IMMUNOCHEMICAL STUDIES OF ANTITOXIN PRODUCED IN NORMAL AND ALLERGIC INDIVIDUALS HYPERIMMUNIZED WITH DIPHTHERIA TOXOID

III. Studies of the Passive Arthus Reaction in Guinea Pigs Using Human Precipitating and Non-Precipitating Diphtheria Antitoxin*

By WILLIAM J. KUHNS, M.D.

(From the Hospital of The Rockefeller Institute for Medical Research)

Plate 53

(Received for publication, December 18, 1952)

In a previous study (1) it was shown that Schick-negative human subjects responded to booster doses of purified diphteria toxoid by forming either precipitating or non-precipitating antitoxin or a mixture of both varieties. Immunological properties of the two contrasting types of human antisera, each containing a high titer of diphtheria antitoxin, were investigated in some detail (2). It was found that non-precipitating antitoxin possesses properties of atopic reagin or skin-sensitizing antibody, and behaves as "univalent" antibody (3, 4). Precipitating antitoxin, on the other hand, possesses some of the properties of allergic blocking antibody (5). These findings suggested the use of the diphtheria system as a model in studies of allergy of the hay-fever type. Diphtheria toxin and toxoid possess advantages over less well characterized materials such as pollen extracts, because they are available in a high state of purity and are reasonably well characterized proteins. In addition, the rabbit intracutaneous test provides a method for accurate measurement of both toxin and antitoxin even when they are present in low concentration.

The diphtheria system is used in the present study to determine the effect of measured amounts of toxoid and antitoxin in another form of experimental allergy, the Arthus reaction.

The Arthus phenomenon, named after the investigator who first described it, occurs when rabbits are given repeated local injections of an antigenic substance, such as horse serum (6). The reaction to early injections consists of transient edema, then of hyperemia and edema, and following many injections becomes hemorrhagic and necrotic. Studies of Rich and Follis (7) and Abell and Schenck (8) demonstrated

* This research was done during the tenure of a Life Insurance Medical Research Fellowship with a supplementary grant from the Helen Hay Whitney Foundation.

that local sensitization of capillaries was necessary for this sequence of events which presumably occurred following the local interaction of antibody with the immunizing agent (9). Observations made in rabbits sensitized with horse serum showed that a characteristic series of histological changes occur following the introduction of antigen subcutaneously: (*a*) blood vessel contraction, (*b*) increased adherence of leucocytes to the walls of capillaries and vessels, (*c*) intravascular aggregation of platelets and leucocytes resulting in cellular thrombosis of capillaries and veins in the area of injection (8). An abnormal metabolic process, as reflected by increased aerobic glycolysis, is observed in the injected skin area (10).

It has been shown that the capacity to react can also be passively transferred to normal animals by injection of the serum from actively sensitized animals (11). The effectiveness of sera in passive transfer is related directly to the precipitin level (12, 13). Quantitative studies have established the amounts of antibody required for the production of passive Arthus reactions in the rabbit. Thus, 1 to 2 mg. of antibody protein sensitizes the skin to a reaction upon subsequent challenge with antigen.

Because of its superior ability as a producer of precipitins against various antigens, the rabbit has been the most widely used animal for the production of Arthus reactions. However, Cannon and Marshall (14) and later Benacerraf and Kabat (15) showed that this reaction could be produced in guinea pigs if they possessed sufficiently high precipitin titers. Benacerraf and Kabat (15) sensitized guinea pigs passively by the intravenous route and caused increasingly severe Arthus reactions with amounts of antibody ranging from 0.09 to 0.92 mg. N. Rabbit and guinea pig antiovalbumin and rabbit antipneumococcus type III serum were found to be equally effective per unit weight. Equivalent weights of non-precipitating ("univalent") rabbit antiovalbumin did not cause Arthus reactions comparable in severity to those produced by precipitating antibody.

The present study was undertaken to determine the amounts of diphtheria toxoid and antitoxin required for the production of passive Arthus reactions in guinea pigs. Because Benacerraf and Kabat (15) found that "univalent" antibody was unable to produce severe Arthus reactions, it also seemed important to determine the comparative roles played in this phenomenon by measured equivalent amounts of human precipitating and non-precipitating diphtheria antitoxin. Additional evidence relating to the importance of aggregation and precipitation in this phenomenon is presented in experiments using heated precipitating antitoxin and certain protein fractions of antitoxic serum.

Materials and Methods

Guinea pigs weighing 300 gm. ± 50 gm. were used in these experiments. Each animal was used for only one experiment. Human sera used for sensitizing guinea pigs were obtained from subjects O'D and Hu and have been previously described (2). Precipitating antitoxic serum obtained from subject O'D contained 110 units per cc. by the rabbit skin test and 240 μg. per cc. of specifically precipitable antitoxic nitrogen. Non-precipitating antitoxic serum from subject Hu contained 70 units per cc. by the rabbit intracutaneous test. Serum Hu could be coprecipitated with known amounts of toxin and precipitating antitoxin, and by this method, 1 unit was equivalent to 1.6 to 1.7 μg. N.

In testing the effect of heat on the antisera, samples of the sera were held at 56°C. for 5 hours as previously described. This procedure causes a loss of antitoxic potency of from 10 to 15 per cent as measured by the rabbit skin test. Precipitating sera treated in this fashion also lose the ability to form precipitates with diphtheria toxoid. It has been shown, however, that the antitoxin present can be carried down quantitatively and specifically with mixtures of appropriate amounts of toxoid and unheated antitoxin. This makes it possible to compare on a quantitative basis the ability of measured amounts of heated and unheated O'D antitoxic serum to cause Arthus reactions.

In sensitizing animals with the various preparations, the desired amounts of antitoxin were injected intravenously in a volume of 4 cc. in borate buffer at pH 7.8.

Serum O'D was fractionated under rigidly controlled conditions of pH, ionic strength, ethanol concentration, protein concentration, and temperature (0 to −8°C.). The procedure followed was essentially that described by Deutsch *et al.* (16). Assay of the reconstituted lyophilized fractions showed that 60 to 70 per cent of the antibody was recovered and 50 per cent of the original unitage was recovered in the γ_2-globulin fraction. A portion of the dried γ_2-globulin fraction was dissolved in borate buffer to contain 275 units per cc. by rabbit skin test. The amount of specifically precipitable nitrogen in this solution was 0.65 mg. per cc.

All guinea pigs were injected intradermally 24 hours after sensitization with serum or serum fractions at a shaved site on the flank with amounts of toxoid ranging from 20 to 340 Lf (0.01 to 0.17 mg. N). Reactions in sensitized animals varied from mild to severe. Those of lesser intensity were characterized (Fig. 5) by only a slight or moderate pink edema and a few or no petechiae. The maximal intensity of such reactions was observed 2 to 6 hours following intracutaneous toxoid. At 24 hours the edema had largely subsided, leaving a small amount of pink discoloration about the area of injection. Severe reactions made their first appearance in a matter of minutes following the local injection of diphtheria toxoid (Fig. 1). This consisted first of a puffy white edema in the center of which was a small dark hemorrhagic inner core surrounded by a blanched zone. A thin red halo was usually present at the periphery of the decolorized area. As the reaction became more intense, the inner and outer colored portions became confluent and assumed a darker hue (Fig. 2). The fully developed reaction was a slightly pink or colorless edema measuring about 30 by 30 mm. which surrounded a dark mottled to homogeneous purple or black area about 10 to 15 mm. in diameter. This remained at maximal intensity for somewhat less than 24 hours and then gradually faded in the course of the next few days. The reaction was graded from ± to ++++ depending on the severity at the time of maximal intensity following intracutaneous toxoid.

EXPERIMENTAL

The relationship between amounts of toxoid and antitoxin injected and the severity of the Arthus reaction is summarized in Table I. The severest reactions were produced in guinea pigs sensitized intravenously with 0.48 mg. N of precipitating antitoxin and injected intradermally 24 hours later with 0.05 or 0.17 mg. toxoid N (Figs. 7 and 8). Reactions of lesser intensity were produced when smaller amounts of either toxoid or antitoxin were injected (Figs. 5 and 6). Only mild reactions occurred in animals sensitized with 0.06 mg. antitoxin N.

The comparative abilities of human precipitating and non-precipitating diphtheria antitoxins to cause Arthus reactions are summarized in Table II. The intravenous administration of 0.48 mg. precipitating antitoxin followed at 24 hours by 0.05 mg. toxoid intradermally produced an intense Arthus reaction

with a characteristic sequence of skin changes (Fig. 7). On the other hand this type of reaction could not be produced in guinea pigs injected with an equivalent quantity of non-precipitating antitoxin. There occurred instead a

TABLE I

Relationship between Severity of the Arthus Reaction and the Amounts of Intravenously Injected Human Precipitating Diphtheria Antitoxin and Intracutaneously Injected Purified Diphtheria Toxoid

24 hour Interval between Sensitizing and Local Injection.

Antitoxin N injected intravenously	Purified diphtheria toxoid N injected intracutaneously		
	0.010	0.050	0.170
mg.			
0.48	++	+++	+++
	++	++++	+++±
		+++±	
		++++	
		+++±	
0.24	+±	+±	
	+		
0.06	±	+	
	±	+	

TABLE II

Comparative Role of Human Precipitating and Non-Precipitating Diphtheria Antitoxin in Passive Arthus Reaction

Type of antitoxin	Amount of antitoxin	0.05 mg. of purified diphtheria toxoid injected intradermally 24 hours following sensitization with antitoxin
		Intensity of reaction
	mg.	
Precipitating	0.48	Severe
Precipitating, heated 56°C. 5 hrs.●	0.44–0.48	Intermediate
Non-precipitating	0.48	Mild
Non-precipitating, heated 56°C. 5 hrs.	0.48	Mild

diffuse pink-colored edema without the hemorrhagic component characteristic of the reaction obtained with precipitating antitoxin (Fig. 9). However, the degree of edema was more intense than that observed when toxoid was given intradermally to control, unsensitized guinea pigs (Fig. 10). The gross appearance somewhat resembled reactions caused by the injection of small amounts of precipitating antitoxin (0.06 mg.), except that edema was more marked and

no central hemorrhagic area was present. Similar reactions were produced in guinea pigs sensitized with 0.48 mg. non-precipitating antitoxin heated at 56°C. for 5 hours. Lesions were similar using 0.5 or 0.17 mg. intradermal toxoid N.

Precipitating antitoxin, which has been heated for 5 hours at 56°C., is no longer precipitable by diphtheria toxoid. However it will be recalled that this antibody remains capable of causing a Danysz effect (2) indicating that antitoxin heated at 56°C. retains the ability to produce aggregates with toxoid

TABLE III

Relationship between Severity of the Arthus Reaction and the Ability of Antitoxin to Produce Aggregates with Toxoid as Measured by Specific Precipitation and the Danysz Reaction

Type of antitoxin	Precipitation of antitoxin by toxoid	Danysz reaction	Arthus reactions	
			Antitoxin used to sensitize	0.05 mg. diphtheria toxoid injected intradermally 24 hrs. after sensitization
				Intensity of reaction
			mg.	
Precipitating	++++	Marked	0.48	Severe
Precipitating, heated 56°C. 5 hrs.	0	Intermediate	0.44–0.48	Intermediate
Precipitating γ_2 globulin heated 56°C. 5 hrs.	++++	Not done	0.48 0.42	Severe Severe
Precipitating γ_2 globulin + crude albumin heated 56°C. 5 hrs.	0	Not done	0.48 0.42	Intermediate Intermediate
Non-precipitating	0	0 or slight	0.48	Mild
Non-precipitating, heated 56° C. 5 hrs.	0	Not done	0.48	Mild

when mixed with appropriate quantities of this antigen. The relation of aggregation and of precipitation to the production of severe Arthus reactions was demonstrated by experiments in guinea pigs using heated precipitating antitoxin. When animals were sensitized with 0.48 mg. antitoxin N, local injections of 0.05 mg. toxoid N resulted in reactions of lesser intensity than those caused by equivalent amounts of unheated precipitating antibody. However, in contrast to the behavior of non-precipitating antitoxin, the reactions caused by heated precipitating antitoxin possessed a hemorrhagic component.

The effect of heat at 56°C. in modifying the intensity of Arthus reactions produced by precipitating antitoxin was further demonstrated by the behavior of certain protein fractions obtained by ethanol fractionation. Cohn and

Pappenheimer (17) showed that a solution of the purified γ_2-globulin fraction in borate buffer at pH 7.4 did not lose the ability to precipitate with appropriate amounts of toxin, even when heated at 56°C. The precipitability of γ_2-antitoxic globulin was again made sensitive to heat when crude albumin obtained by fractionation from the same serum was mixed with it. These results were repeated in the present experiments using γ_2-globulin and crude albumin fractions from precipitating serum O'D. Guinea pigs were sensitized with (*a*) O'D γ_2-globulin heated 5 hours at 56°C. and (*b*) a similarly heated mixture of O'D γ_2-globulin and approximately 50 mg. of the crude albumin fraction. In each instance, the amount of antibody was 0.42 or 0.48 mg. N and intracutaneous toxoid given at 24 hours was equivalent to 0.05 mg. N. Heating the purified γ_2-globulin at 56°C. did not impair its ability to produce a severe Arthus reaction. On the other hand, injection of the heated γ_2-globulin–crude albumin mixture resulted in a less intense reaction (Figs. 3 and 4, Table III).

DISCUSSION

The severity of passive Arthus reactions in the guinea pig is dependent upon the sensitizing dose of human precipitating antitoxin and the challenge dose of toxoid. Mild reactions consisting only of edema and hyperemia were caused by 0.06 mg. antitoxin N and severe reactions were produced by 0.48 mg. antibody N (Fig. 7). Benacerraf and Kabat (15) obtained analogous results using rabbit and guinea pig antiovalbumin and rabbit antipneumococcus type III serum.

Sensitization of guinea pigs with 0.48 mg. N of non-precipitating antitoxin (Hu) did not produce the severe reaction observed in animals injected with the same amounts of precipitating antitoxin (O'D). Although non-precipitating antitoxin in these amounts produced a rather marked edema at the site of toxoid challenge, there occurred no visible hemorrhagic or petechial reaction such as was caused by equivalent amounts of precipitating antibody. Non-precipitating antitoxin, therefore, seemed to behave as "univalent" antibody (15). It is to be recalled that both varieties of antitoxin possess an approximately equal capacity to cause anaphylaxis in appropriately sensitized guinea pigs, a finding similar to that of Kabat (14) who used other antigen-antibody systems. As these authors previously demonstrated, considerably more antibody was required to cause severe Arthus reactions than was needed to sensitize animals to fatal anaphylactic shock.

From the present experiments and those referred to previously (17), it is possible that heat at 56°C. for 5 hours may cause the formation of a complex between γ_2-precipitating antitoxic globulin and a component of the crude albumin fraction which is associated with loss of precipitability and impairment of the ability to cause an Arthus reaction. This is suggested by the comparative behavior of whole serum containing precipitating antitoxin and of purified γ_2-antitoxic globulin which have been heated at 56°C. for 5 hours. Heat abolishes the precipitability of whole antitoxic serum with toxoid and

also reduces the ability to cause severe Arthus reactions. Mixtures of heated precipitating γ_2-globulin and the crude albumin fraction exhibit a similar behavior. On the other hand, γ_2-globulin which has been heated alone remains precipitable by appropriate amounts of toxoid and also causes a severe Arthus reaction in the guinea pig. Experiences with the precipitin reaction and the Danysz phenomenon (2), (Table III) using unheated and heated precipitating and non-precipitating antitoxic sera suggest that differences in severity of the Arthus reaction produced by these preparations reside in their comparative abilities to form intravascular aggregates with appropriate quantities of toxoid.

SUMMARY

The Arthus reaction was studied in guinea pigs passively immunized with human diphtheria antitoxin. Diphtheria toxoid was given intradermally 24 hours following the intravenous administration of antitoxin and the subsequent reactions were graded and measured.

The intensity of Arthus reactions was dependent upon the relative amounts of precipitating antitoxin and toxoid used. Severe lesions were caused by intravenous sensitization with 0.48 mg. precipitating antitoxin N and intradermal challenge with 0.05 or 0.17 toxoid N. Reactions of lesser intensity were caused by smaller amounts of antitoxin and toxoid.

The nature of the antibody used for sensitization was of importance in the severity of Arthus reactions. In contrast to the behavior of precipitating antitoxin, amounts of non-precipitating antitoxin equivalent to 0.48 mg. N did not cause severe Arthus reactions when 0.05 or 0.17 mg. toxoid N was given intradermally.

Precipitating antitoxic whole serum is altered by heating at 56°C. for 5 hours so that its precipitability is lost without any appreciable loss of antitoxic strength. This modified antitoxin produced Arthus reactions of only intermediate severity in guinea pigs sensitized with 0.48 mg. antitoxin N. When fractions of precipitating antitoxic serum were obtained using a cold ethanol technique described by Deutsch (16), a mixture of the purified γ_2-globulin and the crude albumin fraction heated together at 56°C. for 5 hours behaved similarly to whole serum. However, γ_2-globulin alone was not affected by the heating procedure, remained precipitable by toxoid, and was able to cause a severe Arthus reaction following sensitization with 0.48 mg. antitoxin N.

The author wishes to thank Dr. Merrill Chase of the Rockefeller Institute for his kindness in preparing the colored photographs.

Purogenated diphtheria toxoid was supplied through the courtesy of Dr. Henry Piersma of Lederle Laboratories, Pearl River, New York.

BIBLIOGRAPHY

1. Kuhns, W. J., and Pappenheimer, A. M., Jr., *J. Exp. Med.*, 1952, **95,** 363.
2. Kuhns, W. J., and Pappenheimer, A. M., Jr., *J. Exp. Med.*, 1952, **95,** 375.

3. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62,** 697.
4. Kabat, E., and Benacerraf, B., *J. Immunol.*, 1949, **62,** 97.
5. Cooke, R. A., Barnard, J. H., Hebald, S., and Stull, A., *J. Exp. Med.*, 1935, **62,** 733.
6. Arthus, M., and Breton, M., *Compt. rend. Soc. biol.*, 1903, **55,** 1478.
7. Rich, A. R., and Follis, R. H., Jr., *Bull. Johns Hopkins Hosp.*, 1940, **66,** 106.
8. Abell, R. G., and Schenck, H. P., *J. Immunol.*, 1923, **8,** 19.
9. Opie, E. L., *J. Immunol.*, 1923, **8,** 19.
10. Stetson, C. A., Jr., *J. Exp. Med.*, 1951, **94,** 347.
11. Opie, E. L., *J. Immunol.*, 1924, **9,** 231.
12. Culbertson, J. T., *J. Immunol.*, 1932, **23,** 439.
13. Seegal, D., Seegal, B. C., and Jost, E. L., *J. Exp. Med.*, 1932, **55,** 155.
14. Cannon, P. R., and Marshall, C. E., *J. Immunol.*, 1941, **40,** 127.
15. Benacerraf, B., and Kabat, E., *J. Immunol.*, 1950, **64,** 1.
16. Deutsch, H. F., Alberty, R. A., and Gosting, L. J., *J. Biol. Chem.*, 1946, **21,** 165.
17. Cohn, M., and Pappenheimer, A. M., Jr., *J. Immunol.*, 1949, **63,** 291.

EXPLANATION OF PLATE 53

Figs. 1, 2, 3, 6, 9, and 10 are one-sixth actual size. Figs. 4, 5, 7, and 8 are between one-fourth and one-fifth actual size.

FIG. 1. 0.48 mg. N intravenous precipitating antitoxic γ_2-globulin heated at 56°C. for 5 hours. 0.05 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 20 minutes.

FIG. 2. 0.48 mg. N intravenous precipitating antitoxic γ_2-globulin heated at 56°C. for 5 hours. 0.05 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 2 hours.

FIG. 3. 0.48 mg. N intravenous precipitating antitoxin γ_2-globulin heated at 56°C. for 5 hours. 0.05 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 4 hours.

FIG. 4. 0.48 mg. N intravenous precipitating antitoxic γ_2-globulin and 50 mg. crude albumin fraction heated at 56°C. for 5 hours. 0.05 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 4 hours.

FIG. 5. 0.24 mg. N intravenous precipitating antitoxin. 0.05 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 4 hours.

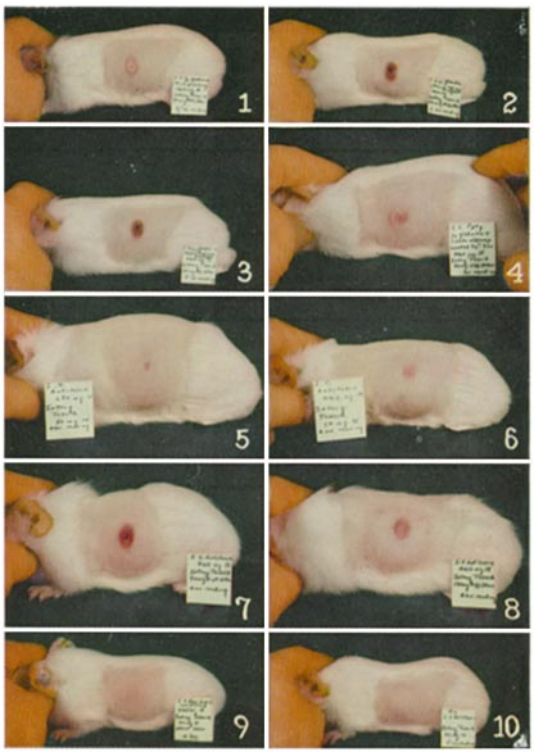
FIG. 6. 0.48 mg. N intravenous precipitating antitoxin. 0.01 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 4 hours.

FIG. 7. 0.48 mg. N intravenous precipitating antitoxin. 0.05 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 4 hours.

FIG. 8. 0.48 mg. N intravenous precipitating antitoxin. 0.17 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 4 hours.

FIG. 9. 0.48 mg. N intravenous non-precipitating antitoxin. 0.05 mg. N intracutaneous toxoid at 24 hours. Reaction observed at 4 hours.

FIG. 10. No intravenous antitoxin. 0.05 mg. N intracutaneous toxoid. Reaction observed at 4 hours.



(Kuhns: Immunochemical studies of antitoxin. III)