# ANTIBODY FORMATION*

## II. The Specific Anamnestic Antibody Response

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It has been shown that passively administered antibody can inhibit the primary antibody response to protein antigens in several species of experimental animals (1). For example, injection of diphtheria toxoid-antitoxin precipitates formed in antitoxin excess into guinea pigs results in suppression of antitoxin formation for 3 to 7 weeks, depending in large part on the species origin of the antitoxin. Following this period of antibody suppression, serum antitoxin usually appears, in certain instances at a rate and magnitude indicative of a secondary type of antitoxin response. It was suggested that this delayed antibody response is due to "free" toxoid which dissociates from specific complexes and then stimulates an immune system already prepared for an anamnestic antitoxin response.

Previous studies of guinea pigs sensitized by injection of antigen-antibody complexes have shown that challenge with a sufficient dose of specific antigen transiently abolishes delayed-type skin reactivity and simultaneously induces an accelerated type of antibody response (2). Scharff *et al.* (3) demonstrated that guinea pigs with delayed-type hypersensitivity to bovine serum albumin show an immune elimination of specific antigen whereas normal guinea pigs do not. Similar results have been obtained by Sell and Weigle (4) and by Salvin (5).

In the studies reported here, it has been shown that diphtheria toxin-antitoxin precipitates formed in antitoxin excess can sensitize guinea pigs, rats, and rabbits for a highly efficient secondary antitoxin response. This sensitization may occur without detectable antitoxin formation to the primary antigenic stimulus.

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## *Materials and Methods*

*Antigens.*—All the antigens have been described in the preceding paper (1): diphtheria toxoids (To) KP59A and PT 55, diphtheria toxins No. 5 and No. 8, egg albumin (Ea) 5 times recrystallized, bovine gamma globulin, and guinea pig gamma globulin (GPGG). In addition, fluid tetanus toxoid (Te), Lot No. 434, 35 Lf/ml., from the New York City Department of Health was employed. Horse gamma globulin (HGG) was prepared by precipitation from normal horse serum with 34 per cent saturated ammonium sulphate.

*Antisera.*—Horse tetanus antitoxic serum, 1100 units/ml., and horse diphtheria antitoxic globulin, 2,000 units/ml., were obtained from Lederle Laboratories, Pearl River, New York. Rabbit diphtheria antitoxins I (65 units/ml.), II (85 units/ml.), and III (90 units/ml.), guinea pig diphtheria antitoxins I (70 units/ml.), II (100 units/ml.), and III (30 units/ml.), and rat diphtheria antitoxin (90 units/ml.) were the same antisera used in the preceding study.

*Immunization.*—Specific precipitates were prepared in antibody excess as previously described (1). Hartley albino guinea pigs, 400 gm., white rabbits, 1 to 2 kg., and albino rats, 150 gm., were employed unless otherwise stated. The antigens (either "free" or in the form of specific precipitates) were usually given in complete Freund's adjuvant (8.5 parts arlacel A, 1.5 parts bayol F, and 2 mg./ml. *Mycobacterium butyricum*). In several experiments, mycobacteria were omitted from the adjuvant (incomplete adjuvant). Antigens were also absorbed on aluminum phosphate ($ALPO_4$) gel as described by Holt (6).

*Antitoxin Determinations.*—Serum was obtained by bleeding from the retro-orbital space of guinea pigs and rats and from the marginal ear vein of rabbits. Antitoxin titers were determined by toxin neutralization in the skin of rabbits using toxin No. 8 as described by Fraser (7).

## RESULTS

*Secondary Diphtheria Antitoxin Response in Rats.*—Groups of 12 rats were immunized with 3 μg. of either diphtheria toxoid (To), To–rabbit antitoxin III precipitate (To–RGG III), or To–rat antitoxin precipitate (To–Rat GG). 24-days later, 6 animals in each group were bled of 2 ml. before all animals were challenged with 30 μg. To. After 12 days, serum was obtained from all animals for antitoxin determinations. All the injections were given in 0.5 ml. of complete Freund's adjuvant, intraperitoneally.

The results of two individual experiments are shown in Table I. It can be seen that in both experiments To–Rat GG prepared rats for a highly efficient antitoxin response (priming). The second experiment indicated that To-RGG was also highly effective in priming. Of particular interest was the finding that the secondary antitoxin response after primary immunization with either precipitate appeared significantly greater in both experiments than the secondary antitoxin response produced in the conventional manner by 2 injections of To. The latter observation could not be accounted for by differences in serum antitoxin levels at the time of reimmunization, since the pooled serum of each group in Experiment No. 2 had approximately 0.1 units/ml. at this time.

In the second experiment (Table I), the antitoxin levels of both the To and

To–Rat GG groups were approximately twice those obtained in the first experiment. The cause of such variations is not known. They may be due to changes in the stability of the immunizing emulsions or may be related to the animals themselves. In any event, such variations indicate that only striking and reproducible differences obtained from simultaneously performed experiments can be considered significant.

Six rats were skin tested with 3 μg. To 2 weeks after immunization with To–Rat GG and 4 reaction sites were biopsied at 24 or 48 hours. No delayed inflammatory reactions were demonstrated.

TABLE I

*Secondary Antitoxin Response in Rats*

| Experiment No. | Immunizing Agent* | Number of animals | Serum antitoxin‡, § in units/ml. | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Individual sera | | | | | Pooled serum |
| | | | 0 to 5 | 6 to 10 | 11 to 20 | 21 to 30 | >30 | |
| 1 | To | 9 | 7 | 2 | 0 | 0 | 0 | 5 |
| | To–Rat GG | 8 | 2 | 2 | 2 | 2 | 0 | 15 |
| 2 | To | 7 | 2 | 4 | 0 | 1 | 0 | 10 |
| | To–Rat GG | 9 | 1 | 1 | 1 | 1 | 5 | 40 |
| | To–RGG III | 8 | 2 | 0 | 1 | 1 | 4 | 30 |

* Animals were immunized with 3 μg. To ("free", or as a precipitate) and were reimmunized 3½ weeks later with 30 μg. To. All injections were given intraperitoneally in 0.5 ml. of complete adjuvant.

‡ Sera obtained 12 days after reimmunization.

§ The primary response to the injection of specific complex at 5 weeks is less than 1 unit/ml. The primary response to the reimmunization injection is approximately 0.1 units at 12 days.

These experiments indicate that To-antitoxin precipitates formed in antitoxin excess can prepare rats for a highly efficient secondary antitoxin response.

*Secondary Antitoxin Response in Guinea Pigs and Rabbits.*—Groups of 8 to 18 guinea pigs were immunized with 3 μg. of either To or a specific toxoid-antitoxin precipitate. In one experiment, only 0.03 μg. of To was used in an attempt to see if priming could be achieved with a small amount of antigen. 1 or 2 months later, the animals were challenged with 3 to 30 μg. of To and after 2 weeks, serum was obtained for antitoxin determinations. In several experiments, serum was also obtained just before reimmunization. The injections were given intramuscularly, or in the foot-pads in 0.5 ml. of complete or incomplete adjuvant as shown in Table II.

Table II summarizes the results of four individual experiments. In Experi-

ments 1 and 2, it can be seen that To-GPGG, To–Rat GG, and To all prepared guinea pigs for a secondary antitoxin response to 3 μg. of To; To-RGG did not. To-RGG is only relatively inefficient in priming since a secondary anti-

TABLE II

*Secondary Antitoxin Response in Guinea Pigs*

| Experiment No. | Immunizing agent* | Number of guinea pigs | Serum antitoxin‡ in units/ml. | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | | Individual sera | | | | | Pooled serum |
| | | | 0 to 5 | 6 to 10 | 11 to 20 | 21 to 30 | >30 | |
| 1 | To–RGG I | 4 | 4 | 0 | 0 | 0 | 0 | 0.5 |
| | To–RGG II | 7 | 6 | 1 | 0 | 0 | 0 | 1.5 |
| | To–RGG III | 7 | 7 | 0 | 0 | 0 | 0 | 0.75 |
| | To–Rat GG | 7 | 5 | 2 | 0 | 0 | 0 | 7.5 |
| | To–GPGG I | 7 | 2 | 1 | 1 | 2 | 1 | 11.0 |
| | To–GPGG II | 8 | 2 | 0 | 1 | 2 | 3 | 25.0 |
| | To | 8 | 0 | 2 | 1 | 3 | 2 | 15.0 |
| 2 | To–RGG I | 10 | 9 | 1 | 0 | 0 | 0 | 1.5 |
| | To–GPGG I | 10 | 0 | 1 | 4 | 3 | 2 | 15.0 |
| | To | 6 | 0 | 0 | 1 | 2 | 3 | 25.0 |
| 3 | To–GPGG II (0.03 μg.) | 6 | — | — | — | — | — | <0.05 |
| | To (0.03 μg.) | 5 | — | — | — | — | — | <0.05 |
| 4§ | To–GPGG I | 10 | — | — | — | — | — | 14.0 |
| | To | 10 | — | — | — | — | — | 10.0 |
| 5§, ‖ | To–GPGG I | 13 | 0 | 1 | 2 | 7 | 3 | 35.0 |
| | To | 8 | 0 | 0 | 3 | 5 | 0 | 20.0 |

—, not done.

* Unless otherwise indicated animals were immunized with 3 μg. To ("free" or as a precipitate) and were reimmunized one month later with 3 μg. To. All injections were given in 0.5 ml. of complete adjuvant.

‡ Serum obtained 2 weeks after reimmunization.

§ Immunized in incomplete adjuvant.

‖ Interval before reimmunization was 2 months.

toxin response can be elicited in such animals if the challenge dose is increased to 100 μg. In several experiments, To-GPGG primed slightly more efficiently than To, but the differences were probably not significant. It is of interest that 0.03 μg. of To (either "free" or as a precipitate) in complete adjuvant was not capable of priming.

The capacity of To-antitoxin precipitates to prepare rabbits for a secondary type of antitoxin response was also tested. 5 rabbits were injected into the foot-pads with 9 μg. of To–GPGG II in complete adjuvant. 4 weeks later, all were rechallenged with 1 mg. To intravenously. 1 week later, at a time that unchallenged rabbits have 1 to 2 units/ml., the pooled serum of this group contained 25 units of antitoxin/ml.

Guinea pigs and rabbits were skin tested with 3 and 100 μg. To, respectively, 2 to 3 weeks after immunization. Typical delayed type hypersensitivity skin reactions were elicited in almost all animals.

These experiments indicate that To-antitoxin complexes can prime guinea pigs and rabbits for a secondary antitoxin response. In guinea pigs, the magnitude of this response depends in large part on the species origin of the antibody used in the complex.

*Antigenic Competition.*—It was thought that the relative inefficiency of To-RGG as an immunizing agent in the guinea pig could be due either to its relatively low *in vivo* dissociability (1), or to the antigenicity of the RGG itself. In the latter case, the antigenic functions of the antibody would compete with those on the toxoid molecule for the antibody response, presumably because both sets of antigenic determinants would be presented to the same cell. If this hypothesis is correct, prior immunization with antigenic gamma globulin should enhance anti-gamma globulin production and might be expected to further decrease antitoxin production. In the first experiment to test this hypothesis, we attempted to alter the antitoxin response in rabbits to To-GPGG by prior immunization with GPGG.

Groups of 8 rabbits were sensitized to GPGG or Ea by intramuscular injection with 0.5 mg. of specific antigen in 1 ml. of complete adjuvant. 3 weeks later, both groups of animals were reinjected in the foot-pads with 9 μg. To–GPGG II in complete adjuvant. Serum was obtained from immunized animals 2, 3, and 4 weeks after To–GPGG immunization for antitoxin determinations.

As can be seen from Fig. 1, there was no striking difference between the antitoxin levels of pooled serum in the group previously immunized with GPGG compared to the control group (Ea-immunized).

In a second experiment, an attempt was made to diminish the *secondary* antitoxin response in To–RGG–immunized guinea pigs by rechallenge with RGG *before* To. 20 guinea pigs were immunized with both 3 μg. To–RGG and 3 μg. tetanus toxoid–horse (Te–HGG) antitoxin. Each animal received successive intraperitoneal injections of each precipitate suspended in 0.5 ml. complete adjuvant. 1 month later, one group of 10 animals was rechallenged with 100 μg. RGG and the control group of 7 animals with 100 μg. HGG. 3 days later, both groups were reimmunized with 100 μg To. All reimmunization injections were given intraperitoneally and contained 0.5 ml of $ALPO_4$ gel. 2 weeks later serum was obtained for antitoxin determinations.

As can be seen in Table III, the secondary antitoxin response was not significantly different in the two groups. These experiments, therefore, offer no

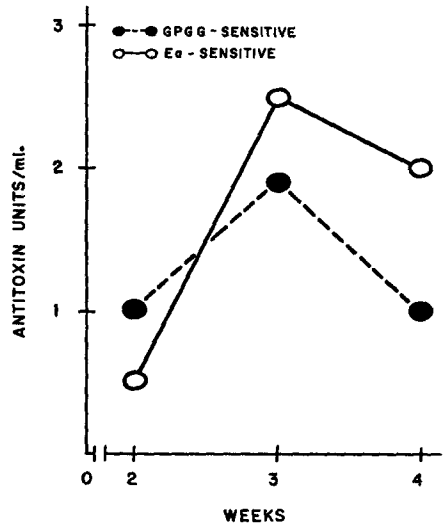


FIG. 1 Effect of prior immunization with GPGG on the antitoxin response to To-GPGG in rabbits. Groups of 8 rabbits were immunized with 1 mg. of either Ea or GPGG 3 weeks before all were reinjected with 9 $\mu$g. of To-GPGG.

TABLE III

*The Effect of Rechallenge with Antigenic Globulin upon the Secondary Antitoxin Response*

| Immunizations | | | Serum antitoxin‖ |
|---|---|---|---|
| 1st* | 2nd‡ | 3rd§ | |
| | | | *units/ml.* |
| To-RGG + Te-HGG | HGG | To | 14 |
| | RGG | | 15 |

* 16 guinea pigs received 3 $\mu$g. each of To and Te in the form of their precipitates in 0.5 ml. of complete adjuvant intraperitoneally.

‡ 1 month later received 100 $\mu$g. of specific antigen ($ALPO_4$ gel).

§ 3 days after second immunization all were reimmunized with 100 $\mu$g. To-$ALPO_4$ gel.

‖ Pooled serum obtained 2 weeks after third immunization.

further support for an antigenic competition between functions on the To molecule and those on the antigenic globulin.

*Suppression of the Secondary Antitoxin Response.*—In the previous study, it was shown that passively administered antitoxin can inhibit the primary

antitoxin response (1). The capacity of antitoxin to inhibit the secondary antitoxin response in guinea pigs was investigated in the following experiment.

41 guinea pigs were sensitized intramuscularly with 3 μg. of To–RGG II in 0.5 ml. of complete adjuvant. 1 month later, groups of 8 animals were challenged intramuscularly with 3 μg. of either To and RGG, To–RGG II, or To–GPGG II and RGG. A control group of 5 sensitized animals was not rechallenged. All animals were bled at weekly intervals for 4 weeks after reimmunization for antitoxin determinations.

As shown in Fig. 2, the pooled serum of the control group that was rechallenged with To had 5 units of antitoxin/ml. at 1 week and a peak level of 17.5.

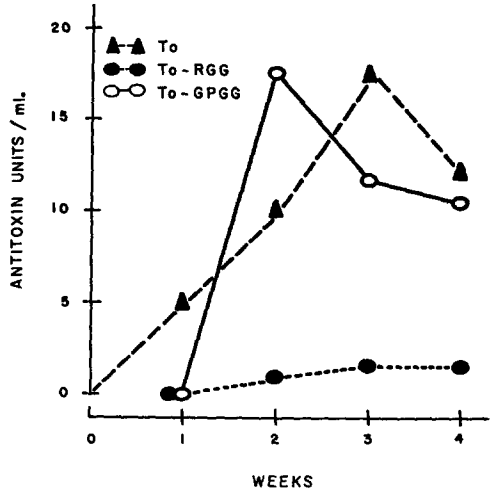


FIG. 2. Suppression of the secondary antitoxin response in guinea pigs. 41 animals were immunized with 3 μg. To–RGG 1 month before groups of 8 were rechallenged with either 3 μg. of either To, To–RGG, or To-GPGG.

units/ml. at 3 weeks. In contrast, the group reimmunized with To-RGG developed a peak level of only 1 unit of serum antitoxin/ml. during the 4 weeks of observation. The group challenged with To-GPGG did not produce detectable serum antitoxin at 1 week, but by 2 weeks their pooled serum contained 17.5 units/ml. The sensitized group that was not rechallenged showed trace amounts of antitoxin ($< 0.05$ units) between 4 and 8 weeks after primary immunization.

These studies indicate that the anamnestic antitoxin response can also be inhibited by passively administered antitoxin, but far less efficiently than the primary response. As expected from previous studies (1), rabbit antitoxin is considerably more effective than guinea pig antitoxin in the guinea pig in this regard. This latter finding together with the lag in secondary antitoxin response

following challenge with specific complexes clearly indicate that it is "free" antigen released from the complex rather than the complex itself which elicits the secondary response. The decreased capacity of antibody to inhibit the secondary as compared to the primary response is undoubtedly due in large part to the more efficient utilization of antigen in primed animals. On the other hand, antigen may also be released more rapidly from specific complexes in sensitized animals, since preliminary unpublished experiments suggest that toxin-GPGG complexes dissociate more rapidly in guinea pigs previously immunized with To-RGG.

*Suppression of the Secondary Antitoxin Response by Administration of Excess Antitoxin After Reimmunization.*—It was previously shown (1) that excess

TABLE IV

*Effect of Injecting Horse Antitoxin after Secondary Challenge with Toxoid**

| Interval between challenge injection of To and horse antitoxin‡ | Antitoxin content of pooled serum in units/ml. | |
|---|---|---|
| | 3 wks. | 4 wks. |
| *days* | | |
| No antitoxin§ | 15 | 10 |
| 0 | 1 | 0.5 |
| +4 | 7.5 | 4 |

* Groups of 6 guinea pigs were immunized with 3 μg. To-RGG in complete adjuvant intramuscularly. 5½ weeks later all were rechallenged with 100 μg. To-($AlPO_4$ gel) intraperitoneally.

‡ 400 units of horse antitoxin intravenously.

§ 12 mg. BGG intravenously.

antitoxin can effectively suppress the primary antitoxin response in guinea pigs as long as 5 days after immunization. The effect of injecting excess antitoxin after *reimmunization* with To was investigated.

Groups of 7 animals were immunized intramuscularly with 3 μg. To-RGG in complete adjuvant. 6½ weeks later, all the animals were rechallenged with 100 μg. To-$ALPO_4$ gel, intraperitoneally. At the same time, one experimental group received 400 units of horse antitoxin and the control group 12 mg. of bovine gamma globulin intravenously. The second experimental group received 400 units of horse antitoxin intravenously 4 days later. All animals were bled for antitoxin determinations 3 and 4 weeks after rechallenge with To. In addition, several immunized animals were injected with 5 M.L.D. of diphtheria *toxin* either 1 or 4 days after rechallenge with To.

As can be seen in Table IV, the pooled serum of the control group had 15 units of antitoxin/ml. at 3 weeks, falling to 10.0 units/ml. at 4 weeks. As expected, the administration of excess antitoxin at the same time as reimmunization resulted in striking suppression of antitoxin formation. However,

a partial but definite suppression of the secondary antitoxin response could be demonstrated when antitoxin was injected 4 days *after* To challenge. The pooled serum of this group had only 7.5 units/ml. at 3 weeks and 4 units/ml. at 4 weeks. Injection of 5 M.L.D. of diphtheria toxin 1 day after reimmunization killed all of 3 guinea pigs, but 4 days after reimmunization only 1 of 3 died. Antitoxin production, therefore, had already begun in the majority of animals.

This experiment indicates that the secondary antitoxin response can be partially suppressed by excess heterologous antitoxin although antitoxin production is already in progress.

## DISCUSSION

These studies indicate that the preparation for a secondary diphtheria antitoxin response (priming) can be achieved by injection of toxoid-antitoxin precipitates, formed in antitoxin excess, into guinea pigs, rats, or rabbits. Depending on the precipitate-animal combination, this preparation can occur with or without a detectable primary antitoxin response. This latter finding suggests that the immune response may be a two step process. After injection of a sufficient amount of "free" antigen, both priming and detectable antibody formation usually occur. In the presence of excess antibody, however, it is possible to separate these two stages, *i.e.*, priming in the absence of detectable serum antibody. This was accomplished when toxoid–rabbit antitoxin precipitates were injected into guinea pigs. Thus, the secondary antitoxin response could be studied without the complication of measurable serum antitoxin persisting from the primary response.

A two stage process in antibody formation has been suggested by other workers in the past (8–10), and more recently by Sercarz and Coons (11) and by Pappenheimer *et al.* (12). The latter have postulated that priming may be the development of a stereospecific mechanism on the cell surface which allows cells destined to produce antibody to efficiently capture and transport specific antigen into the cells. It has been suggested that it is this mechanism which underlies the delayed type of hypersensitivity. Our data are, in general, consistent with this latter hypothesis but offer no further support for it. It has been possible for us to demonstrate the delayed type of skin reactivity in primed guinea pigs and rabbits, but not as yet in primed rats.

The mechanism by which specific precipitates induce priming is not yet understood. The observation, that toxoid–rabbit antitoxin precipitates are less effective than toxoid–guinea pig antitoxin precipitates in priming guinea pigs, may provide an insight into this problem. This difference between the two precipitates could be due to antigenic competition from rabbit antibody and/or might be related to the smaller *in vivo* dissociation of toxoid from toxoid–rabbit antitoxin precipitates. Dissociation appears to be the more important factor, since: (*a*) the hypothetical antigenic competition could not be increased

by either preimmunization or rechallenge before "boosting" with antigenic gamma globulin, and (*b*) toxoid–rat antitoxin was capable of priming guinea pigs. If priming depends upon dissociation, it is possible that the precipitate merely acts as a storehouse which continually releases small amounts of "free" antigen. Under such circumstances the precipitate *per se* may not even enter immunologically competent cells. It will be of considerable interest, therefore, to investigate the role of dissociation of antigen-antibody precipitates in delayed type hypersensitivity, since it is known that such complexes are capable of inducing (13), eliciting (2), and abolishing (2) this immune response.

In rats, the priming capacity of toxoid-antitoxin precipitates was superior to the conventional method of using "free" toxoid for the preparative injection. In contrast, in the guinea pig, there was little difference between efficacious precipitates and "free" antigen. The mechanism(s) responsible for the increased priming efficiency of precipitates in rats is not known. There are at least 3 possibilities: (*a*) Precipitates may form a more efficient emulsion than "free" antigen for purposes of immunization. The importance of adjuvants for antibody formation, particularly in the rat, is well known (14). (*b*) Precipitates may gain access to immunologically competent cells more readily than soluble antigen, since specific precipitates are more easily phagocytized by leucocytes (15), and are more rapidly removed from the circulation (16) than soluble antigen. (*c*) Antibody may protect antigen from catabolism. It is known that antibody can protect protein antigens from digestion by various proteolytic enzymes *in vitro* (17), and it is possible that an analogous process occurs *in vivo*. Such a concept may, at first, appear inconsistent with the accepted role of antibody which is to accelerate removal of foreign material from the circulation of the host. On the other hand, there is ample evidence to suggest that only a small proportion of injected antigen enters cells destined to produce antibody (12). In these cells (as opposed to others), the protection of antigen by antibody would be of biological value.

In any event, the immunizing efficiency of specific precipitates suggests a possible role for such complexes in human immunization, particularly in those instances in which a brisk secondary antibody response is required. Such complexes were, in fact, used in the early part of this century when large numbers of school children were successfully immunized with relatively small amounts of underneutralized diphtheria toxin–horse antitoxin mixtures (18–20).

The practice of passive immunizations is still prevalent in clinical medicine. Although our experimental findings confirm that injection of large amounts of antiserum simultaneously with active immunization may prevent development of immunity, they also suggest that such a regimen may prime for a highly efficient secondary type of antibody response. As has been suggested by previous workers (21, 22) it would appear desirable to prime individuals

receiving tetanus and diphtheria antitoxin with simultaneous immunizing doses of specific toxoid followed later by reimmunization with toxoid.

SUMMARY

Diphtheria toxoid-antitoxin precipitates formed in antitoxin excess can prepare guinea pigs, rats, and rabbits for a secondary type of antitoxin response. Priming may occur without the development of detectable serum antibody. In rats, toxoid-antitoxin precipitates are more efficient than "free" toxoid in priming, whereas in guinea pigs, the magnitude of the anamnestic response varies with the precipitate employed. The possibility that priming is due to "free" antigen released from the specific precipitate rather than the precipitate itself is discussed. The anamnestic antitoxin response can be inhibited by passive antitoxin, but less efficiently than primary antitoxin formation. Partial suppression of the secondary antitoxin response was accomplished by injection of excess horse antitoxin as long as 4 days after reimmunization with toxoid. The importance of these findings for the understanding of passive-active immunization in the human is discussed.

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