for at least 6 weeks with To, 3 to 6 weeks with BSA, and 2 to 3 weeks with Ea and BGG. These findings indicate that, as expected, antibody suppression is usually less efficient with the more antigenic proteins such as Ea and BGG. An exception to this is the observation that antitoxin formation is more easily suppressed than anti-BSA formation.

Dose of Antigen.—Groups of 8 guinea pigs were immunized with varying

amounts of To-GPGG II prepared in antitoxin excess (30 to 0.003 μg. of To). Serum was obtained for antitoxin determinations at 3, 4, and 6 weeks.

As shown in Fig. 4, antitoxin suppression became less effective as the dose of precipitate was increased. By comparison of Fig. 4 (3 μg. curve) with Fig. 2 (To-GPGG$_6$), it can be seen that guinea pig antitoxin II did not suppress

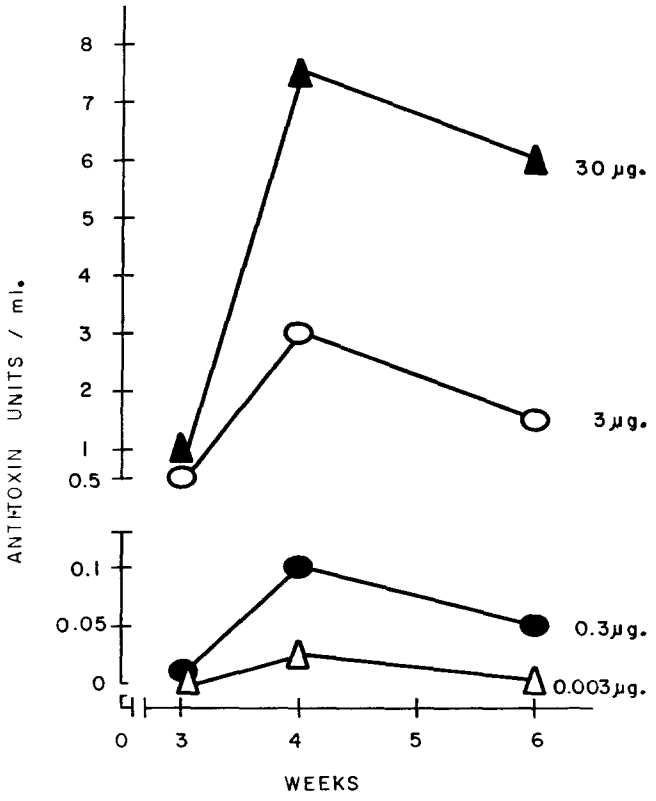

FIG. 4. Effect of dose of antigen as a specific precipitate on the suppression of antitoxin formation. Groups of 8 guinea pigs were immunized with varying amounts of To-GPGG.

antitoxin formation as well as guinea pig antitoxin I. This observation, which was confirmed in later experiments, indicates that the particular antiserum used also affects the efficiency of inhibition.

The use of adjuvants with specific precipitates was also shown to affect antibody suppression. Two groups of 6 guinea pigs each were immunized with 3 μg. Ea-RGG, either suspended in saline or absorbed onto aluminum phosphate gel. When chal-

lenged 18 days later with 3 mg. Ea intravenously, none of the precipitate-in-saline group showed anaphylaxis, whereas all 6 of the precipitate-on—aluminum phosphate gel group exhibited severe anaphylaxis which terminated fatally in 5.

Complement.—Since complement has the capacity to bind to certain types of antigen-antibody precipitates, it seemed possible that the presence of C′ in the precipitate would influence the extent of antibody suppression.

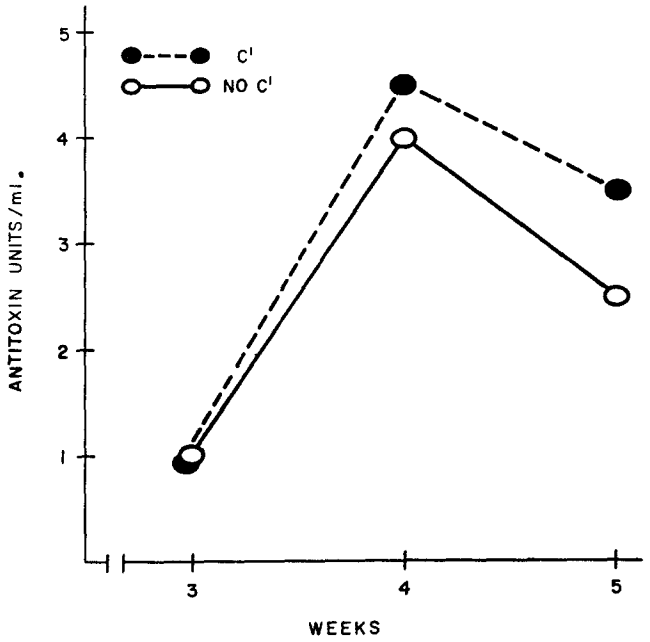

FIG. 5. Effect of complement on the suppression of antitoxin formation. Groups of 8 guinea pigs were immunized with To-GPGG prepared either in the presence or absence of complement.

For preparation of a precipitate containing C′, 0.5 ml. of fresh guinea pig complement containing 90 units (31) was added to 0.7 ml. of "aged" guinea pig antitoxin I before precipitation with toxoid. The control precipitate was prepared similarly except that heat-inactivated C′ was added to the antiserum and the mixture was decomplemented by addition of Ea–RGG (100 μg. Ea). Groups of 8 guinea pigs were immunized intramuscularly with 3 μg. of precipitate, with or without C′. The animals were bled at 3, 4, and 5 weeks, and the antitoxin titers determined.

As shown in Fig. 5, the presence or absence of C′ during precipitation did not significantly influence the antitoxin response in guinea pigs.

Antibody to the Antigenic Globulin.—The possibility that antibody directed against the antigenic globulin of a complex might affect the extent of anti-

body suppression to the antigen was also investigated. 18 guinea pigs were immunized with 3 μg. Ea–RGG 1 hour after one-half of them had been injected intravenously with 0.5 ml. of guinea pig anti-RGG (900 μg. antibody). Studies of the anti-Ea response 14 and 21 days later, using the PCA test on serum dilutions and elicitation of systemic anaphylaxis, failed to reveal any difference between the two groups.

Injection of Antibody After Immunization.—Previous studies (26) have demonstrated that antitoxin formation can be suppressed in guinea pigs by successive separate injections of excess horse antitoxin and To. It was assumed that the binding between To and antitoxin had occurred *in vivo*, since such

TABLE II

*Effect of Injecting Horse Antitoxin after Immunization with Toxoid**

Interval between injection of To and horse antitoxin‡	Antitoxin at 4 wks.	
	Negative Schick test	Pooled serum
days		*units/ml.*
No antitoxin§	8/8	0.640
0	0/9	0.005
+3	0/9	0.005
+5	0/9	0.005

* Groups of 9 guinea pigs were immunized with 15 μg. To in incomplete adjuvant intramuscularly.

‡ Received 400 units of horse antitoxin intravenously.

§ Received 12 mg. normal horse gamma globulin intravenously.

guinea pigs had no detectable antitoxin after elimination of the antigenic horse antitoxin. This experiment suggested that the rate of development of the capacity to form antitoxin could be studied by injecting an antigenic antitoxin at various times *after* the injection of To.

Forty-eight guinea pigs were immunized intramuscularly (right leg) with 15 μg. To in 0.5 ml. of incomplete adjuvant (bayol F and arlacel A, without mycobacteria). Three groups of 9 animals each were then injected intravenously (left leg) with 400 units of horse antitoxic digested gamma 1 globulin (12 mg. protein) either at the same time as the To immunization or 3 or 5 days later. A control group of To-immunized animals was injected intravenously with 12 mg. of normal digested horse gamma 1 globulin at the same time as the To immunization. 1 month after To immunization, all the animals were bled for antitoxin determinations and were then Schick tested. (Preliminary experiments had established that, by this time, the antigenic horse antitoxin is eliminated.) In addition, 12 To-immunized animals were set aside for skin testing and additional antibody studies.

Table II shows that each of the animals in the control group was Schick-negative and that the pooled serum of this group had 0.64 units/ml. of antitoxin. In contrast, none of the animals in the other three groups receiving

antitoxin, either simultaneously or 3 or 5 days after immunization, produced significant amounts of antitoxin. All 27 of these animals were Schick-positive and the serum pools each contained 0.005 units of antitoxin/ml. The pooled serum of the control group and the group injected with horse antitoxin simul-

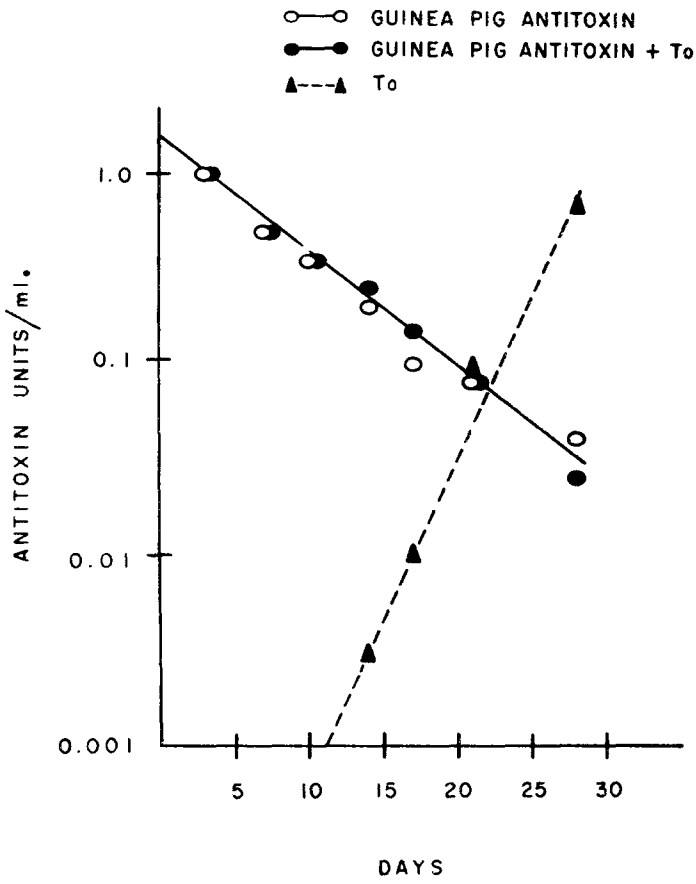

FIG. 6. Suppression of antitoxin formation in guinea pigs by the injection of guinea pig antitoxin 5 days after To immunization. Groups of 5 to 8 animals were injected with either To, guinea pig antitoxin, or both.

taneously with To were examined for anti-HGG by PCA. The highest dilution of each serum which showed antibody by this technique was 1:10. Thus, the control group had received an antigenic stimulation with digested HGG which was analogous to that of the experimental groups.

Two additional immunologic observations were made on To-immunized animals that were not injected with horse antitoxin: (*a*) 3 of 4 To-im-

munized animals already showed large delayed type skin reactions by the 5th day; (*b*) serum antibody was detected in the skin by active cutaneous anaphylaxis on the 12th to 13th day.

A second experiment of this type was carried out using homologous antitoxin. Groups of 5 to 8 guinea pigs were injected with either: (*a*) 15 μg. To; (*b*) 15 μg. To followed 5 days later by 30 units of guinea pig antitoxin (III) intravenously; or (*c*) 30 units of guinea pig antitoxin (III) intravenously. To was given in 0.5 ml. of incomplete adjuvant, intramuscularly.

As can be seen in Fig. 6, passively administered homologous antitoxin disappeared at the same rate in both groups, suggesting that significant antitoxin formation had not occurred in the group previously immunized with To. The To-immunized group that received no passive antitoxin formed 0.17 units of antitoxin by 4½ weeks.

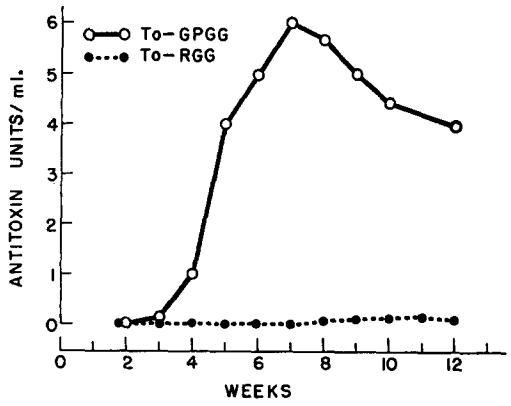

FIG. 7. Comparison of suppressive effects of rabbit and guinea pig antitoxin in guinea pigs. Groups of 8 guinea pigs were immunized with either To-GPGG and Ea-RGG, or To-RGG and Ea-GPGG.

These two experiments indicate that suppression of antitoxin formation in guinea pigs is virtually complete if excess antitoxin (homologous or heterologous) is administered as late as 5 days after To immunization.

Species Origin of Antibody.—Earlier in this study, it was observed that suppression of antitoxin formation in guinea pigs was more effectively accomplished by rabbit than by guinea pig antitoxin (see Figs. 1 and 2). In order to investigate this observation more fully and, in particular, to determine whether or not the antigenicity of the antibody was responsible for the observed difference, the following experiment was undertaken.

Specific precipitates were prepared of To-GPGG I and To-RGG I in 2½ times antitoxin excess, and of Ea-RGG and Ea-GPGG in 4 times antibody excess. 8 guinea pigs and 5 rabbits each received both To-RGG and Ea-GPGG. Another group of 8 guinea pigs and 5 rabbits each received To-GPGG and Ea-RGG. Each animal in the experiment, therefore, received the same 4 proteins. In one group of guinea pigs and rabbits, the To was bound to homologous antibody and the Ea to heterologous antibody, whereas in the other group of

animals, the To was bound to heterologous antibody and the Ea to homologous antibody. Each animal received 3 μg. of antigen (as a precipitate) in 0.5 ml. containing complete Freund's adjuvant/400 gm. body weight. Guinea pigs were injected intramuscularly with one precipitate in each hind leg, and rabbits intramuscularly with each precipitate into a front and hind leg. Serum was obtained at weekly intervals for antibody determinations.

Fig. 7 shows the serum antitoxin levels of the two groups of guinea pigs. The group injected with To-GPGG had no antitoxin detectable in their serum for approximately 3 weeks. Serum antitoxin then appeared and reached a level of 5 units/ml. at 6 weeks. In contrast, none of the To-RGG group showed

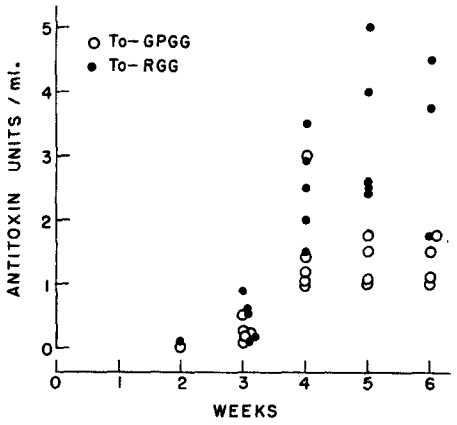

FIG. 8. Comparison of suppressive effects of guinea pig and rabbit antitoxin in rabbits Groups of 5 rabbits were immunized with either To-GPGG and Ea-RGG, or To-RGG and Ea-GPGG.

detectable antitoxin at 6 weeks. Indeed, 3 of the 8 animals showed no detectable antitoxin (< 0.001 units/ml.) during the 3 months of observation.

The serum antitoxin levels of individual immunized rabbits are shown in Fig. 8. In contrast to the guinea pigs, both groups of rabbits produced detectable antitoxin at approximately 2 weeks, and there appeared to be no significant difference between the two groups until 4 to 6 weeks, at which time the To-RGG group appeared to have slightly higher antitoxin levels.

Additional serologic tests performed upon the sera of these animals are summarized in Table III. The PCA test was used for guinea pig antiserum; PCA and double Preer agar diffusion for rabbit antiserum. In guinea pigs, anti-Ea was detectable at 2 weeks in all 8 of the animals receiving Ea-GPGG, but in none of the 8 receiving EA-RGG. In rabbits, there was no difference in the numbers of animals showing anti-Ea at 2 weeks. Both guinea pigs and rabbits usually produced antibody against the heterologous gamma globulin by 4 weeks as demonstrated by PCA and/or double Preer agar diffusion performed with heterologous serum. That the specificity of these antibodies was directed to the heterologous gamma globulin itself and not to other heterologous serum

proteins which nonspecifically absorbed to the initial immunizing precipitate was confirmed by immunoelectrophoresis with whole heterologous serum and purified heterologous gamma globulin. No antibody was detected against homologous gamma globulin, either in guinea pigs or rabbits. It is known that intensive immunization with homologous gamma globulin can stimulate antibody formation in rabbits (32) and delayed hypersensitivity in guinea pigs (33). In the experiments reported here, however, relatively small amounts of pooled homologous gamma globulin were used.

This experiment indicated that the species origin of the globulin played an important role in determining the extent of antibody suppression in guinea pigs. This finding was not due to the qualities of a particular antigen or antiserum, since analogous results were obtained with To- and Ea-specific precipitates. The species to be immunized also influenced the results, since these same precipitates injected into rabbits showed little difference in antibody suppression. The small difference between the two rabbit groups

TABLE III

Additional Antibody Studies in Guinea Pigs and Rabbits Immunized with To and Ea Precipitates

Species	Immunization	Antibody* detectable to		
		Ea	RGG	GPGG
Guinea pig	To-RGG + Ea-GPGG	8/8	8/8	0/8
	To-GPGG + Ea-RGG	0/8	8/8	0/8
Rabbit	To-RGG + Ea-GPGG	3/5	0/5	4/5
	To-GPGG + Ea-RGG	3/5	0/5	4/5

* Serum antibody looked for by PCA and double Preer agar diffusion.

that was observed was the "reverse" of that observed in the guinea pigs and could have been accounted for by the antigenicity of the antibody gamma globulin. The small difference, however, could easily have been caused by uncontrolled minor variables.

A second experiment of this type was therefore carried out using a different species combination, guinea pig and rat. Groups of 8 guinea pigs and 8 rats were injected with either To–rat gamma globulin (To–Rat GG) or To-GPGG I in complete adjuvant, intraperitoneally. The groups that received homologous antibody were given an additional injection of 30 μg. of RGG so that each animal received 2 antigenic proteins. Animals were bled at weekly intervals for antitoxin determinations.

Fig. 9 shows the results of the antitoxin determinations in guinea pigs. Rat GG was significantly more effective than GPGG in suppressing antitoxin formation in guinea pigs (see also Fig. 7). In Fig. 10, which shows the results of the rat immunizations, the antitoxin levels were extremely low; therefore, the scale of the ordinate was reduced. In this species, GPGG appeared somewhat more effective than Rat GG in suppressing antitoxin formation. Immunoelectrophoresis indicated that the guinea pigs which received To–Rat GG had made antibodies against Rat GG by 4 weeks. Precipitating rat anti-GPGG, however, was not detected.

In these two experiments, therefore, involving 3 species, the homologous antibody was less effective than the heterologous in suppressing antibody formation. The most striking difference, however, was observed between guinea pigs injected with To-RGG and To-GPGG. If this difference had been due solely to the antigenicity of RGG, than To–Rat GG would have been expected to behave similarly, since both these gamma globulins are highly antigenic for guinea pigs. However, in the guinea pig, To–Rat GG did *not* suppress antitoxin formation as efficiently as To-RGG (see Figs. 7 and 9), sug-

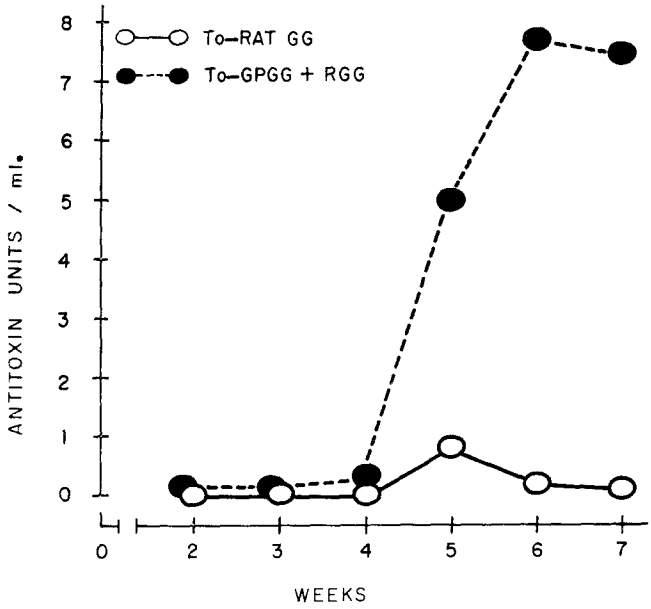

FIG. 9. Comparision of suppressive effects of guinea pig and rat antitoxin in guinea pigs. Groups of 8 guinea pigs were immunized with either To-Rat GG, or To-GPGG and RGG.

gesting that differences other than the antigenicity of the antibody may have played a role in the foregoing results.

Dissociation.—The preceding experiments suggested the possibility that the differences in antibody suppression between specific precipitates could be accounted for in part by differences in dissociability of precipitates. The ultimate appearance of serum antibody might depend upon stimulation of the immune mechanism by "free" antigen which had dissociated from the specific complex. In order to test this possibility, it was necessary to inject a labelled protein whose release from the specific complex could be detected.

The use of the biologic activity of diphtheria toxin as a tracer presented several advantages: (*a*) the native molecule could be used; (*b*) extremely minute amounts of dissociated antigen could be detected (approximately 0.002μg. N); (*c*) only 1 protein was

labelled (the toxin itself). It was possible, however, that dissociated diphtheria toxin might not have its original biologic activity due to irreversible alterations in the molecule resulting from the prior binding with antitoxin. In order to determine the amount of biologic activity that could be recovered from toxin-antitoxin precipitates after dissociation, 10 Lf of toxin was precipitated with excess guinea pig antitoxin in a total volume of 0.6 ml. After washing 4 times with 3 ml. cold saline, the precipitate was

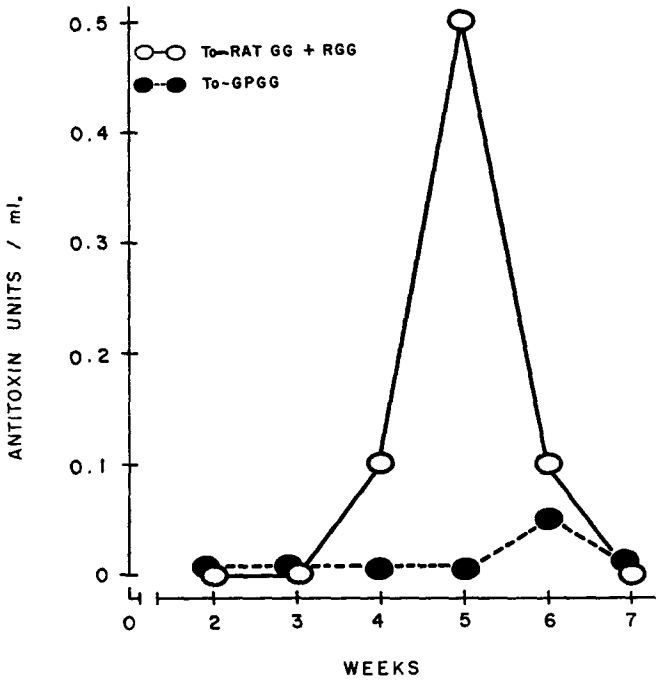

FIG. 10. Comparison of suppressive effects of guinea pig and rat antitoxin in rats. Groups of 8 rats were immunized with either To-Rat GG and RGG, or To-GPGG.

homogenized in a Teffler blender and diluted to contain 1 Lf/ml. 0.1 Lf of precipitate was then added to 1400 Lf *toxoid* contained in 1 ml. in order to solubilize the precipitate (34). The mixture was kept at 27°C. for 3 hours with gentle agitation and at 4°C. for 24 hours. The mixture was then centrifuged at 2000 R.P.M. for 20 minutes and the toxigenicity of the supernatant tested. As a control, 10 Lf toxin was mixed with normal guinea pig serum and was treated similarly except for the homogenization and washings. The toxigenicity of the experimental supernatant as determined by dermonecrotic activity in rabbit skin was 70 per cent of the control toxin. This represents a minimum figure since the manipulations of the experiment (particularly the solubility of the precipitate in the washings) and/or incomplete solubilization of the toxin-antitoxin precipitate in the toxoid might well have contributed to the 30 per cent "loss" of toxicity. The M.L.D. content of this toxin containing 282 Lf/ml. was determined by the usual guinea pig testing and was found to be 28 M.L.D./Lf.

325 Lf of diphtheria toxin was then precipitated with either 650 units of guinea pig antitoxin II or 555 units of rabbit antitoxin II. After standing at 37°C. for 30 minutes and at 4°C. for 24 hours, the precipitates were washed 4 times with 20 ml. cold saline. They were suspended in saline to contain 25 Lf or 700 M.L.D. toxin as a precipitate/0.5 ml. The suspension was non-toxigenic for rabbit skin and the supernatants of both precipitates showed substantial titres of excess neutralizing antitoxin, confirming that the precipitates had been formed in the zone of antitoxin excess.

Groups of 4 animals, 300 gm. guinea pigs or 800 to 1000 gm. rabbits, were injected intravenously with 0.5 ml. of suspension (700 M.L.D. toxin) per guinea pig or 1 ml. per rabbit. The animals were observed daily for signs of diphtheria intoxication and were weighed twice weekly.

The animals appeared well for the 1st week. As shown in Table IV, however, guinea pigs injected with Toxin-GPGG developed typical diphtheritic paralysis usually followed by death 2 to 3 weeks after injection. Guinea pigs, injected with Toxin-RGG which had a slightly greater *in vitro* dissociability than that of the guinea pig antitoxin, did not show signs of intoxication. Rabbits developed diphtheritic paralysis whether injected with Toxin-RGG or Toxin-GPGG.

This experiment demonstrates that dissociation of antigen can occur from specific precipitates formed in the zone of antibody excess. Moreover, the pattern of dissociation parallels that which was predictable from the results of the preceding immunization experiments, *i.e.*, antigen-GPGG complexes dissociate more readily than antigen-RGG complexes in the guinea pig, but not in the rabbit.

The possibility that the absence of toxicity in guinea pigs injected with Toxin-RGG was due to the production of whole body immunity[2] (not reflected in the serum) was also excluded since such animals, 3 weeks after receiving Toxin-RGG, were shown to be fully susceptible to the lethal effects of diphtheria toxin.

Since the antitoxins used in the preceding experiments were relatively inefficient in terms of neutralization (see Materials and Methods) toxin precipitates were formed with the same antitoxins used in the immunization experiments. Similar conditions were employed except that the precipitates were formed in only slight antitoxin excess. 2 of 3 rabbits injected with Toxin–RGG I and 3 of 4 guinea pigs which received Toxin–GPGG I developed paresis of the extremities with eventual recovery. These findings indicate that the *pattern* of dissociation shown in Table IV is not dependent upon the neutralizing capacities of the diphtheria antitoxic sera that are employed. Rosenberg *et al.* (35) have obtained similar results from studies of the dissociation of antigen from soluble antigen-antibody complexes (formed in extreme antigen excess). Using the PCA reaction in guinea pig skin, they have shown that dissociation occurs when guinea pig but not rabbit antibody is used.

Such observations could be explained if "normal" guinea pig gamma globulin exchanged with the antibody gamma globulin of antigen-GPGG complexes, but not with the antibody gamma globulin of antigen-RGG complexes. To investigate this possibility, well washed Toxin-GPGG and Toxin-RGG precipitates (both formed in

[2] Ipsen has shown (personal communication) that, under certain conditions, specifically immunized rabbits may have partial immunity to challenge with tetanus toxin although they lack detectable serum antitoxin.

antitoxin excess) were incubated in either normal guinea pig or rabbit serum, or peptone water; and, after subsequent centrifugation, the supernatants were injected into normal rabbit skin. In this experiment, dissociation of toxin from Toxin-GPGG could be easily demonstrated in guinea pig serum, but not in either rabbit serum or peptone water, thus explaining, in part, the dissociability of Toxin-GPGG in guinea pigs. However, Toxin-RGG also appeared to dissociate slightly in guinea pig serum, but not in peptone or rabbit serum, so that the pattern of dissociation of these toxin-antitoxin precipitates in guinea pigs and rabbits cannot be entirely explained by our *in vitro* studies. The studies of Weigle and Dixon (28), however, may help to elucidate our observations. They found that RGG was unusual in its capacity to resist degradation as compared to other heterologous and homologous serum proteins in the guinea pig.

TABLE IV

Dissociation of Toxin-Antitoxin Precipitates in Guinea Pigs and Rabbits

Species injected*	Origin of antibody globulin	Avidity‡ of antibody	Paralyzed or dead at wk.			
			1	2	3	4
Guinea pig	Guinea pig	1.5	0/4	2/4	4/4	4/4
	Rabbit	2.0	0/4	0/4	0/4	0/4
Rabbit	Guinea pig	1.5	0/4	4/4	4/4	4/4
	Rabbit	2.0	0/4	3/4	4/4	4/4

* Guinea pigs received 25 Lf and rabbits 50 Lf of diphtheria toxin intravenously in the form of complexes with RGG II or GPGG II.

‡ A measure of *in vitro* dissociation.

In order to determine the approximate amount of toxin which dissociates in guinea pigs injected with Toxin-GPGG, groups of 3 guinea pigs were injected with 0.1 to 0.5 M.L.D. toxin intravenously. It was found that 0.25 to 0.5 M.L.D. toxin caused a syndrome similar to that seen in guinea pigs receiving 25Lf Toxin-GPGG II.

The percentage of "free" toxin that dissociates from Toxin-GPGG in guinea pigs could, therefore, be calculated from the amount of complex injected, the biologic activity of dissociated toxin, and the approximate amount of toxin absorbed by the animals. For example, in guinea pigs injected with Toxin-GPGG II, approximately 0.05 per cent of the toxin injected dissociates. This figure can only be considered an approximation, since the amount of toxin released in the precipitate-injected animals necessary to produce paralysis may be different from that which results in paralysis after a single injection of "free" toxin. Moreover, the amount of "immunizing" toxin and "lethal" toxin released from the complex may not be identical. For example, toxin complexed to 1 molecule of antitoxin may be non-toxic, but capable of stimulating antibody formation.

In order to obtain information about the rate of toxin release from specific precipitates, a Toxin-GPGG II precipitate was prepared as previously described. 0.5 ml. of the precipitate suspension (700 M.L.D. of toxin) was injected intravenously into 20 guinea

pigs. Three groups of 5 each were injected intravenously with 100 units of horse antitoxin at various times before or after the precipitates were injected.

As shown in Table V, excess horse antitoxin completely prevented diphtheria intoxication whether given simultaneously or 1 hour after the precipitates were injected. It was of particular interest, however, that antitoxin given 5 hours later could still provide substantial but not complete protection, since specific antigen-antibody aggregates of the type used in this experiment are completely cleared from the circulation within several hours by cells of the reticuloendothelial system (36). Since the release of toxin from the specific complex is in progress at this time, it would appear that the host is actively participating in the dissociation.

TABLE V

*Effect of Excess Horse Antitoxin on In Vivo Dissociation of Toxin-Antitoxin Precipitates**

Time of administration of antitoxin‡	Dead or paralyzed		
	2 wks.	3 wks.	4 wks.
None given	3/5	4/5	5/5
−5 min.	0/5	0/5	0/5
+1 hr.	0/5	0/5	0/5
+5 hrs.	0/5	0/5	2/5

* Each guinea pig was injected intravenously with 25 Lf (700 M.L.D.) diphtheria toxin–50 units GPGG II.

‡ 400 units of horse antitoxin, intravenously.

DISCUSSION

The studies reported here have suggested that the capacity of passively administered antibody to suppress antibody formation is mainly dependent upon the intensity of the antigenic stimulation and the dissociability *in vivo* of the specific complex. Thus, the degree of antigenic stimulation which is determined by the dose and nature of antigen, type of immunization procedure, and the species selected for immunization is, in general, inversely related to the efficiency of antibody suppression. Similarly, antibody suppression is less effective with complexes that readily dissociate *in vivo*. The release of antigen from specific complexes is not only dependent upon the nature of the antigen and species to be immunized, but is also related to the antigen to antibody ratio, and the species origin and particular characteristics of the antiserum employed.

Perhaps the most striking finding to emerge from these studies, however, is that inhibition of antitoxin formation in guinea pigs was virtually complete following intravenous injection of homologous or heterologous antitoxin 5 days *after* toxoid immunization, although delayed-type hypersensitivity to toxoid had already developed. It is probable that even if the interval between

toxoid immunization and subsequent antitoxin injection had been increased, partial inhibition could have been demonstrated. This experiment, therefore, suggests that antibody formation may inhibit itself by the binding and inactivation of antigen that would otherwise stimulate further antibody production. Although the mechanism responsible for inhibition is obscure, our studies suggest that inhibition does not depend upon antigenic competition or the binding of all major antigenic functions, since suppression can be effectively accomplished with fewer homologous antibody molecules than the minimum number of known major determinant sites on the antigen molecule.

The site at which this "feedback" mechanism operates is not known. Inhibition may occur in the plasma cell itself after antigen has initiated antibody production. If such is the case, then antibody production may represent an important mechanism for the termination of antibody synthesis. In support of this view is our subsequent finding that significant inhibition of the anamnestic antitoxin response in guinea pigs can be achieved *after* antitoxin formation has begun (37). Alternatively, antibody may simply intercept antigen *before* it initiates antibody synthesis by preventing antigen from reaching an intracellular template (instructive theories), or from interacting with its "corresponding" pre-plasma cells (selective theories). Under these circumstances, it is possible that this "feedback" mechanism operates only under the artificial conditions of hyperimmunization.

As yet, there is no obvious analogy between this type of inhibitory mechanism and reported "feedback" mechanisms concerned with induced enzyme synthesis in bacteria. In the latter, only products of a metabolic pathway have been shown to act as the suppressors (38, 39).

Another known inhibitory function of antibody is its capacity to prevent the rejection of certain tumors by mice (40). The hypothesis that antibody "coats" certain specific antigens of the tumor inoculum and thus prevents them from stimulating an effective immune response has been considered (41–43). One reason offered for the rejection of this hypothesis is the finding of Kaliss (43), that antiserum can cause enhancement even when administered as long as 10 days after inoculation of C57 Bl/6KS mice with SA 1. Since such tumors begin to regress after the 12th day in unenhanced mice, it seemed probable that the immunologic capabilities of such mice were already well developed by the 10th day and could not have been significantly influenced by the injected antibody. This interpretation appears to be supported by the findings of Mitchison (44) that tumor immunity can be passively transferred by lymph nodes taken from mice 3 to 10 days after the donors had been inoculated with tumors. The data in this paper deal with antibody formation to purified protein antigens rather than with homograft reactions to cellular antigens. Nevertheless, our findings that in the former system suppression of the host response can be accomplished long *after* immunization, indicates a

need for re-evaluation of the development of the immune response in the SA 1–C57 Bl/6KS system. It seems possible that the maturation of the immune response between the 10th and 12th day may be necessary for the rejection of the tumor transplant in this delicately balanced system, and that administration of antiserum on the 10th day may still be able to block an effective immune response. A study of the immune response after passive transfer of lymph nodes from donor mice 10 days after SA 1 inoculation and immediately after enhancement (injection of antiserum) might help to clarify this point. The concept of a block in the "affector" side of the immune mechanism would be supported if passive immunity is not provided by such enhanced immune cells, but is successfully accomplished by the transfer of analogous but unenhanced lymph node cells. Recent studies by Snell support the "affector-block" hypothesis (45).

The findings that diphtheria toxin-antitoxin precipitates formed in the zone of antitoxin excess are capable of causing diphtheritic intoxication 2 or more weeks after their injection into experimental animals was unexpected. Previous studies concerned primariy with the immunizing capacities of toxin-antitoxin mixtures have emphasized the lack of harmful effects of "neutralized" mixtures, *i.e.*, mixtures formed at equivalence (1). Our studies indicate that dissociation of toxin from specific precipitates of composition Toxin-Ab_5 (formed in antitoxin excess) may occur. Thus, it would appear that more antibody is required for neutralization of antigen *in vivo* than *in vitro* in the toxin-antitoxin system. The dissociation of antigen from the precipitate depends not only on solubilization in the body fluids, but also upon active participation of the host as suggested by the following findings: (*a*) under certain conditions there may be a striking lack of correlation between *in vitro* and *in vivo* dissociation (see Table IV); (*b*) the pattern of dissociation of specific precipitates depends upon the species injected (see Table IV); (*c*) excess horse antitoxin can partially protect guinea pigs 5 hours after injection of toxin-antitoxin precipitates (see Table V). At this time, precipitates of this type have been essentially eliminated from the circulation and are being catabolized by the host (36).

The means by which antigen "escapes" from the specific precipitate is not known. It is possible that the readily available antibody molecules are removed first, thereby exposing antigenic determinants. If such is the case, our findings indicate that a minimum of 3 antibody gamma globulin molecules must be removed (at least in part) from a complex of To-RGG_5 before it is capable of stimulating a significant antitoxin response. Some of this dissociated or undigested antigen probably enters immunologically competent cells, since there is an excellent correlation between the dissociation of toxin from specific precipitates and the capacity of such precipitates to eventually immunize (Table IV, Figs. 6 and 7). The amount of antigen that dissociates from antigen-antibody precipitates has been shown to be relatively small, perhaps as little as

0.001 μg. of "free" antigen in guinea pigs receiving 3 μg. of To-GPGG or To-RGG. Nevertheless, such tiny amounts of antigen appear capable of stimulating a vigorous antibody response many weeks after injection of specific precipitates (see Figs. 7 and 9). Such observations suggest that the immune mechanism of the host has been prepared for a specific anamnestic antibody response. This prediction is verified by quantitative studies of the secondary antibody response presented in the following paper.

SUMMARY

The suppression of antibody formation by passively administered antibody is influenced by the dose and nature of the antigen, type of immunization procedure, ratio of antibody to antigen, species origin and characteristics of the antiserum used, as well as the species selected for immunization. In guinea pigs, diphtheria antitoxin formation can be effectively suppressed by an intravenous injection of excess homologous or heterologous antitoxin as long as 5 days after toxoid immunization and after delayed-type hypersensitivity to toxoid has developed. Following the period of antibody suppression which lasts 2 to 7 weeks, serum antibody can usually be demonstrated. It is proposed that this delayed immunization results from dissociation of antigen, since diphtheritic paralysis and death can be produced in guinea pigs and rabbits by the intravenous injection of toxin-antitoxin precipitates formed in antitoxin excess. This syndrome is prevented by injection of excess horse antitoxin 1 hour after injection of the toxin-antitoxin complexes.

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