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

VI. Further Investigations on the Identity and Specificity of Non-Precipitating Skin-Sensitizing Antitoxin

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One of the difficulties encountered in studying allergies of the hay-fever variety is a lack of available techniques for specific characterization of the component antigen-antibody system or systems implicated in these conditions. This in turn may result in failure to identify wheal activity of passively transferred reaginic sera with the interaction between a given skin-sensitizing antibody and its corresponding antigen. Additional experimental methods for the study of the wheal phenomenon would be more feasible if a greater number of quantitative immunological procedures could be applied to the above problems. Thus it was felt that more precise information relating to the interactions between a reagin and its related antigen could be obtained using diphtheria skin-sensitizing antitoxin and toxoid as a model system (1) since immediate wheal reactivity as measured by passive transfer techniques was, in several hyperimmune sera, directly related to antitoxin content as measured by the rabbit skin test (2) which provided a second independent and sensitive means of determining antibody activity.

In the course of recent studies of certain sera from hyperimmunized subjects, it was possible to apply immunological techniques which were of unusual value in their application to problems of antibody specificity and had not previously been used to characterize skin-sensitizing antitoxin. These included (*a*) the agar diffusion technique of Oudin and (*b*) a previously unpublished method which demonstrates that skin-sensitizing antitoxin remains at intracutaneous sites and neutralizes the delayed specific toxic effects of subsequently injected Schick test reagent. The present report describes experiences with both techniques as well as with the usual serological and passive transfer procedures using sera from eight subjects possessing marked immediate skin reactivity to toxoid.

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Further evidence will be presented to show that immediate wheal reactions are caused by skin-sensitizing antitoxin, and that reactions following the injection of toxin or toxoid in sensitized sites occur because of specific interaction between the antigen and this variety of antitoxin.

Materials and Methods

Materials used in the Schick test, evaluation of immediate reactions to Schick toxoid, and methods of hyperimmunization of Schick-negative subjects are described in previous publications (1). Preparation and properties of the diphtheria toxin and toxiod used and the techniques employed in carrying out the intracutaneous neutralization test in rabbits are described elsewhere (3, 4).

TABLE I

Skin-Sensitizing Antitoxin Formed in Eight Individuals Hyperimmunized with Diphtheria Toxoid

Name	Immediate skin reactivity to toxoid		Rabbit skin titer		Precipitation with toxoid		Fixation of guinea pig complement (Antitoxin needed to fix 2 units of C')
	Before immunization	After immunization	Before immunization	After immunization	Capillary	Tube	
			Units/cc.	*Units/cc.*			
Hu	0	++++	1	80	0	0	>8 units
Au	++++	++++	10	12	0		>1 unit
Ra	+	++++	0.1	10	0		>1 unit
Fr	++++	++++	1	4	0		No C'F
Gr	0	++++	0.01	2	0		No C'F
Chr	±	++++	0.01	50	0	0	>5 units
Ma	0	++++	0.01	3	0		No C'F
Wi	±	++++	0.1	10	0		>1 unit

Human Non-Precipitating Skin-Sensitizing Sera.—Eight sera were selected from specimens available from 160 subjects who had been given a single booster dose of diphtheria toxoid; the subjects were the same as those used in previous studies (1, 2, 5). All eight individuals were adults and all but one gave a history of having had previous injections of diphtheria toxoid 2 months to 15 or more years before the present booster dose of toxoid (Table I). None gave a personal or family history of allergy. Seven received 37.5 Lf of fluid toxoid and one (Hu) received 200 Lf of alum toxoid. Six showed no or slight immediate skin reactivity to Schick toxoid prior to immunization and two (Au and Fr) demonstrated marked immediate reactivity prior to booster toxoid. All eight subjects showed marked immediate reactivity when tested with intradermal Schick toxoid 9 days following booster toxoid. The antitoxin titers as determined by the rabbit intracutaneous test ranged from 0.01 units/cc. to 10 units/cc. prior to immunization and from 2 units/cc. to 80 units/cc. subsequent to booster toxoid. All of the sera possessed little or no ability to fix guinea pig complement even when undiluted. None showed precipitable antitoxin when undiluted serum was mixed with varying amounts of purified toxin or toxoid using the capillary precipitin technique (6) and, in some instances, the tube technique (1).

Agar Diffusion Technique.—The Oudin technique (7) as modified by Munoz and Becker (8) was employed with minor alterations. The 0.6 per cent agar and glassware were prepared as

described by these authors. Agar-coated glass tubes of 5 mm. inner diameter and 70 mm. length were used. Specimens of precipitating and of non-precipitating antitoxin containing 0.02 per cent merthiolate were incorporated into serum-agar mixtures using equal amounts of antitoxic serum or serum dilutions heated to 48°C. and 0.6 per cent melted agar which had been cooled to 48°C. Approximate 1 cc. portions of the serum-agar mixtures were placed in the prepared tubes and cooled at 4°C. for 1 hour. Each mixture was then overlayed with the appropriate antigenic material. The following substances were used: crude and purified diphtheria toxoid from the same lot,[1] and a 1 per cent solution of P-protein.[2] It was felt that the use of crude preparations would expose the antisera to relatively high concentrations of antigens other than toxin and thereby possibly provide evidence for the existence of precipitating systems unrelated to diphtheria toxin and antitoxin. The strength of toxoid, when used, was from 55 Lf/cc to 115 Lf/cc. For control purposes, a specimen of each serum-agar mixture was overlayed with borate buffer at pH 7.8 which had been used as a diluent for the toxoid. All filled tubes were sealed with plasticene to prevent evaporation. They were kept at 37°C. and observed for a period of 2 to 3 weeks during which time migration of the leading edge of the precipitating band from the antigen-serum-agar meniscus was observed and measured.

The number of units in serum-agar mixtures varied and was dependent upon the titer of the original undiluted sera. In the case of high titered sera, dilutions were made in such a way that the overlying toxoid contained more than the equivalent amount of antitoxin present in the mixture beneath. Some specimens of skin-sensitizing antitoxic sera possessed only very low antitoxin titers (as low as 2 units/cc.). In order to ascertain the possible existence of precipitating bands in the presence of only small amounts of antitoxin it was necessary to determine the limit of reliability of this technique using a known precipitating serum of low titer. Therefore, mixtures containing a range of dilutions of precipitating antitoxin were tested. By this means it was demonstrated that precipitating antitoxic sera diluted to contain amounts as small as 1.2 units/cc. showed a visible band following the addition of crude and purified toxoid.

Passive Transfer Experiments in Human Skin

Measurement of Immediate Skin Reactivity Using Diphtheria Toxoid.—The dilution method and the neutralization technique were used as described in an earlier publication (2), except for slight modifications. Recipients were individuals who were Schick-positive and who gave no immediate wheal reaction to intradermal toxoid. In carrying out the dilution tests, sites were injected intradermally with 0.1 cc. amounts of antitoxin dilutions, and 48 hours later injected with 0.1 Lf of toxoid in 0.02 cc. of saline. Skin-sensitizing activity was expressed in terms of the amount of antitoxin which upon challenge with toxoid yielded reactions greater than a toxoid control (0.1 Lf at an unprepared skin site). In the neutralization tests, mixtures of constant antitoxin and varying amounts of toxoid contained in 0.1 cc. were placed in unprepared sites and the immediate reactions read. Subsequent toxoid challenge of each site was made at 48 hours using 0.1 Lf in 0.02 cc. Neutralization was presumed to have occurred at the site or sites where mixtures caused a significant wheal reaction leaving insufficient residual antitoxin to cause a further reaction upon later toxoid challenge.

Tests Using Schick Toxin at Intradermal Sites Containing Skin-Sensitizing Antitoxin.—Additional studies were based on the ability of skin to retain intradermally injected skin-sensitizing antitoxin and thus neutralize the toxic effects of Schick toxin in Schick-positive subjects. Skin sites were prepared initially with tenfold dilutions of the antitoxic sera (including

[1] The author is indebted to Dr. J. A. McComb and L. Levine of the Massachusetts Antitoxin Laboratory, Jamaica Plains, for this material.

[2] Dr. A. M. Pappenheimer, Jr., Department of Microbiology, New York University College of Medicine, kindly supplied the P-proteins which were used in these experiments.

one dilution equivalent to the amount of toxin subsequently injected) and control sites were injected with buffer diluent at pH 7.8. 2 to 7 days later, the same sites were injected with 0.02 MLD (0.0004 Lf) of Schick toxin. They were examined again 2 and 4 days thereafter for the presence of reactions caused by toxin or for degrees of inhibition of this reaction due to neutralization of toxin by intradermal skin-sensitizing antitoxin. Experiences with this technique have shown that neutralization of Schick toxin by skin-sensitizing antitoxin may be demonstrated in human skin at levels of antigen and antibody which are insufficient for the production of immediate wheal reactions demonstrable by the usual methods of passive transfer (Table III).

In order to facilitate interpretations in subsequent sections, we designate skin tests employed to evaluate properties of skin-sensitizing antitoxin as follows:—

Procedure I. Dilution Test.—Titration of wheal and erythema activity of sera; intradermal serum dilutions were challenged in 48 hours using intradermal toxoid.

Procedure II. Neutralization Test.—Measurement of amount of toxoid required to abolish wheal and erythema activity of a constant amount of antitoxin; sites containing mixtures of different amounts of toxoid and constant antitoxin were challenged in 48 hours with intradermal toxoid.

Procedure III. Measurement of Capacity of Antitoxin, Previously Injected into the Skin, to Neutralize Diphtheria Toxin.—Antitoxic activity of skin-sensitizing antitoxin was measured directly by intradermal injection of serum followed 2 to 7 days later by an intradermal injection of Schick test reagent. Readings 2 and 4 days thereafter were compared with control.

RESULTS

Absence of Precipitating Antibody.—The eight skin-sensitizing sera were tested for precipitating bands in the presence of crude and purified diphtheria toxoid using the agar diffusion method. No bands were demonstrable by this sensitive technique following prolonged incubation at 37°C., a finding which suggested the absence of precipitating antibody. This result confirms earlier negative findings with capillary and precipitin tests.

Passive Transfer Experiments in Human Skin.—The result of a typical series of passive transfer experiments is shown in Table II. The skin-sensitizing serum used was that of subject Gr. When appropriate dilutions of this serum were placed intradermally in a Schick-positive recipient and the latter was challenged with toxoid (Procedure I), it was found that an amount as small as 0.01 unit of antitoxin caused a consistent increase in the intensity of reaction upon comparison with unprepared sites receiving toxoid. When mixtures consisting of constant antitoxin (0.05 units) and varying toxoid (0.005 to 0.5 Lf) were injected at unprepared sites (Procedure II), the immediate reactions were stronger in the presence of larger amounts of toxoid (0.05 to 0.5 Lf). Subsequent challenge of each site 48 hours later with toxoid caused reactions only at sites containing residual antitoxin (*i.e.*, mixtures which had less than equivalent amounts of toxoid). This indicated that neutralization of skin-sensitizing antitoxin by toxoid had taken place and that 1 Lf of toxoid was equivalent to about 1 unit of antitoxin. Similar results obtained with serum Hu have been described in an earlier publication (2). A total of six of the eight sera in the present series, in-

cluding those of Gr and Hu, reacted similarly upon passive transfer when tested by the methods of dilution and neutralization by toxoid. Two sera (Fr and Wi) on passive transfer gave results which were not typical of those shown by the other six sera and indicated that the titer obtained by tests in human skin did not parallel the degree of neutralization as shown by the rabbit skin test. Both sera were less potent than Gr in their abilities to sensitize human skin by the

TABLE II

Passive Transfer Tests in Human Skin Using Serum Gr Containing Skin-Sensitizing Antitoxin (Procedures I and II)

Dilution technique			Neutralization technique		
Sensitizing dose (units of antitoxin in 0.1 cc.)	Challenge toxoid at 48 hrs. (Lf in 0.02 cc.)	Immediate skin reaction (15–30 min.)	Lf toxoid mixed with 0.1 unit antitoxin	Immediate skin reaction (15–30 min.)	Reaction to challenge toxoid (0.1 Lf) at 48 hrs.
1.0	0.1	++++	1.0	+++	±
0.3	0.1	++++	0.3	+++	±
0.1	0.1	+++	0.1	++±	+
0.03	0.1	++	0.03	+±	+++±
0.01	0.1	+±	0.01	+	++++
Control		±			

TABLE III

Intracutaneous Neutralization of Schick Toxin by Serum Gr Containing Skin-Sensitizing Antitoxin (Procedure III)

Amount of antitoxin in skin (units in 0.1 cc.)	Amount of Schick toxin at 4 days (Lf − μg. N in 0.1 cc.)	Immediate wheal reaction (15 min. after intracutaneous toxin)	Delayed reaction to toxin at 96 hrs.	Neutralization of toxin by antitoxin
0.04	0.0004 − 0.0002	+	0	Complete
0.004	0.0004 − 0.0002	±	0	Complete
0.0004	0.0004 − 0.0002	0	±	Partial
Control	0.0004 − 0.0002	0	+	

dilution technique; the degree of neutralization by toxoid observed in other instances did not occur when these sera were similarly tested (Table IV).

Neutralization of Schick Toxin by Skin-Sensitizing Antitoxin.—(Procedure III) Appropriate dilutions of antitoxin were given intradermally to Schick-positive recipients and challenged with Schick toxin at from 2 to 7 days as described under Methods. Immediate readings of wheal reactions were made 15 minutes after injection of toxin, and delayed toxic reactions were measured at 96 hours. Since the amount of toxin used (0.02 MLD or 0.0004 Lf) was considerably less than the amount of toxoid (0.1 Lf) employed in other passive

transfer tests, immediate reactivity of skin-sensitizing antitoxin using this level of antigen was less marked.

The abilities of serum Fr and serum Gr to neutralize Schick toxin were tested in two experiments (Tables III and V). In each instance, three sites were prepared with amounts of antitoxin ranging from 0.0004 to 0.04 unit. and were challenged with toxin 4 days thereafter. Sites injected with Fr showed less immediate reactivity to toxin than those receiving Gr. A similar difference in

TABLE IV

Passive Transfer Tests in Human Skin Using Serum Fr Containing Skin-Sensitizing Antitoxin (Procedures I and II)

Dilution technique			Neutralization technique		
Sensitizing dose (units of antitoxin in 0.1 cc.)	Challenge toxoid at 48 hrs. (Lf in 0.02 cc.)	Immediate skin reaction (15–30 min.)	Lf toxin mixed with 0.1 unit antitoxin	Immediate skin reaction (15–30 min.)	Reaction to challenge toxoid (0.1 Lf) at 48 hrs.
1.0	0.1	+++	1.0	++±	0
0.3	0.1	+++	0.3	++	±
0.1	0.1	++	0.1	+	±
0.03	0.1	+	0.03	+	±
0.01	0.1	0	0.01	+	+
Control		±			

TABLE V

Intracutaneous Neutralization of Schick Toxin by Serum Fr Containing Skin-Sensitizing Antitoxin (Procedure III)

Amount of antitoxin in skin (units in 0.1 cc.)	Amount of Schick toxin at 4 days (Lf – μg. N in 0.1 cc.)	Immediate wheal reaction (15 min. after intracutaneous toxin)	Delayed reaction to toxin at 96 hrs.	Neutralization of toxin by antitoxin
0.04	0.0004 – 0.0002	0	0	Complete
0.004	0.0004 – 0.0002	0	±	Partial
0.0004	0.0004 – 0.0002	0	+	No
Control	0.0004 – 0.0002	0	+	

titer of these two sera had been shown when tested by the ordinary method of passive transfer (see Tables II and IV). Comparison of the delayed reactions to toxin indicated that 0.0004 unit of serum Gr when injected intradermally neutralized 0.1 cc. of toxin only to a slight extent. Similar tests of skin-sensitizing serums Hu and Au showed that 0.0004 unit. neutralized 0.1 cc. of Schick toxin almost completely. Sera from Fr (Table V) and Wi diluted to contain 0.0004 unit. failed to neutralize Schick toxin. Only a slight neutralization of toxin was given by 0.004 unit of serum Fr, while complete protection by Fr or Wi required 0.04 unit. The results using sera Fr and Wi were comparable

with those obtained by other methods of passive transfer and they provided further evidence that the antitoxin titer demonstrated by these methods did not parallel the titer as demonstrated by rabbit skin test.

DISCUSSION

Vaughan and Kabat (9) have studied the immunological and biological properties of rabbit antibodies formed against recrystallized ovalbumin and showed that the immediate skin reaction in passively sensitized human subjects was caused by antibodies to trace substances carried over from egg white rather than to ovalbumin itself. On the basis of their findings with rabbit antibodies produced against egg albumin, these authors suggested that the immediate reactions elicited by purified diphtheria toxoid in human subjects hyperimmunized with purified toxoid might be due to "reagins" whose specificity was directed toward impurities remaining in the toxoid itself. However, the original investigations of skin-sensitizing antitoxin carried out by Kuhns and Pappenheimer (2, 10) suggested that skin-sensitizing antitoxin reacted specifically with toxin or toxoid itself and not with some impurity. The following observations gave support to this opinion:-

1. Passive transfer studies showed that the amount of toxoid needed to neutralize skin-sensitizing antitoxin was equivalent to the antitoxin titer as obtained by the rabbit skin test.

2. Skin-sensitizing antitoxin from one subject (Hu) caused skin to react to injections of crude diphtheria proteins (P-proteins), although fifty times as much anti–P-protein as antitoxin was needed to cause sensitization. P-proteins were prepared the same way as toxin except that 1 mg. of iron per liter was added to the medium and presumably the mixture contained all of the nontoxic substances that might have contaminated the purified toxin. Electrophoretic diagrams of both materials showed a difference only in the toxin component.

3. It was possible to co-precipitate skin-sensitizing antitoxin by adding it to appropriate amounts of toxin and precipitating antitoxin. Tests carried out on the supernatant obtained from these precipitated mixtures revealed no demonstrable skin-sensitizing antitoxin and negligible amounts of toxin. Passive transfer experiments using this material showed that it possessed no immediate wheal and erythema activity.

Additional experimental support for the specificity of this variety of antitoxin was suggested by its physical behavior. When sera containing skin-sensitizing antitoxin were separated by electrophoresis, the antitoxin migrated as a γ_1-globulin. Eluates of γ_1-globulin containing skin-sensitizing antitoxin when tested for wheal activity by the method of passive transfer were found to possess the same activity as equivalent amounts of sensitizing antitoxin contained in whole serum (11). Precipitating antitoxin separated from appropriate

sera by electrophoretic analysis was found to migrate as a γ_2-globulin. When both antitoxins were present together in sera, it was possible using this method to separate them without loss of the properties by which each could be characterized when existing singly in whole serum.

The present observations provide further evidence for the specificity of reaction between skin-sensitizing antitoxin and purified diphtheria toxoid. In addition, the experiments demonstrated that antitoxin from individuals sensitive to toxin remained in the skin of the recipient Schick-positive subjects for many days following passive transfer and that it retained an undiminished capacity to neutralize the toxic effects of Schick test reagent.

The ability to remain at intradermal sites is a distinctive property of skin-sensitizing antibody, and in this respect it is readily distinguished from "blocking" antibodies which merely inhibit wheal reactions (12–14). In view of the fact that a form of "blocking" non-precipitating antitoxin exists which does not sensitize skin or remain at skin sites, it is important to consider the implications of these differences when confronted with atypical sera such as Fr and Wi. Sera containing both varieties of non-precipitating antitoxin may show a lack of parallelism between titers as obtained by rabbit skin tests and upon passive transfer tests in human skin. This is because the latter techniques, in contrast to the rabbit test which is carried out using toxin-antitoxin mixtures, depend upon the ability of antibody to remain in human skin until subsequent antigen challenge. Therefore sera which contain sensitizing and non-sensitizing antitoxins will demonstrate a titer upon rabbit skin test which represents the sum of both antitoxins, but only sensitizing antitoxin will be demonstrated when titered by the method of passive transfer. In view of the discrepancies observed upon testing sera Fr and Wi, it is possible that they contained a mixture of non-precipitating antitoxins, one of them skin-sensitizing antitoxin, and the other antitoxin devoid of both the wheal and erythema property and of the ability to remain at intradermal sites.

The use of a toxic antigen in skin tests is both simpler and more exact than a test based on measurement of a wheal reaction after injection of reagin and antigen. The fact that skin-sensitizing antitoxin remains fixed at intradermal sites makes it possible to devise a specific test for skin-sensitizing antitoxin, based on the neutralization of subsequently injected toxin. It has been found that the neutralizing power of a given quantity of intradermal antitoxin remained relatively constant regardless of recipient (providing he was Schick-positive), and that the end points were easily read since they were given by delayed reactions which change only slowly in size, rather than by evanescent wheals. In practice the different tests provide information based upon the known biological properties of skin-sensitizing antitoxin which would not be available using a single test. Thus, Procedures I and II quantitate wheal and erythema activity, whereas Procedure III is based upon the ability of this variety of antibody to remain in the skin. The results of these tests are in turn directly re-

lated to the titer obtained by rabbit skin test in the presence of only skin-sensitizing antitoxin.

Because it is possible to utilize Schick toxin intradermally in conjunction with skin-sensitizing antitoxin as outlined above, the determination of survival time of intradermal reagin should now be possible. This technique may also find application in comparative studies of intradermal survival time of the different varieties of antitoxin.

SUMMARY

Studies were carried out on sera from eight subjects hyperimmunized with toxoid who developed marked immediate skin reactivity to toxoid associated with circulating non-precipitating antitoxin. With the use of the rabbit skin test, the agar diffusion technique, and three different methods for passive transfer of skin sensitivity, it was possible to obtain detailed qualitative and quantitative data relating to the antitoxin in these sera. It was found that specimens from six individuals contained only skin-sensitizing antitoxin. Two sera showed a lack of parallelism between antitoxin titers as obtained by rabbit skin test and titers as demonstrated by tests in human skin. It was presumed that these sera contained two different varieties of non-precipitating antitoxins, and that only one of them was skin-sensitizing antitoxin.

A new technique is described for measurement of skin-sensitizing antitoxin. Its specificity is based upon the ability of this antitoxin to remain at skin sites and later neutralize the delayed specific toxic effects of intradermal Schick test reagent.

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