STUDIES ON NON-PRECIPITATING ANTIBODY

II. Circulating Antibody-Antigen Complex Following Repeat Injections of Antigen*

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When a single intravenous injection of foreign protein is given to rabbits, large amounts of antibody-antigen complex appear in the circulation. The complex usually is first detected on the 7th day, at a time when free circulating antigen is still present. It reaches a maximum on the 12th day, when free antigen disappears. After the 12th day antibody-antigen complex slowly declines, while free precipitating antibody may be found in the circulation (1).

It appeared interesting to study the formation of antibody-antigen complex after repeated injections of antigen and to correlate the data with the levels of free antigen and free precipitating antibody and with the appearance of free non-precipitating antibody (2). The present paper is a report of such studies.

Methods

Flemish Cross and Albany giant male rabbits, 6 months old, which had been free of infection since birth, were given biweekly intravenous injections of equal doses of foreign protein. Crystalline bovine plasma albumin, Armour Laboratories, (CBPA) was used in doses of 100 mg./kg. body weight and bovine plasma gamma globulin, Armour (BPγG) in doses of 100, 50, 10, and 5 mg. respectively, per kg. body weight. Blood was obtained before each injection and at frequent intervals after injection. The sera were stored in the manner described previously (1).

Circulating Antibody-Antigen Complex.—The sera were treated at pH 12.6 at 1°C. for 6 minutes as previously described and antibody-antigen complex was estimated by nitrogen analysis of the precipitates obtained (1). Tests for specificity were applied as described.

Free Antigen and Free Precipitating Antibody.—Determinations were carried out by the method of Heidelberger and Kendall (3, 4) in the manner of reference 1.

Detection of Non-Precipitating Antibody.—Non-precipitating antibody was considered to be present if alkali-treated serum was able to precipitate more antigen than the corresponding untreated serum (2). Following the separation of antibody-antigen complex from treated serum by centrifugation, the supernate was collected. The supernate from centrifuged

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untreated serum was also obtained. One ml. portions of the supernates were mixed with 1.0 ml. of merthiolated saline[1] containing 1.6, 3.2, 6.4, and 12.8 μg. antigen N respectively. The tubes were left at 1°C. for 4 days and centrifuged at 1000 g at 1°C. for 20 minutes. The new set of supernates thus obtained was tested for excess antigen with a strong precipitating antiserum (prepared with Freund's complete adjuvants (5, 6) in the proportions given in (7)).

If antigen was found in all the supernates of the untreated serum but not in all the supernates of the alkali-treated serum, the serum was considered to contain non-precipitating antibody and to be devoid of precipitating antibody. When the number of tubes free of antigen in the supernates of the treated serum exceeded the number of tubes free of antigen in the supernates of the untreated serum, the serum was considered to contain both, precipitating and non-precipitating antibody. If the number of tubes free of antigen in the supernates of the treated serum equalled the number of tubes free of antigen in the supernates of the untreated serum, the serum was considered to contain precipitating antibody but to be devoid of non-precipitating antibody. If all the tubes of the supernates of the treated and the untreated serum contained antigen, the serum was considered to be devoid of both, precipitating and non-precipitating antibody.

EXPERIMENTAL RESULTS

Circulating Antibody-Antigen Complex in Rabbits after a Repeated Intravenous Injection of 100 Mg./Kg. of Crystalline Bovine Plasma Albumin.—

Six rabbits were given two intravenous injections of CBPA 14 days apart each in doses of 100 mg./kg. body weight.

Fig. 1 shows some of the alkali-treated and untreated sera obtained from one of these rabbits, typical of 4 of the group. The first tube shows the untreated serum obtained before injection. The second tube shows the same serum after alkali treatment. No precipitate developed in this treated serum. This is the expected result with normal preinjection serum (1). The third tube shows the untreated serum obtained on the 14th day after the first injection. The fourth tube represents the same serum after alkali treatment. A precipitate of antibody-antigen complex developed in the treated serum. The fifth tube shows the untreated serum obtained 1 hour after the second injection, 14 days after the first injection. No precipitate developed in this tube. Upon alkali treatment however, a large amount of precipitate separated (6th tube). It is evident that more antibody-antigen complex separated from the treated serum obtained immediately after reinjection than from the treated serum obtained before reinjection. The test for specificity of the precipitate obtained in tube 6 is illustrated in Fig. 1 of reference 2.

Two of the six rabbits in this experiment failed to show any response either to the first or the second injection of 100 mg. CBPA/kg. body weight. When larger intravenous doses of CBPA are given (1 gm./kg.) a poor immune response seems to be the rule (1).

[1] 0.16 N sodium chloride solution containing "merthiolate" 1:10,000.

Immune Response of Rabbits after Repeat Injections of 100 Mg./Kg. of Bovine Plasma Gamma Globulin.—

Nine intravenous injections of BPγG in amounts of 100 mg./kg. body weight were given to each of two rabbits at 14 day intervals. The succeeding changes of free antigen, free precipitating antibody and circulating antibody-antigen complex were followed.

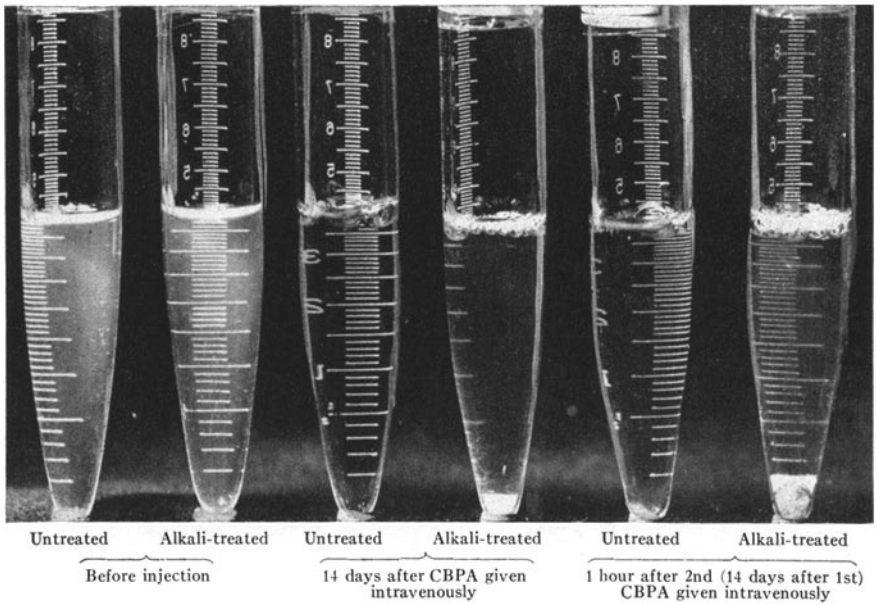

FIG. 1. The immediate formation of circulating antibody-antigen complex after a repeated intravenous injection of 100 mg. CBPA per kg. body weight.

Fig. 2 shows for one rabbit that after the first injection free antigen persisted until the 7th day. Free antibody was not detected. Eighteen μg. N of antibody-antigen complex per ml. of serum appeared on the 7th day, at a time when free antigen still was found in circulation. Only a trace of antibody-antigen complex was detected from the 9th to the 14th day.

After the second injection on the 14th day antigen disappeared more rapidly as expected in a secondary response (8, 9). No antigen was found on the 18th day, 4 days after reinjection. Free antibody appeared in this secondary response, though in trace amounts only. It was first detected on the 21st day, 7 days after the 2nd injection. Antibody-antigen complex rose as early as the 2nd day after reinjection. A maximum of 302 μg. N/ml. was obtained on the 21st day, 7 days after reinjection. At this time free antigen had disappeared from circulation and free antibody was first detected.

After the third and subsequent injections an additional response was encountered. The serum obtained 1 hour after injection contained more antibody-antigen complex than the serum obtained before injection. (This immediate rise of antibody-antigen complex also occurs after a second injection of antigen as shown in Fig. 1 for the response to CBPA. In the present experiment no blood was obtained 1 hour after the 2nd injection.) On the 28th day before the 3rd injection the serum contained 43 μg. N/ml. of antibody-antigen complex. 1 hour after injection 147 μg. were obtained. On the 98th day before the 8th injection the level of antibody-antigen complex was 45 μg. N/ml. and 1 hour

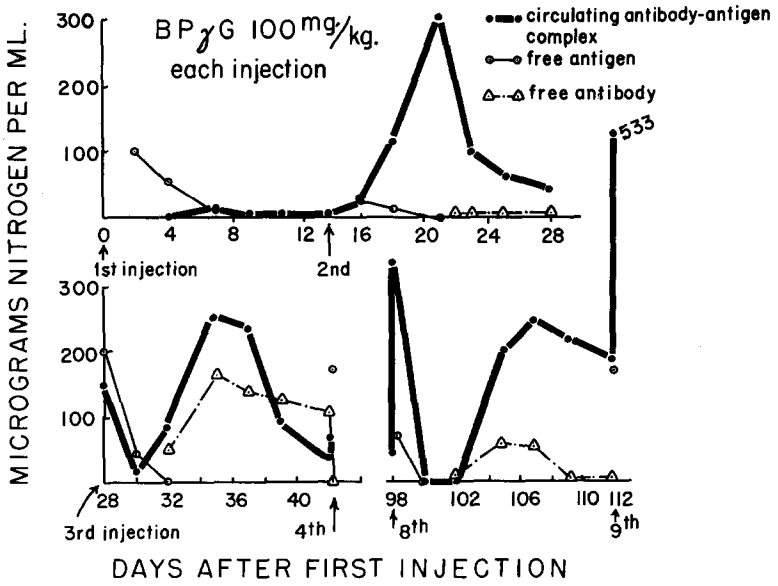

FIG. 2. Immune response after repeated intravenous injections of 100 mg. BPγG per kg. body weight.

after injection it was 342 μg. N/ml. Again, before the 9th injection on the 112th day the serum contained 190 μg. N of complex per ml. 1 hour after injection 533 μg. N of antibody-antigen complex was isolated. The sera obtained after injection, like all other sera, never formed a spontaneous precipitate without alkali treatment. This indicates that the antibody-antigen complex precipitated from the treated sera was derived from non-precipitating factors in the untreated sera. It did not represent a compound of the antigen injected with precipitating antibody. Indeed, it seems that the immediate appearance of antibody-antigen complex upon reinjection of antigen bears an inverse relationship to the presence of free precipitating antibody before injection. Thus, before the 3rd, 8th, and 9th injection, which elicited a rise of antibody-antigen complex, the serum was either free of precipitating antibody or contained only

traces of it. After the 4th injection on the 42nd day antibody-antigen complex rose only from 37 to 65 μg. N/ml. The serum obtained before this injection contained 108 μg. N of free precipitating antibody per ml.

The antibody-antigen complex that appeared immediately after reinjection persisted for a short time only. On the 2nd day after reinjection both free antigen and antibody-antigen complex disappeared from circulation. The complex reappeared on the 32nd day, 4 days after th 3rd injection, and on the

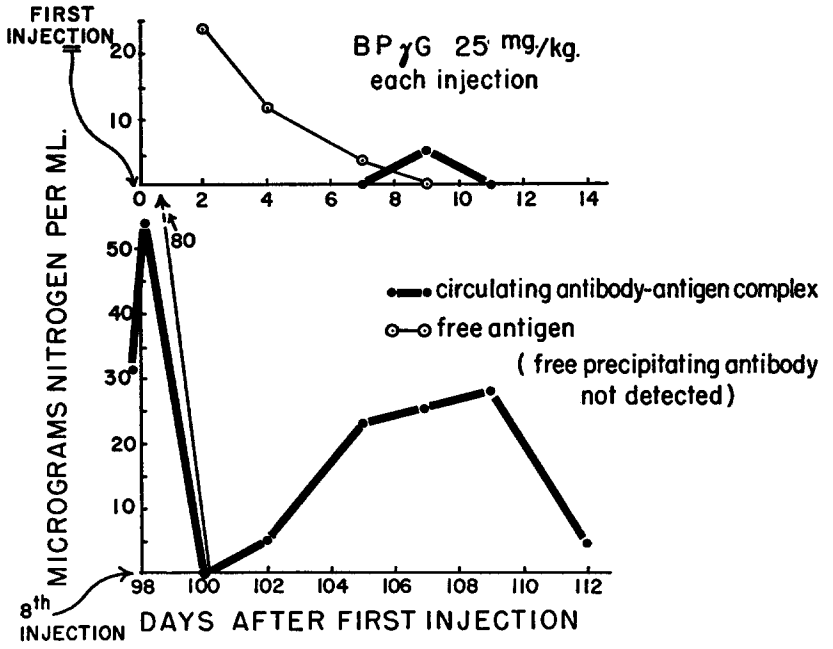

FIG. 3. Immune response after the 1st and 8th intravenous injection of 25 mg. BPγG per kg. body weight.

105th day, 7 days after the 8th injection. The reappearance of antibody-antigen complex in Fig. 2 correlated with the appearance of free precipitating antibody.

Immune Response of Rabbits after Repeat Injections of 25 Mg./Kg. BPγG.—

The previous experiment was repeated with one rabbit, but the dose of each injection of antigen was reduced to 25 mg./kg. of body weight.

Fig. 3 gives the response to the 1st and 8th injection. The sequence of events after the first injection resembled that observed in the preceding experiment. A trace of antibody-antigen complex was detected only on the 9th day. None persisted on the 11th day.

Free precipitating antibody was not detected in this rabbit in any of the blood samples obtained at frequent intervals after the 1st, 2nd, 3rd, and 8th injection

of antigen. Nevertheless, the response of antibody-antigen complex was similar to that of the preceding experiment. Thus, as shown in Fig. 3, on the 98th day, before the 8th injection, the serum contained 31 μg. N of antibody-antigen complex per ml. 1 hour after injection the complex rose to 54 μg. N/ml. Both, free antigen and antibody-antigen complex, were eliminated on the 100th day, 2 days after the 8th injection. The complex reappeared on the 102nd day, 4 days after injection.

Immune Response of Rabbits after Repeat Injections of 5 and 10 Mg./Kg. BPγG.—

In this experiment the dose of BPγG was further reduced. One rabbit received intravenous injections of BPγG in doses of 5 mg. and the other in doses of 10 mg./kg. body weight each 2 weeks. Each rabbit received 15 injections. Blood samples were obtained before the first injection and before and 1 hour after the 3rd and 4th, and 8th to 15th injection. Blood was also obtained at occasional intervals between the 1st to 3rd and 8th to 15th injection.

TABLE I

Relationship of the Presence of Precipitating and Non-Precipitating Antibody before Injection to the Formation of Circulating Antibody-Antigen Complex after Injection

Presence of precipitating antibody before injection	−	+	+	−
Presence of non-precipitating antibody before injection	+	+	−	−
No. of cases with antibody-antigen complex 1 hr. after injection	6	2	0	1
No. of cases without antibody-antigen complex 1 hr. after injection	0	1	6	4

With these small doses of antigen the sera obtained before reinjection usually were devoid of antibody-antigen complex. 1 hour after reinjection complex reappeared in some instances, but failed to appear in others. An attempt was made to correlate the appearance of antibody-antigen complex 1 hour after injection to the presence of precipitating and non-precipitating free antibody before injection. Table I shows that in all the 6 instances in which non-precipitating antibody was present but precipitating antibody absent before reinjection, antibody-antigen complex appeared 1 hour after reinjection. When both, precipitating and non-precipitating antibody, were found in the serum before reinjection, antibody-antigen complex appeared after injection in 2 out of 3 cases. In none of the 6 instances showing precipitating antibody before reinjection, but lacking non-precipitating antibody, did antibody-antigen complex form 1 hour after reinjection. When neither precipitating nor non-precipitating free antibody were detected before reinjection, 4 out of 5 cases failed to form antibody-antigen complex after reinjection. Apparently the presence of non-precipitating antibody before injection is necessary for the immediate formation of circulating antibody-antigen complex after reinjection.

DISCUSSION

When antigen is added to non-precipitating antibody *in vitro* and the mixture treated by alkali in the cold, antibody-antigen complex precipitates (2). Similar results were now obtained *in vivo* by revealing an increase of antibody-antigen complex upon injection of antigen into immunized rabbits and alkali treatment of the resulting sera. The formation of antibody-antigen complex upon reinjection seems to depend on the presence of non-precipitating antibody before reinjection. The observations suggest the tentative conclusion that circulating antibody-antigen complex is formed *in vivo* by antigen and non-precipitating antibody.

If the serum before reinjection of a small dose of antigen contains precipitating antibody but is devoid of non-precipitating antibody, no complex forms upon reinjection. When the preinjection serum contains both, precipitating and non-precipitating free antibody, apparently part or all the reinjected antigen may be removed from the circulation by precipitating antibody. In such a case only the antigen left in circulation may be available for formation of circulating antibody-antigen complex.

The antibody-antigen complex isolated immediately after injection of BPγG in doses of 25 or 100 mg./kg. body weight is formed in the presence of an excess of free antigen. Both the free antigen and the antibody-antigen complex are eliminated by the 2nd day after reinjection. The increased disappearance rate of antigen upon reinjection compared to its rate of elimination after the first injection seems to indicate an anamnestic response (8, 9) and may depend upon the appearance of precipitating antibody (10). The disappearance of antibody-antigen complex may be due to a similar cause. Precipitating antibody may combine with the antigen of the complex and eliminate it from circulation. Its antibody component (non-precipitating antibody (2)) may be left in solution.

Usually on the 4th day after reinjection of antigen, antibody-antigen complex reappears in circulation. The complex rapidly increases in amount on the subsequent days. Much more complex is encountered after a reinjection than after a primary injection, apparently indicating an anamnestic response of non-precipitating antibody.

The antigen of the antibody-antigen complex usually reappearing on the 4th day after a repeat injection of antigen necessarily comes from the tissues, since no antigen is found in the serum on the 2nd day after reinjection. It seems possible that the transient disappearance of antigen from circulation may have a physiologic significance. Perhaps the antigen undergoes metabolic change in the tissues. It is known that the original antigen injected is rapidly eliminated from circulation in anamnestic responses (8, 9, 11) and that none persists or reappears by the time free antibody is detected (11). Experiments in progress seem to indicate that an antigen-like material may persist, however, in circulation at a time when free antibody is detected. This material may either be a metabolic breakdown product of the original antigen or it may be

a new antigen like material formed by the host in response to antigen. It is not capable of reacting with precipitating antibody, but may react with non-precipitating antibody. Its specificity appears to be not as marked as that of the original antigen. Indeed, such persistence of antigen-like material has been postulated before to explain continued formation of antibody (12–14). McMaster was able to demonstrate antigen specific material in the serum of rabbits for as long as 4 weeks after a single intravenous injection of BPγG (15). Freund (9) suggested that immune globulin arising in the host may give some protection to the antigen against destruction and elimination. Perhaps non-precipitating antibody by its capacity for forming antibody-antigen complex with degraded antigen may eventually prove to be such a protecting factor.

SUMMARY

Reinjection of antigen into immunized rabbits usually results in an immediate increase of circulating antibody-antigen complex. This immediate formation of complex seems to depend upon the presence of non-precipitating antibody before reinjection. The complex disappears by the 2nd day after reinjection and reappears between the 4th to 7th day. This is earlier than its first appearance after a primary injection and may indicate an anamnestic response of the non-precipitating antibody responsible for the formation of circulating antibody-antigen complex.

BIBLIOGRAPHY

1. Sternberger, L. A., Maltaner, F., and DeWeerdt, J., *J. Exp. Med.*, 1953, **98,** 451.
2. Sternberger, L. A., Feinberg, S. M. and Clarke, M., *J. Exp. Med.*, 1956, **103,** 523.
3. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1932, **55,** 555.
4. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.* 1935, **62,** 697.
5. Freund, J., Thomson, K. J., Hough, H. B., Sommer, H. E., and Pisani, T. M., *J. Immunol.*, 1948, **60,** 383.
6. Freund, J., Lipton, M. M., and Thompson, G. A., *Proc. Soc. Exp. Biol. and Med.*, 1954, **87,** 408.
7. Sternberger, L. A. and Pressman, D., *J. Immunol.*, 1950, **65,** 65.
8. Von Dungern, E., Die Antikörper. Resultate früherer Forschungen und neue Versuche, Jena, 1903, cited by Freund (9).
9. Freund, J., *in* The Nature and Significance of the Immune Response, (A. M. Pappenheimer, editor), New York, Columbia University Press, 1953.
10. Talmage, D. W., Dixon, F. J., Bukantz, S. C., and Damin, G. J., *J. Immunol.*, 1951, **67,** 243.
11. Dixon, F., *in* The Nature and Significance of the Immune Response, (A. M. Pappenheimer, editor), New York, Columbia University Press, 1953.
12. Breinl, F., and Haurowitz, G., *Z. physiol. Chem.*, 1930, **192,** 45.
13. Alexander, J., *Protoplasma*, 1931, **14,** 296.
14. Mudd, S., *J. Immunol.*, 1932, **23,** 423.
15. McMaster, P., Kruse, H., Sturm, E., and Edwards, J. L., *J. Exp. Med.* 1954, **100,** 341.