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

II. A Comparison between the Immunological Properties of Precipitating and Non-Precipitating (Skin-Sensitizing) Antitoxins*

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In the preceding paper (1) immediate skin reactions of the wheal and erythema type to purified diphtheria toxin and toxoid employed in the Schick test were described. The strongest reactions were observed in individuals who were Schick-negative and who had either a personal or familial history of allergy or both. Hyperimmunization of selected subjects with purified diphtheria toxoid in a number of instances yielded antisera which contained relatively high titers of *non-precipitating* antitoxin. Such antitoxic sera exhibited marked discrepancies between *in vivo* antitoxin titer as determined by the intracutaneous neutralization test in rabbits, and *in vitro* titer as estimated by quantitative precipitation. The development of skin sensitivity to toxin or toxoid was correlated with the presence of non-precipitating antitoxin.

The present paper describes in some detail, the contrasting immunological properties of precipitating and non-precipitating human diphtheria antitoxin, both *in vivo* and *in vitro*.

Materials and Methods

Preparation and properties of the purified diphtheria toxin and toxoid used and the technics employed in carrying out the intracutaneous neutralization test in rabbits and the quantitative precipitin reaction are described in the preceding paper (1).

Antitoxic Sera.—

Subject 4, Hu.—The preimmunization antitoxin titer as determined by rabbit skin test was between 0.1 and 1 unit/cc. No immediate reaction to the Schick control toxoid was elicited at this time. The subject was then given a single subcutaneous injection of 200 Lf of alum precipitated, purified diphtheria toxoid. There was considerable local reaction (itching, red-

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ness, swelling) at the injection site for the 1st and 2nd days. 7 days after immunization, redness and swelling were again observed at the injection site. Serum collected 10 days after immunization contained 80 units antitoxin per cc. by rabbit skin test. Mixtures of diluted (1:3 in borate buffer, pH 7.6) decomplemented serum with equal volumes of toxin solutions containing 5 to 35 Lf per cc. failed to yield any precipitation whatever, even after remaining in the icebox for 3 months. The same result was obtained with undiluted serum. Additional blood samples were collected subsequently. Following the rise in antitoxin level, the subject was again tested with Schick control toxoid and this time exhibited a severe immediate wheal and erythema reaction with pronounced pseudopodia.

Subject 9, Fo.—The preimmunization titer of serum Fo lay between 0.01 and 0.1 units per cc. At this time there was no immediate reaction to the Schick control toxoid. Following subcutaneous injection of 37.5 Lf fluid toxoid only a mild transient local reaction was observed 24 hours later. Serum collected on the 10th day contained 200 units antitoxin per cc. by rabbit skin test and 465 μg. specifically precipitable antitoxin nitrogen. A sample obtained at 20 days containing 140 units/cc. by rabbit skin test and 249 μg. specifically precipitable nitrogen was used for many of the passive transfer experiments to be described. Following immunization, slight skin sensitivity to toxoid developed.

Subject 21, La.—The serum contained 0.06 units antitoxin per cc. prior to immunization. At this time the immediate wheal and erythema reaction to the Schick control toxoid was strongly positive. La gave a personal history of sensitivity to a variety of grasses, pollens, etc., with hay-fever of 21 years' duration and frequent asthmatic attacks. Following subcutaneous injection of 60 Lf alum precipitated purified toxoid, a mild, local, erythematous reaction was evident at the injection site at 1 and 2 days. Serum collected 10 days after injection contained 60 units antitoxin per cc. by rabbit skin test but only 50 μg. specifically precipitable antitoxin nitrogen per cc. instead of the expected 150 μg./cc. The immediate skin reaction to toxoid remained strongly positive following immunization.

Subject X, OD.—This patient, previously studied by Balch (2), was hospitalized because of chronic alcoholism and frostbite. The serum contained 0.5 units antitoxin per cc. before immunization. The highest antitoxin level reached following subcutaneous injection of 26 Lf fluid toxoid was 250 units per cc. Additional bleedings were taken subsequently, and the serum from one large bleeding which contained 140 units/cc. and 263 μg. specifically precipitable nitrogen was lyophilized. A portion of this serum was reconstituted for use in some of the present experiments. A second portion reconstituted later contained 110 units/cc. and 240 μg. specifically precipitable nitrogen. This serum was used for the studies on passive anaphylaxis.

Passive Transfer (P-K) Experiments in Human Skin.—

Both the serum dilution method and neutralization technic were used. Although it is possible to sensitize skin sites in non-sensitive, Schick-negative individuals to diphtheria toxoid (3), care was taken to use only Schick-positive recipients in the present study. Titrations were usually performed in duplicate, *i.e.*, using 2 recipients. In most instances, antitoxin titrations were performed on the recipient's serum drawn at the time the passive transfer was carried out in order to confirm the *absence* of more than 0.01 unit per cc.

In the serum dilution method, 0.1 cc. of dilutions of the donor's serum (in borate buffer, pH 7.6) was injected into a series of skin sites. The smallest quantity of antitoxic serum required to sensitize a local skin site in the Schick-positive recipient was determined by challenging the sites 48 hours later with 0.02 cc. purified toxoid containing 0.1 Lf which is equivalent to 0.05 μg. toxoid nitrogen.

In the neutralization method, chosen dilutions of sensitizing serum were mixed with equal volumes of progressive dilutions of toxoid. 0.1 cc. of mixture was injected into each skin site.

Immediate wheal and erythema reactions were measured after intervals of 15 to 45 minutes. 2 days later the same sites were injected with the standard dose of toxoid (*i.e.*, 0.1 Lf in 0.02 cc.). Reactions appeared at those sites prepared with mixtures containing insufficient toxoid to neutralize the available antitoxin. In all experiments, a control site (unprepared) was injected with the challenge dose of toxoid.

Complement Fixation.—

Amboceptor and guinea pig complement were standardized so that 0.1 cc. of each contained 2 units. Unit complement or amboceptor was taken as the smallest amount of each necessary for complete hemolysis of 0.1 cc. of a 10 per cent suspension of sheep red blood cells. To 0.1 cc. of the appropriate serum dilutions were added equal volumes of toxoid solution (1 Lf per 0.1 cc.) and of complement. The mixtures were briefly agitated and placed in the water bath for 1 hour at 37°C. To each tube was then added 0.1 cc. each of amboceptor and a 10 per cent suspension of sheep red blood cells. After incubation for an additional hour at 37°C. the tubes were observed for presence or absence of hemolysis.

Passive Anaphylaxis Experiments in the Guinea Pig Using Precipitating and Non-Precipitating Antibody.—

All guinea pigs used in these experiments weighed between 200 and 300 gm. Sensitization was carried out with sera O'D (precipitating) and Hu (non-precipitating). The quantity of precipitating antitoxin required to sensitize animals to fatal anaphylaxis was determined by the intravenous (femoral vein) injection of three series of guinea pigs with 10, 20, and 40 units respectively. (The corresponding antibody N values for O'D and Hu sera are indicated in Tables XII, XIII, and XIV.) The comparative effectiveness of sera O'D and Hu in passively sensitizing guinea pigs was assayed by injecting the desired quantity of antitoxin into a femoral vein. 40 to 50 units was generally used. 2 days later either 50 Lf or 100 Lf (23 or 46 μg. N) of diphtheria toxin or toxoid was injected as the shocking dose into the other femoral vein. Comparative experiments showed that toxin of 85 per cent purity and toxoid of 70 per cent purity were equally effective in provoking shock in sensitized guinea pigs. Immediately following the shocking dose of antigen, animals were observed for symptoms of anaphylaxis which were graded as negative, mild, moderate, severe, or fatal depending on the presence and severity of air hunger, sneezing, convulsions, etc. All animals that died following injection of antigen showed typical postmortem findings of anaphylaxis.

Passive anaphylaxis experiments were also carried out using non-precipitating serum Hu which had been heated at 56°C. for 5 hours.

RESULTS

Experiments in Human Skin

Passive Transfer of Skin Sensitivity to Purified Toxoid.—Only sera containing appreciable amounts of *non-precipitating* antitoxin possessed the property of conferring local skin sensitivity to toxoid in Schick-positive recipients. Table I shows titrations of skin-sensitizing antitoxin using sera Hu and La. When challenged at 48 hours with 0.1 Lf toxoid, sensitivity could be demonstrated in sites prepared with 0.0001 cc. serum (0.006 to 0.016 units/antitoxin) or less. The serum of La was tested before and after injection of the "booster" dose of toxoid. It will be noted that an approximate 1000-fold increase in skin-sensitizing titer occurred which paralleled the antitoxin rise from 0.06 to 60 units per cc.

The sera of other individuals tested behaved similarly. In all cases, the titer of skin-sensitizing antitoxin was low prior to immunization and in no case could sensitization be demonstrated using serum from subjects who were not themselves sensitive to toxoid.

Persistence of Passively Transferred Precipitating and Non-Precipitating Antitoxin in Human Skin.—Both types of antitoxin formed complexes when mixed

TABLE I

Passive Transfer (P-K Reaction) of Sensitivity to Diphtheria Toxoid: Pre- and Postimmunization Reactions

Serum Hu		Serum La		
Sensitizing dose (units antitoxin)	Postimmunization	Sensitizing dose (units antitoxin)	Preimmunization	Postimmunization
8	++++	6	Not done	++++
0.8	+++±	0.6	Not done	+++
0.08	+	0.06		++
0.016	±	0.006	+	+
0.008	0	0.0006	±	Not done
Control	0	Control	0	0

* The sensitizing dose was injected into skin sites in Schick-positive individuals. 48 hours later each site was challenged with 0.1 Lf purified toxoid. Reactions were read approximately 15 minutes later and noted as above. The recipients used for serums Hu and La were different.

TABLE II

Disappearance of Precipitating Antitoxin (Fo) from Injected Skin Sites

Units antitoxin injected into skin	Intensity of wheal and erythema reaction following challenge with 0.1 Lf toxoid		
	40 min.	100 min.	48 hrs.
14	++++	±	0
1.4	+++	0	0
0.14	++	0	0
0.014	++	0	0
Control	0	0	0

with purified toxoid in the test tube which were irritating and caused immediate wheal and erythema reactions when injected intradermally. Precipitating antitoxin, however, disappeared rapidly from skin, and the wheal and erythema reaction could only be evoked by toxoid in prepared sites if challenge was carried out within 100 minutes as shown in Table II. In contrast, non-precipitating antitoxin remained at the local skin site for many weeks (Table III).

Specificity of Skin-Sensitizing Factor.—Three series of cutaneous sites were prepared, each with 0.1 cc. of comparable dilutions of serum Hu. 48 hours later

one series was challenged with 0.05 μg. (0.1 Lf in 0.02 cc.) purified diphtheria toxoid nitrogen. The second series of sites received 0.02 cc. of a solution estimated to contain 0.05 μg. formalinized P-protein (4) nitrogen and 0.0005 μg. toxoid nitrogen. The third series was challenged with 0.0005 μg. toxoid nitrogen. Column 3 of Table IV shows that serum Hu contained some skin-sensitizing anti-P protein, the reactivity of which could not be compared with that of the skin-sensitizing antitoxin present (column 2, Table IV).

TABLE III

Persistence of Non-Precipitating Antitoxin in the Skin

No. of days after sensitization of skin site*	Intensity of immediate skin reaction following challenge of 0.1 Lf toxoid	Reaction at unprepared sites
1	++++	0
4	+++	0
6	+++	0
45	++	0

* Skin sites sensitized with 0.1 cc antitoxin Hu (8 units).

TABLE IV

Specificity of Skin-Sensitizing Antitoxin

Sensitizing dose (units antitoxin injected into skin)	Intensity of wheal and erythema following challenge at 48 hrs. with		
	0.1 Lf toxoid (0.05μg. N)	P-protein* (0.05μg. N)	0.001 Lf toxoid (0.0005μg. N)
(1)	(2)	(3)	(4)
8	++++	++	±
0.8	+++	+	0
0.08	+	0	0
0.16	±	0	0
0.008	0	0	0
Control	0	0	0

* Estimated toxoid content was 0.001 Lf (0.005μg. N).

Column 4 of this table shows that no significant erythema was evoked in sites prepared with serum Hu when challenged with traces of toxoid comparable to the amount estimated to be present in the P-protein solution.

Specific Neutralization of Skin-Sensitizing Antitoxin by Toxoid.—Sites were prepared in the skin of Schick-positive recipients using mixtures of non-precipitating antitoxin and toxoid. In one series, each 0.1 cc. of mixture injected intradermally contained 0.4 units of antitoxin (Hu) and increasing amounts of toxoid between 0.005 and 0.5 Lf. Reactions read 15 to 30 minutes later were maximal with mixtures containing equivalent amounts of toxoid; *i.e.*, 0.4 and

0.5 Lf. Table V shows that when challenged at 48 hours with the standard dose of toxoid, little or no sensitization had occurred at those sites which had been prepared with mixtures containing equivalent amounts of toxoid and antitoxin. These findings provide additional evidence that the reaction in the skin

TABLE V

*Neutralization of Skin-Sensitizing Antitoxin by Toxoid**

Lf toxoid mixed with 0.4 units antitoxin	Immediate skin reaction	Reaction to challenge dose (0.1 Lf toxoid)
0.005	0	++++
0.05	+±	+++
0.1	++	+++
0.2	+++	+±
0.3	+++±	+
0.4	++++	+
0.5	++++	0

* Skin sites were sensitized with mixtures of antitoxin Hu and toxoid as indicated. Skin reactions were recorded 15 to 30 minutes later. The same sites were then challenged at 48 hours with 0.1 Lf toxoid.

TABLE VI

*Immediate Reactions to Fresh and Heated Precipitating and Skin-Sensitizing Antitoxin Mixed with Varying Amounts of Diphtheria Toxoid**

Lf units toxoid mixed with 0.35 units antitoxin	Serum O'D precipitating		Serum Hu skin-sensitizing	
	Fresh	Heated	Fresh	Heated
0.0005	+	0	+	+
0.005	+±	+±	+	+±
0.025	+++	++++	+±	+
0.05	+++	++++	+±	+
0.25	+++	++++	++±	+
0.50	++	++++	+++±	+
1.10	+±	+++±	+++±	+
2.25	+±	+++±	+++	+

* Heated sera were treated at 56°C. for 4 hours. All mixtures were injected into unprepared skin sites and the reactions read at 15 to 30 minutes.

which results in wheal and erythema, is a specific one between toxoid and antitoxin.

Effects of Heating at 56°C.—When non-precipitating antitoxin was heated at 56°C. for 4 hours, its capacity to sensitize local skin sites was entirely lost and no reaction occurred when toxoid was introduced at the sites 48 hours later. Moreover, as shown in Table VI, injection of mixtures of heated Hu serum and

toxoid, caused only slight skin reactions. Heating resulted in insignificant loss of antitoxic potency as determined by skin titration in the rabbit.

The behavior of precipitating antitoxin was altogether different. It can be seen from Table VI that immediate skin reactions with unheated O'D serum and toxoid were produced with mixtures containing antitoxin in considerable excess. Heating serum O'D for 4 hours at 56°C. appeared to *increase* the intensity of the wheal and erythema produced by test tube mixtures, particularly in the region of toxoid excess. Both fresh and heated O'D and Fo antitoxin disappear rapidly from skin sites. It is of interest to recall at this point that human antitoxic sera lose their capacity to specifically precipitate toxin or toxoid following treatment at 56°C. without undergoing appreciable loss in antitoxic potency (5).

TABLE VII

*Inhibition (Blocking) Titer of Heated Non-Precipitating Antitoxin**

Lf units toxoid mixed with 0.4 unit heated antitoxin	Reaction to toxoid-heated antitoxin mixture	Reaction to toxoid-borate buffer control mixture
0.005	0	++±
0.05	±	++++
0.1	±	++++
0.2	++	++++
0.3	++±	++++
0.4	++++	++++
0.5	++++	++++

* Serum Hu heated to 56°C. for 4 hours and mixed with toxoid (as above) was injected into skin sites uniformly sensitized 48 hours previously with 8 units of fresh antitoxin Hu.

It has been demonstrated that exposure of *non-precipitating* antitoxin Hu to 56°C. for 4 hours resulted in a loss of its capacity to sensitize the skin of recipients to subsequent challenge doses of toxoid and that mixtures of heated Hu serum and toxoid no longer caused appreciable wheal and erythema when injected into the skin. The following experiments demonstrate that heated non-precipitating antitoxin remains capable of reacting with approximately the same amounts of toxoid as unheated antitoxin. In the experiment illustrated in Table VII, mixtures containing 0.4 units of heated Hu antitoxin and increasing amounts of toxoid were injected in a volume of 0.1 cc. into skin sites uniformly prepared 48 hours previously with 0.1 cc. (8 units) of unheated Hu antitoxin. At the same time, similarly prepared skin sites were injected with comparable amounts of toxoid in borate buffer, pH 7.6. Table VII shows clearly that heated Hu serum is capable of exerting a *blocking* effect. Only when mixtures containing amounts of toxoid equivalent to or in excess of the heated antitoxin present were injected into sensitized sites, were the wheal and erythema reactions produced comparable in intensity to those observed at the control sites.

Table VIII shows that heated non-precipitating antitoxin retains its capacity to inhibit the reaction caused by fresh toxoid-antitoxin mixtures for at least 48 hours after injection into the skin. In this experiment, mixtures of heated Hu

TABLE VIII

*Persistence of Heated Non-Precipitating Antitoxin in Injected Skin Sites**

Lf units toxoid mixed with 0.4 units antitoxin	Reaction to fresh serum-toxoid mixture	Reaction to heated serum-toxoid mixture	Reaction at sites prepared with heated serum-toxoid mixtures to challenge mixture (48 hrs.)‡
(1)	(2)	(3)	(4)
0.0005	+	+	±
0.005	+	+±	±
0.025	+	+	+
0.05	+±	+	±
0.25	++	+	+
0.50	+++	+	+±
1.10	+++	+	+±
2.25	+++	+	++±

* Sites listed under columns (2) and (3) were prepared with fresh and heated Hu antitoxin-toxoid mixtures. Reactions were read 15 to 30 minutes later. Sites prepared with heated serum-toxoid mixtures as listed under column (3) were challenged at 48 hours by injection of a mixture containing 0.4 unit fresh Hu antitoxin and 0.4 Lf toxoid. Reactions were read 15 to 30 minutes later. These are tabulated in column (4).

‡ The reaction of unprepared sites (column 2) to the challenge mixture was observed at this time.

TABLE IX

*Inhibition Titer of Precipitating Antitoxin**

Lf units toxoid mixed with 0.35 units precipitating antitoxin (O'D)	Reaction to fresh antitoxin-toxoid mixture	Reaction to heated antitoxin-toxoid mixture	Reaction to borate buffer-toxoid control mixture
0.0025	+	++	+++±
0.025	+	+++	++++
0.05	+++	++++	++++
0.10	++++	++++	++++
0.15	++++	++++	++++
0.20	++++	++++	++++

* Skin sites were uniformly sensitized with 0.1 cc. (8 antitoxic units) Hu serum. 48 hours later the sites were challenged as indicated above with mixtures of precipitating antitoxin, fresh and heated, and toxoid.

serum containing 0.4 unit of antitoxin and increasing amounts of toxoid were injected in 0.1 cc. volume into a series of skin sites. 48 hours later, the same sites were challenged with a mixture containing 0.4 unit fresh Hu antitoxin and 0.4 Lf toxoid. Some blocking of the wheal and erythema reaction occurred even at those sites prepared with heated antitoxin containing toxoid in excess.

Inhibiting Effect of Precipitating Antitoxin.—In the experiment illustrated in Table IX, three series of skin sites in Schick-positive recipients were sensitized uniformly with 0.8 unit of skin-sensitizing, non-precipitating antitoxin Hu. 48 hours later one series of skin sites was challenged with 0.35 unit of *precipitating* antitoxin O'D mixed with increasing amounts of toxoid in a total volume of 0.1 cc. Column 2 of Table IX shows that an inhibiting or blocking effect was demonstrable with mixtures containing less than 0.05 Lf toxoid and the reactions observed were considerably less intense than those evoked by the corresponding amounts of toxoid alone (column 4). The blocking effect was considerably diminished following treatment of the precipitating antitoxin at 56°C. for 4 hours (column 3).

Immunochemical Properties of Precipitating and of Non-Precipitating Antitoxin

Specific Coprecipitation of Non-Precipitating Antitoxin.—Although skin-sensitizing antitoxin Hu failed to show any sign of precipitation even after remaining several months in the cold with varying amounts of toxin, it could be coprecipitated in the presence of toxin and precipitating antitoxin as illustrated by the following experiment:

1.0 cc. diluted decomplemented serum Hu containing 14 units antitoxin, was mixed with 1.0 cc. of decomplemented Fo serum diluted to contain *ca.* 100 μg. specifically precipitable antitoxin nitrogen. To the mixture, 45 Lf or 20.7 μg. toxin was added (tube 1). In a control tube (tube 2), 35 Lf toxin (16.1 μg. toxin nitrogen) was added to 1.0 cc. diluted Fo serum containing 100 μg. specifically precipitable nitrogen. The tubes were incubated for 2 hours at 37°C. and then placed in the icebox for 10 days. The precipitates were collected, washed, and analyzed.

The specific precipitate in tube 1 contained 142.5 μg. total specifically precipitable nitrogen or 121.8 μg. specifically precipitable antitoxin nitrogen. The precipitate in tube 2 contained 116.0 μg. total specifically precipitable nitrogen or 99.9 μg. specifically precipitable antitoxin nitrogen. It can be seen, therefore, that 121.8 − 99.9 or 21.9 μg. nitrogen was specifically coprecipitated from antitoxin Hu, equivalent to 1.6 μg. per unit.

The results from several similar experiments are in agreement and indicate that 1 unit of human non-precipitating antitoxin is equivalent to about 1.6 μg. antibody nitrogen.

Complement Fixation Reactions.—Complement fixation reactions were carried out with several sera including (*a*) two sera (Fo and OD) containing precipitating but little or no skin-sensitizing antitoxin, (*b*) two sera (Hu and Mu) containing skin-sensitizing but virtually no precipitating antitoxin, and (*c*) serum La containing a mixture of both types of antitoxin.

Table X shows that in the presence of an appropriate amount of toxoid only 0.2 to 0.3 unit of precipitating antitoxin Fo was required to fix 2 units of guinea pig complement. Heating at 56°C. for 4 hours diminished this titer but slightly despite the fact that the heated antitoxin no longer gave a specific precipitate

with toxin. On the other hand, the non-precipitating, skin-sensitizing antisera showed little or no complement fixation. 8 units of antitoxin Hu (undiluted serum) was required to fix 2 units of guinea pig complement. After heating to 56°C. undiluted serum Hu failed entirely to fix complement. The behavior of serum La was intermediate. It will be recalled that serum La possessed a high titer of skin-sensitizing antitoxin but at the same time showed some precipitating antitoxin. The effect of heating in this instance was especially marked and 3 times as much heated antitoxin was required to fix 2 units of complement as compared with the unheated serum.

The Danysz Effect.—Danysz (6) showed many years ago that far less toxin is neutralized by a given amount of antitoxin if the total amount of toxin is

TABLE X

Complement-Fixing Titers of Certain Human Antitoxic Sera

Serum	Type	Units antitoxin required to fix 2 units of guinea pig complement
Fo	Precipitating	0.2
Fo (heated)	Non-precipitating	0.3
O'D	Precipitating	0.35
O'D (heated)	Non-precipitating	0.47
Hu	Non-precipitating, skin-sensitizing	8
Hu (heated)	Non-precipitating	>8
Mu	Non-precipitating, skin-sensitizing	2
La	Both precipitating and non-precipitating, skin-sensitizing	0.5
La (heated)	Non-precipitating	1.5

added in successive portions than if it is added all at once. It was of interest to find out whether precipitating and non-precipitating human antitoxins differ from one another in this respect. Three specimens of serum were examined: (*a*) precipitating antitoxin Fo, (*b*) antitoxin Fo after heating at 56°C. for 4 hours, and (*c*) unheated, non-precipitating antitoxin Hu.

The experiments summarized in Table XI were carried out in the following manner:

Serum was diluted so as to contain 2.5 units of antitoxin in the case of Fo, and 5.0 units in the case of serum Hu. To aliquots of antitoxin solution were added equal volumes of toxin containing amounts which increased by increments of 0.2 Lf. After standing at 25°C. for 1 hour, 2 cc. of the mixtures were injected subcutaneously into 250 gm. guinea pigs (series A). As seen from Table XI sharp end-points were obtained. With precipitating serum Fo 2.5 units of antitoxin mixed with 3.4 Lf toxin caused only a slight local reaction in the guinea pig while the same amount of antitoxin mixed with 3.6 Lf caused death within 4 days. The end-point was taken as that dose of toxin which when mixed with the fixed amount of antitoxin was sufficient to cause a visible local reaction with or without death of the animal. In series B,

TABLE XI

Danysz Effect

Precipitating antitoxin (Fo)		Precipitating antitoxin heated at 56°C.—4 hrs.		Non-precipitating antitoxin (Hu)	
A. Toxin + antitoxin mixture 1 hr. at 25°C.		A. Toxin + antitoxin mixture 1 hr. at 25°C.		A. Toxin + antitoxin mixture 1 hr. at 25°C.	
Injection mixture	Reaction in guinea pig	Injection mixture	Reaction in guinea pig	Injection mixture	Reaction in guinea pig
2.5 units AT + 3.2 Lf toxin	No reaction	2.5 units AT + 3.0 Lf toxin	No reaction	5.0 units AT + 3.0 Lf toxin	No reaction
2.5 units AT + 3.4 Lf toxin	Slight local reaction	2.5 units AT + 3.2 Lf toxin	Slight local reaction	5.0 units AT + 3.2 Lf toxin	Dead 4 days
2.5 units AT + 3.6 Lf toxin	Dead 4 days	2.5 units AT + 3.4 Lf toxin	Dead 3 days	5.0 units AT + 3.4 Lf toxin	Dead 3 days
Therefore 2.5 units AT $\backsimeq$ 3.4 Lf toxin		Therefore 2.5 units AT $\backsimeq$ 3.2 Lf toxin		Therefore 5.0 units AT $\backsimeq$ 3.2 Lf toxin	
B. 2.5 units antitoxin + 1.1 Lf toxin overnight 0°C. followed by further addition of toxin 1 hr. at 25°C.		B. 2.5 units antitoxin + 1.0 Lf toxin overnight 0°C. followed by further addition of toxin 1 hr. at 25°C.		B. 5.0 units antitoxin + 1.0 Lf toxin overnight 0°C. followed by further addition of toxin 1 hr. at 25°C.	
Injection mixture	Reaction in guinea pig	Injection mixture	Reaction in guinea pig	Injection mixture	Reaction in guinea pig
Mixture + 1.2 Lf toxin	Dead 5 days	Mixture + 1.6 Lf toxin	Local reaction 5 days	Mixture + 1.8 Lf toxin	No reaction
				Mixture + 2.0 Lf toxin	Dead 4 days
				Mixture + 2.2 Lf toxin	Dead 3 days
Therefore 2.5 units AT $\backsimeq$ 2.3 Lf toxin Difference (A–B) 1.1 Lf = *ca.* 60 M.L.D.		Therefore 2.5 units AT $\backsimeq$ 2.6 Lf toxin Difference (A–B) 0.6 Lf = *ca.* 30 M.L.D.		Therefore 5.0 units AT $\backsimeq$ 3.0 Lf toxin Difference (A–B) 0.2 Lf = 10 M.L.D.	

toxin was added in two successive portions to 2.5 units of antitoxin (5.0 units in the case of serum Hu). With unheated serum Fo, 1.1 Lf of toxin was added and the mixture was allowed to stand overnight in the cold. On the following day, additional increments of toxin were added and after 1 hour at room temperature, the mixtures were injected into guinea pigs. As can be seen from Table XI, a total of only 2.3 Lf of toxin sufficed to kill a guinea pig in series B as compared with 3.4 Lf necessary to produce a local reaction in series A. The difference of 1.1 Lf, representing the magnitude of the Danysz effect, is equivalent to approximately 60 M.L.D.

As shown by Table XI, the behavior of antitoxin Hu was entirely different and practically the same end-point was reached, regardless of whether the toxin was added all at once or in successive portions. The difference of 0.2 Lf, equivalent to about 10 M.L.D., might have been due to the presence of a trace of precipitating antitoxin. It should be noted that 5 units of non-precipitating

TABLE XII

Relationship between Sensitizing Dose of Precipitating (O'D) Antitoxin and Severity of Passive Anaphylaxis in the Guinea Pig

Sensitizing dose of antitoxin		Challenge dose of toxin		No. of guinea pigs	Lived				Died
Units	Mg. N	Lf units	Mg. N		0	Mild	Moderate	Severe	
10	0.022	50	0.023	4	2	2			
20	0.044	50	0.023	5		2	3		
40	0.088	50	0.023	4			2	1	1

antitoxin were required to neutralize 3.2 Lf of toxin; *i.e.*, about twice the unitage of precipitating antitoxin required for neutralization of the same amount of toxin. We are unable to offer an explanation for this discrepancy.

It is of interest that heated Fo antitoxin which no longer gave specific precipitates with toxin, still showed a marked Danysz effect (30 M.L.D.).

Passive Sensitization of Guinea Pigs to Anaphylactic Shock.—Results of passive anaphylaxis experiments are summarized in Tables XII, XIII, and XIV. The amount of precipitating antitoxin required to sensitize guinea pigs to severe or fatal shock is 0.088 mg. specifically precipitable antitoxin N or *ca.* 40 units when the shocking dose of toxin is 50 Lf or 0.023 mg. N (see Table XII). Sensitizing doses of 10 and 20 units (0.022 mg. N and 0.044 mg. N respectively) produced mild to moderate symptoms of anaphylaxis upon challenge with 50 Lf of toxin. Table XIII demonstrates the approximately equal effectiveness of precipitating (O'D) and non-precipitating (Hu) antitoxin (40 units) in sensitizing animals to fatal anaphylaxis. Essentially the same results were obtained with shocking doses of 50 Lf (0.023 mg. N) of toxin and 100 Lf of toxoid. The ability of non-precipitating antitoxin (Hu) to sensitize guinea pigs to fatal anaphylaxis was

unimpaired by heating the serum at 56°C. for 5 hours. This is shown in Table XIV. Sensitization was effected with 48 units (0.077 mg. N) of non-precipitating antitoxin and the challenge dose was 50 Lf (0.023 mg. N) of toxin.

TABLE XIII

Effect of Precipitating (O'D) and Non-Precipitating (Hu) Antitoxin in the Production of Passive Anaphylaxis in the Guniea Pig

Type of antitoxin	Sensitizing dose of antitoxin		Challenge dose of toxin		No. of guinea pigs	Lived			Died
	Units	Mg. N	Lf units	Mg. N		Mild	Moderate	Severe	
Precipitating	40	0.088	50	0.023	3			1	2
Precipitating	40	0.088	100*	0.046	6	1		1	4
Non-precipitating	40	0.064	50	0.023	3				3
Non-precipitating	40	0.064	100*	0.046	6	1	2		3

* Toxoid.

TABLE XIV

Effect of Heat at 56°C. upon Ability of Non-Precipitating Antitoxin to Produce Passive Anaphylaxis

Type of antitoxin	Sensitizing dose of antitoxin		Challenge dose of toxin		No. of guinea pigs	Lived			Died
	Units	Mg. N	Lf units	Mg. N		Mild	Moderate	Severe	
Fresh Non-precipitating	48	0.077	50	0.023	3			2	1
Heated (56°C., 5 hrs.) Non-precipitating	48	0.077	50	0.023	4	2			2

DISCUSSION

It has been shown that human non-precipitating diphtheria antitoxin is capable of specifically sensitizing skin sites of Schick positive (non-sensitive) individuals to subsequent challenge with purified diphtheria toxoid. Indeed, non-precipitating, skin-sensitizing antitoxin exhibits most of the properties of *atopic reagin* as encountered in the serum of hay-fever patients and asthmatics sensitive to pollen, grasses, danders, etc. For example, non-precipitating antitoxin is capable of causing sensitization of local skin sites to toxoid which persists for many weeks, and is further characterized by its almost complete failure to fix guinea pig complement in the presence of toxoid. In contrast, precipitating antitoxin disappears rapidly from injected skin sites and fixes complement to a high titer. The two types of antitoxin are further differentiated by the fact that whereas precipitating antitoxin exhibits a pronounced Danysz effect, non-

precipitating antitoxin is capable of neutralizing the same amount of toxin whether the latter is added in portions or all at one time. Both types of antitoxin were equally effective in the ability to sensitize guinea pigs to fatal anaphylaxis, a property which was not affected by heating sera at 56°C. Table XV summarizes the properties of a typical non-precipitating antitoxin (serum Hu) and compares them with those of a typical precipitating antitoxin (serum Fo). It should be emphasized that the two sera studied in greatest detail, whose properties are summarized in Table XV, represent extreme cases. Many of the persons hyperimmunized with diphtheria toxoid developed both types of antitoxin concurrently.

TABLE XV

*Summary of Properties of Precipitating and Non-Precipitating Antitoxins**

Property	Precipitating antitoxin		Non-precipitating antitoxin	
	Fresh	Heated	Fresh	Heated
1. Titer-rabbit skin, test, *units/cc.*	140	120	80	70
2*a*. Specifically precipitable antitoxin nitrogen, *gamma/unit.*	2.5	0	0	0
b. Coprecipitable antitoxin nitrogen, *gamma/unit*	—	*Ca.* 2.5‡	*Ca.* 1.6	Not done
3. Skin-sensitizing titer (P-K)	Nil	Nil	0.0002 cc	Nil
4. Persistence at injection site	Minutes	Minutes	Weeks	Weeks
5. Units required to fix 2 units of guinea pig complement	0.2	0.3	8	8
6. Danysz effect	Marked	Diminished but still pronounced	Absent	Not done
7. Passive sensitization of guinea pigs to anaphylactic shock	Yes	Yes	Yes	Yes

* As exemplified by precipitating antitoxin Fo and non-precipitating antitoxin Hu.

‡ Average values obtained on series of sera containing precipitating antitoxin, M. Cohn.

Table XV also summarizes the properties of precipitating and non-precipitating antitoxic sera which have been heated to 56°C. for 4 hours. It is of interest that although the skin-sensitizing ability of non-precipitating antitoxin is entirely destroyed by heating at 56°C., it remains capable of inhibiting or blocking the wheal and erythema produced by injecting into the skin mixtures of unheated antitoxin and toxoid. Moreover, the magnitude or titer of the blocking effect does not differ appreciably from its neutralization titer before heating. It would appear, therefore, that heating causes a quantitative conversion of skin-sensitizing antitoxin to a modified antitoxin with blocking properties.

The relative lability of skin-sensitizing activity to heat at 56°C. has been previously demonstrated by Jadassohn (7), Schmidt and Lippard (8), and Loveless (9).

The former workers (7, 8) also indicated that heating reagin at 56°C. resulted in relatively greater loss of skin sensitizing properties than of neutralizing powers. Jadassohn (7) who used *Ascaris*-sensitive serum-antigen mixtures, actually showed that heating *Ascaris*-sensitive serum for 24 hours did not destroy its neutralizing ability. These results demonstrate a further parallelism between skin sensitizing antibody and non-precipitating diphtheria antitoxin.

Although Loveless (9) has carried out similar heat experiments using serum from treated pollen-sensitive patients, she has interpreted her results to signify the presence of two distinct antibodies; a thermolabile skin-sensitizing antibody and a thermostable blocking antibody. However, because of the difficulty in measuring either type of antibody quantitatively, it is possible that the results obtained were due to conversion of sensitizing antibody to a form with blocking properties by heating at 56°C.

The present studies show that the capacity to block or inhibit the immediate wheal reaction caused by injection of toxoid into prepared skin sites is possessed by non-precipitating antitoxin heated to 56°C. and, to a lesser extent, by fresh and heated precipitating antitoxin. Mixtures of toxoid with a large excess of precipitating antitoxin cause little reaction in normal skin and probably fail to cause a reaction in the prepared site because the toxoid is saturated by the antitoxin. When precipitating antitoxin-toxoid mixtures contain approximately equivalent amounts of antigen and antibody, the mixture itself is highly irritating and produces wheal and erythema in both normal and prepared human skin sites. On the other hand, mixtures of heated non-precipitating antitoxin and toxoid do not produce wheal and erythema reactions in normal or in sensitized skin sites even when toxoid and antitoxin are present in equivalent amounts.

In summary, then, heating to 56°C. results in almost quantitative conversion of skin-sensitizing antitoxin to a form which possesses the properties of Loveless' thermostable blocking antibody. Furthermore, it has been shown by inhibition technics that the heated form persists at the injection site. Heating to 56°C. does not appear to influence, to any appreciable extent, the less pronounced blocking effect of precipitating antitoxin. In addition, precipitating antitoxin, whether fresh or heated, disappears rapidly from injected skin sites and the irritating effects of mixtures containing appropriate amounts of toxoid are not abolished by heating as in the case of skin-sensitizing antitoxin.

Although heating at 56°C. destroys the precipitating capacity of antitoxic sera, the antitoxin titer as measured by the rabbit skin test remains essentially unchanged. In addition, such heated sera showed a diminished but still pronounced Danysz effect and only a small diminution in complement fixation titer. These observations would seem to indicate that precipitating antitoxin which has been subjected to heat at 56°C. is still capable of undergoing considerable aggregation with toxin or toxoid even though visible aggregation does not occur.

It is suggested that certain of the unusual immunological properties of skin-sensitizing non-precipitating antitoxin depend on its failure to form aggregates with toxin or toxoid. The failure of skin-sensitizing antitoxin to precipitate with toxin and the absence of a Danysz effect indicate that the toxin-antitoxin complex is not aggregated to any significant extent. Skin-sensitizing antitoxin, therefore, exhibits the properties of what has been termed "univalent" or "incomplete" antibody (10). The relatively slight ability of skin-sensitizing antitoxin and toxoid to fix complement may be due to the low powers of aggregation of this complex.

A number of previous workers have demonstrated the ineffectiveness of skin-sensitizing antibody in passively sensitizing guinea pigs to anaphylactic shock. Our experimental results, therefore, may appear somewhat at variance. For example, Prausnitz and Kustner (11), Coca and Grove (12), Jadassohn (7), and Otto and Adelsberger (13) have all demonstrated the inability of various reagin-like substances to produce passive sensitization in the guinea pig. On the other hand, positive results were obtained by Ratner and Gruehl (14) who showed that serum from a patient sensitive to horse dander was capable of sensitizing guinea pigs to anaphylaxis.

Kabat and Benacerraf (15) demonstrated that guinea pigs could be sensitized to fatal anaphylactic shock with approximately the same quantity of non-precipitating or univalent rabbit antiovalbumin as with precipitating anti-ovalbumin. These authors showed that amounts of either antibody equivalent to 0.03 mg. N were required to sensitize guinea pigs to fatal anaphylaxis. In the present experiments it has been found that 10 units (0.025 mg. N) of human precipitating antitoxin caused mild or no symptoms of anaphylaxis and that 40 units of either precipitating or non-precipitating antitoxin produced severe anaphylaxis, frequently leading to death when the shocking dose of antigen was equivalent to 0.023 mg. N. From the studies of Kabat and Benacerraf (15) and those reported here, it is apparent that the amount of antibody required to sensitize guinea pigs to anaphylaxis may be considerably greater than that commonly present in the serum of allergic patients. For example, a number of individuals in our series who showed positive immediate skin reactions against purified Schick toxoid prior to hyperimmunization had extremely low levels (less than $^1/_{50}$ unit) of demonstrable antitoxin. This amount of antitoxin is entirely insufficient to passively sensitize guinea pigs. It is conceivable, therefore, that sera of many allergic patients showing specific skin reactivity might be incapable of sensitizing guinea pigs to anaphylaxis simply because of low circulating antibody titers.

While non-precipitating antitoxin appears similar in almost all respects to atopic reagin, the relationship between precipitating antitoxin and the thermostable blocking antibody studied by Cooke *et al.* (16, 17), Loveless (9), and others in the serum of treated patients and normal subjects immunized with

ragweed pollen, is by no means clear. Precipitating antitoxin disappears rapidly from injected skin sites and when mixed with toxoid in certain proportions is capable of inhibiting the wheal and erythema provoked by toxoid in sensitized skin. In these respects, precipitating antitoxin resembles blocking antibody. However, unlike the blocking antibody from treated hay-fever patients or normal subjects immunized with ragweed pollen, certain mixtures of precipitating antitoxin and toxoid elicit immediate reactions in normal skin. The irritating properties of heated precipitating antitoxin-toxoid complexes appear to be even more pronounced.

In the preceding paper it was shown that a close correlation existed between hypersensitivity of the immediate type to diphtheria toxin or toxoid and a personal or familial history of allergy. A description was also given of the case of a 6 year old boy with both personal and familial history of allergy who suffered a severe asthmatic crisis shortly after receiving a "booster" dose of diphtheria toxoid. His serum contained a high titer (50 units/cc.) of non-precipitating skin-sensitizing antitoxin. In the present study it has been demonstrated that this type of human antitoxin possesses most of the properties described for atopic reagin. For these reasons, it is suggested that the toxin-antitoxin reaction may provide a useful model for studying the type of immunological reactions encountered in allergic conditions of the hay-fever type.

SUMMARY

1. The immunological properties of two contrasting types of human antisera, each containing a high titer of diphtheria antitoxin, have been investigated.

2. Sera which contain only *non-precipitating* antitoxin exhibit most of the properties of atopic reagin-containing sera. This type of antitoxin is capable of sensitizing normal human skin to toxin or toxoid and remains for many weeks in the injected area. It exhibits no Danysz effect, does not fix complement unless very large amounts of serum are used, and can be specifically coprecipitated by addition of precipitating antitoxin and toxin. On the other hand, it is capable of sensitizing guinea pigs to fatal anaphylactic shock. Heating at 56°C. for 4 hours destroys the skin-sensitizing properties and results in almost quantitative conversion to a modified antitoxin which is capable of blocking the wheal and erythema reaction caused by injection of toxoid into sensitized skin. Heating at 56°C. does not result in an appreciable loss of neutralizing power as judged by tests in rabbit and human skin. The anaphylactogenic property of non-precipitating antibody is likewise unaffected by heat at 56°C.

3. *Precipitating* antitoxin is incapable of sensitizing normal skin to toxin or toxoid and disappears rapidly from the injected sites. It fixes complement to high titer and sensitizes guinea pigs to fatal anaphylactic shock. It was possible to demonstrate inhibition of the wheal and erythema reaction in sensitized skin by injecting certain mixtures of precipitating antitoxin and toxoid.

4. The two antitoxic sera studied in greatest detail represented extreme cases. Many persons immunized with toxoid developed both precipitating and non-precipitating antitoxin simultaneously.

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BIBLIOGRAPHY

1. Kuhns, W. J., and Pappenheimer, A. M., Jr., *J. Exp. Med.*, 1952, **95**, 363.
2. Balch, H. H., *J. Immunol.*, 1950, **64**, 397.
3. Lawrence, H. S., personal communication.
4. Lawrence, H. S., and Pappenheimer, A. M., Jr., *Am. J. Hyg.*, 1948, **47**, 226.
5. Cohn, M., and Pappenheimer, A. M., Jr., *J. Immunol.*, 1949, **63**, 291.
6. Danysz, J., *Ann. Inst. Pasteur*, 1902, **16**, 331.
7. Jadassohn, W., *Arch. Dermat. u. Syph.*, 1928, **156**, 690.
8. Schmidt, W. M., and Lippard, V. W., *Am. J. Dis. Child.*, 1937, **54**, 777.
9. Loveless, M., *J. Immunol.*, 1940, **38**, 25.
10. Heidelberger, M., and Kendall, F. E., *J. Exp. Med.*, 1935, **62**, 697.
11. Prausnitz, C., and Kustner, H., *Centr. Bakt., 1 Abt., Orig.*, 1921, **86**, 160.
12. Coca, A. F., and Grove, E. F., *J. Immunol.*, 1925, **10**, 445.
13. Otto, R., and Adelsberger, L., *Z. Hyg. u. Infektionskrankh.*, 1931, **113**, 16.
14. Ratner, B., and Gruehl, H. L., *Proc. Soc. Exp. Biol. and Med.*, 1929–30, **27**, 574.
15. Kabat, E., and Benacerraf, B., *J. Immunol.*, 1949, **62**, 97.
16. Cooke, R. A., Barnard, J. H., Hebald, S., and Stull, A., *J. Exp. Med.*, 1935, **62**, 733.
17. Cooke, R. A., Loveless, M., and Stull, A., *J. Exp. Med.*, 1937, **66**, 689.