DELAYED HYPERSENSITIVITY

II. Induction of Hypersensitivity in Guinea Pigs by Means of Antigen-Antibody Complexes*

By JONATHAN W. UHR,‡ M.D., S. B. SALVIN,§ Ph.D., and A. M. PAPPENHEIMER, Jr., Ph.D.

(*From the Department of Microbiology, New York University College of Medicine, New York*)

(Received for publication, September 22, 1956)

In the previous paper (1) it was reported that guinea pigs infected intradermally with a living toxigenic strain of *C. diphtheriae* developed delayed hypersensitivity to diphtheria toxin even when treated with antitoxin *before* infection. The present paper describes experiments which show that small amounts of diphtheria toxoid or of ovalbumin are capable of inducing a high degree of tuberculin type hypersensitivity in guinea pigs if the antigen is injected intradermally in the form of a complex with excess homologous antibody. Maximum sensitization is achieved several weeks before circulating antibody can be detected.

Materials and Methods

Antigens.—Schick test materials and purified toxoid KP28 were the same as described in the preceding paper (1). Ovalbumin, three times recrystallized, was supplied by Dr. Milton Levy in the form of a dry powder. It was dissolved in saline or in phosphate buffer, filtered, and the protein concentration determined by measuring the absorption at 277 mμ of aliquots diluted in 0.25 N acetic acid.

Antisera.—Rabbit antitoxin 379–380, horse antitoxic gamma globulin 5353AD, and human precipitating antitoxic gamma globulin were the same materials used in the preceding study (1). Human skin-sensitizing antitoxin *Hu* (2) was used without fractionation. This serum was from a recent bleeding taken from subject *Hu* who had received an immunizing dose of alum toxoid 5 years previously. The serum still contained 20 units of non-precipitating, skin-sensitizing antitoxin per ml. The guinea pig antitoxin was prepared in this laboratory by Dr. Melvin Cohn some years ago and had been kept in the lyophilized state. When reconstituted in water, it was found to contain 1.35 mg. specifically precipitable antitoxin protein per ml., equivalent to 80 *in vitro* units per ml.

* This work was aided by a grant from the National Institutes of Health, United States Public Health Service; it was sponsored by the Commission on Immunization, Armed Forces Epidemiological Board.

‡ Aided by a fellowship from the Dazian Foundation for Medical Research.

§ United States Department of Health, Education and Welfare, Public Health Service, National Institutes of Health, National Microbiological Institute, Rocky Mountain Laboratory, Hamilton, Montana.

Rabbit antiovalbumin serum was provided by Dr. C. A. Stetson. This was a pooled serum collected from rabbits which 4 weeks previously had received a single injection into the foot-pads of 2 mg. crystalline ovalbumin in Freund adjuvant containing killed mycobacteria. The serum contained 1.8 mg. antibody protein per ml. specifically precipitable by ovalbumin.

The antibody content of all antisera (except the human non-precipitating) was determined by quantitative precipitation, according to the method of Gitlin (3). The twice washed specific precipitates, after standing 48 to 72 hours in the cold, were dissolved in 0.25 N acetic acid and their protein content determined by absorption at 277 mμ. Antitoxins were also titrated by the rabbit intracutaneous method of Fraser (4).

Antitoxin-Absorbed Toxoid.—Purified toxoid was precipitated by excess human antitoxic gamma globulin, or was mixed with human Schick-positive gamma globulin, as described in the previous paper (1). The supernates were used to demonstrate specificity of skin reactions to toxoid. In animals sensitized with complexes containing human antitoxin, supernates prepared from horse antitoxin were used for demonstrating the specificity.

Method of Sensitization.—Various time-dosage schedules and routes of administration were tested. The method finally adopted was the following: In the case of rabbit, human, or guinea pig antisera, one-half that quantity of antigen required to bring the system to the equivalence point was added. For example, rabbit antitoxin 379–380 contained 40 units per ml. To 1 ml. antitoxin was added 2 ml. of purified toxoid diluted so as to contain 10 Lf per ml. The mixture was allowed to incubate at 37°C. for 1 hour and then placed in the cold overnight. The precipitate was centrifuged, washed twice with 2 ml. changes of cold saline, and finally suspended in 2 ml. of saline (10 Lf combined toxoid per ml.). Toxoid-horse-antitoxin floccules were similarly prepared, except that in this case only slight antitoxin excess was used (430 units antitoxin plus 400 Lf toxoid). Once again, the washed floccules were suspended in saline so as to contain 10 Lf per ml. toxoid in the form of complex. Washed ovalbumin-antiovalbumin precipitates were likewise suspended in saline to contain 30 μg. per ml. ovalbumin in the form of its complex with excess antibody. In all cases the molecular composition of the specific precipitates approximated an antibody-antigen ratio of 4:1.

Guinea pigs (300 to 400 gm.) were sensitized by injection of the specific precipitates suspended in oil-water emulsion. The saline suspensions of antigen-antibody complex were emulsified in a syringe with an equal volume composed of 15 per cent arlacel A and 85 per cent bayol F or with a preparation of Freund adjuvant obtained from Difco Laboratories. Heat-killed *Mycobacterium butyricum*, 1 mg. per ml., was incorporated into some of the suspensions. Guinea pigs were usually sensitized by a single injection of 0.4 ml. of the oil-water emulsion of antigen-antibody complex, with or without mycobacteria, containing a total of 2.5 μg. of antigen; *i.e.*, either purified toxoid or ovalbumin. The injection was distributed into the digits of all four feet.

Control guinea pigs were injected into the foot-pads with the same amounts (2.5 μg.) of the purified toxoid or ovalbumin in oil-water emulsion either with or without killed mycobacteria.

Skin Reactions.—Guinea pigs were usually skin tested 2 to 3 weeks after sensitization with 0.1 ml. of 10-fold dilutions of either purified toxoid (10 Lf per ml.) or ovalbumin (30 μg. per ml.). Dilutions were made in saline containing 1 per cent normal guinea pig serum, in order to prevent surface denaturation. Reactions were usually observed at intervals for the first 6 hours after challenge and again at 24 hours.

Preparation of Leucocytes for Transfer of Hypersensitivity.—Popliteal and axillary lymph nodes from 2 to 5 sensitized guinea pigs were removed and suspended in cold Tyrode's solution and the cells teased out with dissecting needles. Equally satisfactory white cell suspensions were obtained, very rapidly, by squeezing minced lymph nodes through a small garlic press. The leucocyte suspensions were washed twice with 10 ml. cold Tyrode's solution, suspended in Tyrode's solution and injected intraperitoneally into normal guinea pigs. The number of cells injected per recipient usually varied between 10^8 and 10^9. 24 to 72 hours after transfer, the recipient animals were challenged in the skin with 3 μg. of homologous antigen.

RESULTS

Induction of Sensitivity to Toxin and to Ovalbumin by Specific Precipitates.— Table I shows the dimensions of delayed skin reactions to diphtheria toxoid in 4 guinea pigs tested 2 to 3 weeks after injection into the foot-pads of 0.8 Lf (2.5 μg.) toxoid as a specific precipitate formed with excess human antitoxic gamma globulin in adjuvant containing mycobacteria. Skin tests were carried out with crude and purified toxoid before and after specific removal of the toxoid component by precipitation with horse antitoxic gamma globulin. It will

TABLE I

Specificity of Delayed Reactions to Toxoid and to Ovalbumin (Ea) in Guinea Pigs Sensitized by Toxoid-Human Antitoxin or Ea-Anti Ea (Rabbit) Specific Precipitates in Adjuvant Containing Killed Mycobacteria

Guinea pig No.	Test material	Skin reactions in mm. at 24 hrs. to 0.1 ml. antigens			
		Undiluted*	1:10	1:100	1:1000
1	Crude toxoid	26 × 23	21 × 15	18 × 12	16 × 11
	Antitoxin-absorbed supernate	14 × 11	5 × 4	±	±
2	Crude toxoid	30 × 30	18 × 18	14 × 14	9 × 9
	Antitoxin-absorbed supernate	15 × 15	10 × 8	±	±
3	Purified toxoid	37 × 33	20 × 18	17 × 13	11 × 9
	Antitoxin-absorbed supernate	7 × 6	±	±	±
4	Purified toxoid	35 × 30	17 × 15	12 × 10	±
	Antitoxin-absorbed supernate	4 × 4	±	±	±
5	Ovalbumin	30 × 25	20 × 15	10 × 10	Not tested
6	Ovalbumin	18 × 17	12 × 11	9 × 9	Not tested
7	Ovalbumin	20 × 15	12 × 10	6 × 6	Not tested

* Undiluted test materials contained 1 Lf (3 μg.) toxoid or 3 μg. ovalbumin per 0.1 ml.

be noted that the degree of sensitivity attained was as great or greater than that induced by infection (1). Moreover, sensitization was highly specific for toxin itself. The small reactions obtained to the supernate after absorption with antitoxin, could have been due to traces of soluble toxoid-antitoxin complex or possibly to a slight sensitivity to other diphtherial proteins owing to contamination of the human serum used to induce sensitivity with a trace of antibody against P proteins.

Table I shows that animals may be rendered equally sensitive to ovalbumin (Ea) by a single injection of 3 μg. Ea in the form of specific Ea-anti Ea (rabbit) precipitates in complete (*i.e.* mycobacteria-containing) Freund adjuvant.

The skin reactions described in Table I are typical of those seen in a large series (more than 100) guinea pigs sensitized to toxoid, Ea and certain other antigens by the same procedure. As will be discussed below, no detectable circulating antibody is present in the serum 2 to 3 weeks after sensitization provided the precipitates are made from antisera containing the corresponding antibody in excess. When *complete* adjuvant is used, effective sensitization is induced regardless of the route of injection, whether intradermal, subcutaneous, intramuscular, intraperitoneal, or even intravenous.

TABLE II

Effect of Adjuvant and of Killed Mycobacteria on Sensitization to Diphtheria Toxoid (To) by Means of Washed Toxoid-Antitoxin Precipitates

Sensitizing material*	No. of injections‡	No. of guinea pigs	Skin reactions 24 hrs. after challenge with 1 Lf toxoid			
			≧20 mm.	10 to 20 mm.	5 to 10 mm.	0
ToA_4 in saline	2	5	0	4	1	0
ToA_4 incomplete adjuvant	1	7	1	5	1	0
	2	22	5	15	0	2
ToA_4 complete adjuvant with mycobacteria	1	16	13	3	0	0

* Sensitizing dose of toxoid (To) was 2.5 μg. per guinea pig divided among all four footpads. ToA_4 represents approximate molecular composition.

‡ When two sensitizing injections were given, the interval between them was 6 days. Skin tests were carried out 12 days after second injection.

Effect of Mycobacteria.—It is well known that delayed hypersensitivity characteristically follows infection by a variety of bacterial, viral and fungal agents. Nevertheless, the idea has persisted that mycobacteria or their products are endowed with special attributes necessary for induction of the hypersensitive state.

Table II shows that marked sensitivity to diphtheria toxoid can be induced in guinea pigs following a single injection of immune precipitate in oil-water emulsion in the absence of killed mycobacteria. Definite, though less pronounced sensitivity develops after two intradermal injections of saline suspensions of toxoid-antitoxin complex. Thus, while mycobacteria seem to promote a more uniformly high degree of hypersensitivity, neither acid-fast organisms nor indeed adjuvant of any kind is essential. When mycobacteria are omitted, the route of the sensitizing injection assumes considerable importance and satisfactory results are only obtained by the intracutaneous route. It may be noted that man may be rendered exquisitely sensitive to diphtheria toxoid

following one or two intradermal injections of small amounts of toxoid-antitoxin precipitate in saline (5).

Hypersensitivity to toxoid may also be induced in guinea pigs by the separate administration of toxoid and an excess of antitoxin. Table III describes the skin reactions in guinea pigs which had received 3 weeks previously a relatively large dose of either horse or guinea pig antitoxin intraperitoneally, followed 24 hours later by 2.5 μg. toxoid in oil-water emulsion (without mycobacteria). In this case, also, the choice of the intradermal route was important and no skin reactions of the delayed type could be elicited on challenge of those

TABLE III

Sensitization of Guinea Pigs Given Antitoxin Intraperitoneally Followed 24 Hrs. Later by Intradermal or Intramuscular Injection of 2.5 μg. Toxoid in Oil-Water Emulsion

Antitoxin		Toxoid, route of injection	Reaction to 1 Lf toxoid*	Serum antitoxin units per ml.
Species	Units injected			
Horse	1000	Intradermal	12 × 6, 12 × 11, 16 × 15	<0.001, <0.001, <0.001
Guinea pig	70	Intradermal	20 × 15,‡ 15 × 15,‡ 22 × 16‡	>0.01, >0.01, >0.01
Horse	1000	Intramuscular	0, 0	<0.001, <0.001
Guinea pig	70	Intramuscular	25 × 18§ 20 × 20§, 13 × 11§	>0.01, >0.01, >0.01
Human‖ (non-precipitating)	2	Intradermal	17 × 17, 12 × 12, 6 × 6	<0.01, <0.01, <0.01

* Challenge dose given 2 weeks after sensitizing injection.

‡ Combined Arthus-tuberculin type. Lymph node cells from these 3 animals transferred to a normal guinea pig. Recipient showed 8 × 8 mm. reaction at 24 hours to 1 Lf challenge.

§ Predominantly Arthus type reaction. Cell transfer was unsuccessful.

‖ Given as complex into foot-pads in two injections 72 hours apart.

animals who received their sensitizing dose of toxoid intramuscularly. Animals which received homologous guinea pig antitoxin did show skin reactions of both Arthus and of the delayed type due to the persistence of circulating antitoxin. Cell transfer, however, was successful only in the case of the guinea pigs sensitized by the intradermal route.

The species of antibody used to form the specific immune precipitate does not appear to influence the sensitization process. Precipitates formed with horse, rabbit, human, and homologous guinea pig antitoxin are all equally satisfactory in inducing sensitivity to diphtheria toxin. Finally, as shown in Table III, injection of 2.5 μg. toxoid in combination with excess non-precipitating "skin-sensitizing" human antitoxin (2) induces the hypersensitive state. Thus it does not appear that aggregation or precipitation of the complex is required for sensitization.

Duration of Induced Hypersensitivity.—Pronounced delayed skin reactions to a challenge dose of 1 Lf (3 μg.) toxoid can be evoked in guinea pigs on the 3rd or 4th day following a primary sensitizing injection of toxoid-antitoxin complex in incomplete adjuvant. When mycobacteria are present in the primary sensitizing mixture, sensitivity persists undiminished for at least 10 weeks.

Sensitizing Dose.—Table IV describes skin reactions to toxoid in groups of guinea pigs sensitized 7 to 14 days previously by single decreasing doses of toxoid-antitoxin (rabbit) complex in oil-water emulsion. The degree of sensitivity induced by 0.1 Lf (0.3 μg.) is less than that induced by 1 Lf of complex. Further increase to 10 Lf of the sensitizing dose does not appear to influence the degree of hypersensitivity attained.

TABLE IV

Skin Reactions to Purified Diphtheria Toxoid (To) in Guinea Pigs 7 to 14 Days after 1 Intradermal Injection of ToA_4 (Rabbit) Complex in Incomplete Adjuvant

No. of guinea pigs	Sensitizing dose		Skin reactions in mm. at 24 hrs. to 1 Lf (3 μg.) To
	μg	Lf	
7	30	10	17 × 17, 7 × 6, 15 × 13, 14 × 14, 8 × 8, 12 × 12, 10 × 10
7	3	1	10 × 10, 23 × 22, 13 × 12, 10 × 10, 7 × 6, 12 × 10, 14 × 12
7	0.3	0.1	10 × 10, 15 × 15, 7 × 7, 10 × 9, 8 × 7, 10 × 10, 7 × 6

Relation of Circulating Antibody to Hypersensitivity.—Numerous studies have shown that excess passively administered antibody is capable of blocking the active antibody response to injected antigen (Glenny and Sudmersen (6), Hartley (7), Mason *et al.* (8)). In only 2 guinea pigs, out of more than 60 tested 2 to 3 weeks after sensitization with toxoid-antitoxin (heterologous) complex, could as much as 0.001 unit per ml. antitoxin (0.0025 μg. antitoxin N per ml.) be detected, although antitoxin was actively formed within a few days after skin testing the same animals with 1 Lf of toxoid. Table V shows that no signs of anaphylaxis were elicited after intravenous challenge of sensitized animals with 0.3 to 2.4 mg. homologous antigen. On the other hand, when guinea pigs were sensitized with the same amount (3 μg.) of *free* ovalbumin or toxoid in adjuvant given as a single dose into the footpads, significant amounts of circulating antibody were formed. 19 of 21 animals in Table V, sensitized with free antigen, showed anaphylactic signs following intravenous challenge with antigen 2 to 3 weeks later and fatal shock occurred in 13 of them. Antitoxin titers of serum taken from guinea pigs 2 to 3 weeks after a single intradermal injection of 2.5 μg. *free* toxoid in adjuvant, with or without mycobacteria, varied from 0.02 to 0.3 units per ml.

In Hartley's experiments referred to above (7), guinea pigs were immunized with toxin-antitoxin floccules formed with excess horse antitoxin. Hartley showed that small amounts of circulating antitoxin could be detected in serum collected from these animals 80 to 90 days after the immunizing injection. It is probable, therefore, that antibody formation is not completely suppressed. In the present study, some of the guinea pigs tested 8 to 10 weeks after sensitization with toxoid-antitoxin precipitates showed low titers of antitoxin in confirmation of Hartley's early work. Furthermore, unpublished experiments have shown that injections of toxoid-antitoxin complexes into rabbits and into man, also cause some antibody production, particularly if given in repeated doses or as a single booster injection.

TABLE V

Anaphylaxis in Animals Sensitized to Egg Albumin (Ea) and to Toxoid (To)

No. of animals	Approximate molecular composition of sensitizing material	Reagent in excess	Shocking antigen	Result			
				Negative	Moderate	Severe	Death
14	Ea	—	Ea	2	1	1	10
6	EaA_2	Antigen	Ea	1	2	1	2
7	EaA_4	Antibody	Ea	7	0	0	0
6	To	—	To	0	1	2	3
7	ToA_4	Antitoxin	To	7	0	0	0
3	ToA_4	Antitoxin	RGG*	0	1	1	1
2	EaA_4	Antibody	RGG	0	0	0	2
5	EaA_2	Antigen	RGG	0	0	0	5

* RGG, rabbit gamma globulin.

When guinea pigs are sensitized with *free* antigen incorporated in *complete* adjuvant, they develop both delayed and immediate types of hypersensitivity. Such animals show skin reactions following intradermal challenge with antigen which combine features of both Arthus and "tuberculin" types of reactions (Freund (9)). Sensitization of animals by intradermal injection of specific precipitates containing slight antigen excess without mycobacteria also induces both the immediate and the delayed types of hypersensitivity.

The guinea pigs used in the experiments summarized in Table V were sensitized with specific precipitates formed with rabbit antibody. When skin tested with rabbit gamma globulin, RGG, these animals showed inflammatory reactions. That these skin reactions were of the Arthus rather than the delayed type is suggested by the fact that they appeared early (2 hours) and were usually receding at 24 hours. This impression was confirmed by the failure to effect transfer of sensitivity to RGG by means of cells and by demonstration of circulating antibody. All of 10 animals challenged intravenously with RGG showed signs of anaphylaxis which in 8 instances terminated in death. This is

in keeping with observations of others (Treffers and Heidelberger (10), Adler (11)) that the heterologous globulin portion of immune precipitates is highly antigenic.

The following experiment is of interest in connection with the failure of guinea pigs to develop significant delayed sensitivity to the globulin portion of the specific precipitates.

6 guinea pigs were sensitized in the usual manner to antitoxic horse gamma globulin (HGG) by injection of HGG-rabbit anti-HGG specific precipitates in complete Freund adjuvant. One week later the animals were challenged in the skin with 0.5 and 5 μg. amounts of HGG and of RGG. While strong reactions appeared at all test sites, those to HGG were of the delayed type whereas those to RGG appeared earlier and were presumably of the Arthus type. 10 mg. HGG were injected intravenously into 3 of the animals and failed to produce any of the signs of anaphylaxis. The same 3 guinea pigs were then given 5 mg. RGG and all succumbed to fatal anaphylactic shock within 3 minutes.

Transfer of Sensitivity by Means of Cells.—It is characteristic of the delayed hypersensitive state that sensitivity can be transferred to normal animals by leucocytes but not by serum from sensitive donors (12–14). Table VI summarizes cell transfer experiments using donor guinea pigs sensitized to toxoid or to ovalbumin 2 to 3 weeks previously by means of specific precipitates. Cells were obtained from lymph nodes of donors 1 to 4 days after skin test and the recipient guinea pigs were skin-tested 48 to 72 hours after transfer. While there can be no doubt that the induced delayed sensitivity has been successfully transferred to recipient animals,[1] nevertheless experiments of the type described in Table VI must be interpreted with caution. Only 2 of the recipient animals were tested for circulating antitoxin and one of these showed about 0.02 unit/ml. In this animal, therefore, the skin reaction observed could have been due in part to an Arthus type of reaction. In this instance the donor animals were skin-tested with 1 Lf toxoid 4 days before removal of lymph nodes for transfer and antitoxin-forming cells were undoubtedly present in the suspension used for transfer. The experiments of Wager and Chase (15), Harris, Harris, and Farber (16), and Stavitsky (17) have shown that lymph node cells from immunized animals continue to form antibody when transferred to a normal host. Because of the similarity between the Arthus and tuberculin type skin reactions in guinea pigs, therefore, it is necessary to demonstrate that the recipient animal contains less circulating antibody than that required for an Arthus reaction.[2] The first recipient listed in Table VI showed less than

[1] Sensitivity to diptheria toxoid, induced by means of intradermal injection of toxoid-antitoxin precipitates, has also been successfully transferred from man to man using *extracts* of leucocytes taken from peripheral blood of the sensitized donors (Lawrence, H. S., and Pappenheimer, A. M., Jr., data unpublished; Good, R. W., personal communication).

[2] Skin reactions of the Arthus type have been observed in guinea pigs immunized with toxoid, whose serum contained as little as 0.02 units of antitoxin per ml. (0.05 μg. antitoxin N per ml.). This concentration of circulating antibody appears to be less than would be expected from the minimal amount of guinea pig antiovalbumin which Benacerraf and Kabat (18) found necessary to passively sensitize guinea pigs to the Arthus reaction.

0.001 units antitoxin per ml. of serum and therefore the 20 x 15 mm. delayed reaction to 1 Lf toxoid can be regarded with reasonable certainty as being of the delayed type.

As mentioned in the preceding section, guinea pigs sensitized to toxoid and to Ea by administration of specific precipitates containing RGG, showed skin reactions to RGG which were considered to be of the Arthus type. A number of recipient guinea pigs were tested with rabbit gamma globulin following cell transfer. Some of the recipients showed small positive reactions when seen at 24 hours. Here again, it is probable that these reactions were of the Arthus type, since intravenous challenge with RGG provoked immediate signs of

TABLE VI

Cell Transfer of Induced Sensitivity

No. of donors	Sensitizing material*	Sensitivity of donors. Average diameter in mm. to 3 μg. To at 24 hrs.	No. of cells‡ × 10^6	Hrs. after transfer	Recipients' skin reactions to 3 μg. To in mm. at 24 hrs.	Antitoxin units per ml.
4	ToA_4 (mycobacteria)	22	950 (4)	72	20 × 15	<0.001
2	ToA_4 (mycobacteria)	29	480 (1.5)	72	12 × 11	—
4	ToA_4 (mycobacteria)	19	450 (4)	72	15 × 13	0.016
3	EaA_4	?	450 (2)	72	12 × 12	—
2	ToA_4§ (mycobacteria)	22	400 (1)	48	9 × 9	—
2	ToA_4	18	160 (1)	72	10 × 9	—
3	ToA_4§ (mycobacteria)	20	130 (1)	48	0	—
3	To	Arthus	380 (1)	72	0	—

* Antigen-antibody of approximate molecular composition AgA_4 in adjuvant. Mycobacteria added where noted.

‡ Figures in parentheses indicate days after skin test that cells were collected.

§ Donors received 1.8 and 2.7 mg. To intravenously 4 hours prior to removing lymph nodes.

anaphylaxis. Presumably, anti-RGG forming lymph node cells continued to form antibody after transfer to the recipient host.

Some Characteristics that Distinguish Arthus and Tuberculin Type Reactions in Guinea Pigs.—Both in the gross and microscopically, the Arthus and the delayed tuberculin type skin reactions resemble one another closely in guinea pigs. The same small amounts of specific antigen suffice to elicit either type of skin reaction in appropriately sensitized animals. However, in the Arthus reaction circulating antibody is required, whereas if antibody of any kind is necessary for the delayed reaction it must be of a type that is bound to cells. Since only small amounts of circulating antibody suffice to render guinea pigs extremely sensitive to the Arthus reaction (see footnote 2), it is probable that many of the delayed tuberculin type reactions studied by others in sensitized guinea pigs were of the "combined" type. When only one type of sensitivity is predominant we have been able to differentiate the two types of skin reac-

tions by the timing of their appearance and disappearance. In our experience, Arthus reactions in the guinea pig consistently make their appearance within 2 hours of injection and are maximal at 4 to 6 hours. Skin reactions of the delayed type, however, do not become discernible before the 4th or 5th hour, are usually maximal at 24 hours and may persist for a considerable time thereafter. At 24 hours, small Arthus type reactions are rapidly diminishing in intensity and may have faded almost entirely. However, more severe Arthus reactions persist and at 24 hours may be indistinguishable in appearance from tuberculin type reactions. Table VII summarizes the properties of the two types of reaction in guinea pigs, using the toxoid-antitoxin system as an illustration.

TABLE VII

Characteristics of Arthus and Tuberculin Type Reactions in Guinea Pigs Sensitized by Intradermal Injection of Purified Toxoid

Sensitizing material	Skin test material	Type of skin lesion	Time of appearance of lesion (hrs.)	Skin reaction		Central hemorrhage necrosis	Serum antitoxin (units per ml.)	Cell transfer
				at 4 hrs.	at 24 hrs.			
Toxoid-rabbit antitoxin ppt. in oil	Toxoid	Tuberculin	5	None	++++	0	<0.001	+
Toxoid-rabbit antitoxin ppt. in oil	Rabbit gamma globulin	Arthus	2	++++	++	0	+	0
Toxoid mycobacteria in oil	Toxoid	Combined	2	++++	++++	+	0.02-0.3	+
Toxoid in oil	Toxoid	Arthus	2	++++	++	+	0.02-0.3	0

DISCUSSION

The induction of the delayed hypersensitive state by means of an antigen-antibody complex is not a new observation. In 1922, before the introduction of toxoid as an immunizing agent, Copeman *et al.* (19) noted that Schick-positive children immunized with under-neutralized toxin-antitoxin mixtures showed pseudoreactions when retested some weeks later with Schick test materials. The following year, Zingher and Park (20) reported on the immunization of school children with toxin-antitoxin and remarked "at Schick retest, many of these children showed a moderate to marked pseudoreaction." Apparently the early workers did not suspect that the sensitivity reactions which they induced were specifically directed against diphtheria toxin itself.

It has been shown that a single intradermal injection of only 0.3 μg. diphtheria toxoid or of ovalbumin in the form of a washed specific precipitate incorporated in oil-water emulsion will induce delayed hypersensitivity in the guinea pig to toxoid or ovalbumin respectively. It seems probable that even smaller quantities will suffice to render animals sensitive if killed mycobacteria

are added to the adjuvant. Provided that the immune precipitate is formed in the region of excess antibody, hypersensitivity develops at least 2 to 3 weeks before the active formation of demonstrable serum antibody. The fact that neither adjuvants nor killed mycobacteria are required for induction of delayed hypersensitivity is confirmed by recent experiments in man (5) in which four Schick-positive subjects were rendered highly sensitive to purified diphtheria toxin by intradermal injection of a saline suspension of washed toxoid-antitoxin specific precipitates.[3]

These experiments help to clarify the intimate relationship between infection and the development of hypersensitivity. As noted above, the amounts of specific protein antigens (in the form of immune precipitates) required for inducing the delayed hypersensitive state are very small and indeed may be equivalent to the amounts of protein which are released from bacteria during an infectious process. It seems reasonable to suggest that much of the antibody formed during the early stages of an infection is used up in reacting with antigens of the invading organism. The antigen-antibody complexes so formed could then induce the hypersensitive state. It seems likely that the degree of hypersensitivity induced will be increased if complex formation takes place at foci of inflammation surrounding the infectious agent.

Very little can be said at this time regarding the mechanism of induction of delayed hypersensitivity by means of immune precipitates. At least three factors are of importance in determining the degree of sensitization attained. They are: (*a*) the intradermal route of injection, (*b*) injection of antigen in the form of a complex with antibody, and (*c*) the cellular reaction in response to the sensitizing injection. While all three of these factors are of importance, no one of them can be regarded as essential.

The importance of skin in the development of delayed hypersensitivity was suggested by the early studies of Jones and Mote (21) and of Simon and Rackemann (22). These workers noted delayed reactions to rabbit and to guinea pig serum in persons who had received repeated intradermal injections of these antigens. The importance of the intradermal route is further indicated by studies on sensitization to simple chemicals (Landsteiner and Chase (23); Eisen *et al.* (24)). Sensitization is induced following topical application in olive oil to guinea pig or to human skin of substances such as picryl chloride and certain 2-4-dinitrophenyl compounds. Parenteral injection of these compounds is far less effective. Recently Porter (25) and Good (26) have shown that congenital agammaglobulinemic patients are readily sensitized to dini-

[3] These results have been further extended by Dr. Robert W. Good (personal communication) of the University of Minnesota, who has successfully sensitized 13 children and adults to toxin by intradermal injection of washed toxoid-antitoxin floccules. It is of particular interest that Dr. Good's series included one acquired and three congenital agamma globulinemic patients.

trochlorobenzene by application of this chemical to the skin. Their experiments prove that antibody formation (in the conventional sense) is not essential for induction of the delayed hypersensitive state even though the present studies with *protein* antigens have shown that their injection as a complex with antibody is a far more effective means of induction than injection of free antigen alone. Perhaps the proteins of skin which conjugate with these simple reactive chemicals play a role comparable to that of antibody.

The importance of the intradermal route suggests that introduction of a protein in the form of a large complex with antibody causes antigen to be retained in the skin, and thereby facilitates sensitization. However, if the role of the antibody portion of the complex were merely to hold antigen in the skin, one would expect sensitized animals to show delayed skin reactivity to heterologous globulin used to form the specific precipitate. This does not seem to be the case. It has *not* been possible to demonstrate delayed sensitivity to horse, rabbit, or human gamma globulin in guinea pigs sensitized to toxoid by means of specific precipitates formed with antitoxins from these species, despite the fact that the animals develop circulating antibody against the corresponding globulins. On the other hand, we have already seen that sensitivity to horse gamma globulin is readily induced in guinea pigs following injection of HGG-anti HGG (rabbit) precipitates. We can only suggest that antibody (even of the non-precipitating type) hinders antigen from reaching the site of conventional antibody formation within the cell, but favors in some undetermined manner its access to different sites involved in sensitization.

In a recent review on the mode of action of adjuvants, Freund (9) has discussed the importance of the cellular reaction to killed acid-fast bacilli in bringing about sensitization to a foreign protein. The present studies show that when immune precipitates are used to induce sensitivity, acid-fast organisms are not required. However, their incorporation into the adjuvant results in increased sensitivity perhaps because of the intensity and type of the cellular reaction which they cause.

It seems probable that all methods of active immunization will induce the tuberculin type of sensitization to some extent, however slight, since actively formed antibody will combine with antigen to form complexes. The present studies imply that the chances of developing hypersensitivity during immunization will be increased considerably following booster doses of antigen, particularly if adjuvants are employed, if the booster dose is administered intracutaneously and if the individual already possesses a sufficiently high titer of circulating antibody.

In conclusion, it has been shown that only 1 or 2 μg. of a protein antigen, when complexed with antibody and injected intradermally, will produce maximal sensitization of the delayed type in experimental animals. When very much larger amounts of protein (a milligram or more) are used, as seems to

have been the case in previous studies, the probability of inducing sensitivity at the same time to traces of impurity becomes very great. Since older methods of inducing the hypersensitive state also lead to a high circulating antibody titer, the reactions observed were often of complex etiology and difficult to interpret. Now that a simple method is available for rendering animals highly sensitive to single proteins without antibody formation, analysis of the mechanisms underlying the delayed hypersensitive state may perhaps be facilitated.

SUMMARY

A general method for induction of the delayed hypersensitive state directed against single protein antigens is described. The method consists of intradermal injection of minute amounts of washed immune precipitates containing the antigen in question. Provided the specific precipitates are formed in the region of antibody excess, maximal sensitivity develops at least 2 to 3 weeks before detectable circulating antibody is formed in guinea pigs against the sensitizing antigen. Neither adjuvant nor killed acid-fast bacteria are required for induction of the delayed hypersensitive state although the degree of sensitization is considerably increased when the sensitizing material is incorporated in Freund's complete adjuvant. Characteristics of the "delayed" as opposed to the "immediate" hypersensitive states in the guinea pig are described and implications of the findings are discussed.

BIBLIOGRAPHY

1. Uhr, J. W., Pappenheimer, A. M., Jr., and Yoneda, M., Delayed hypersensitivity. 1. Induction of hypersensitivity to diphtheria toxin in guinea pigs by infection with *C. diphtheriae*, *J. Exp. Med.*, 1957, **105**, 1.
2. Kuhns, W. J., and Pappenheimer, A. M., Jr., 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, *J. Exp. Med.*, 1952, **95**, 375.
3. Gitlin, D., Use of ultraviolet absorption spectroscopy in the quantitative precipitin reaction, *J. Immunol.*, 1949, **62**, 437.
4. Fraser, D. T., The technic of a method for quantitative determination of diphtheria antitoxin by a skin test in rabbits, *Tr. Roy. Soc. Canada*, 1931, sec. 5, **25**, 175.
5. Lawrence, H. S., and Pappenheimer, A. M., Jr., Transfer of delayed hypersensitivity to diphtheria toxin in man, *J. Exp. Med.*, 1956, **104**, 321.
6. Glenny, A. T., and Sudmersen, H. J., Notes on the production of immunity to diphtheria toxin, *J. Hyg.*, 1921, **20**, 176.
7. Hartley, P., The antigenic properties of precipitates produced by the interaction of diphtheria toxin and antitoxin, *Brit. J. Exp. Path.*, 1925, **6**, 112.
8. Mason, J. H., Robinson, M., and Christensen, P. A., Active immunization of

guinea pigs passively immunized with homologous antitoxic serum, *J. Hyg.*, 1955, **53**, 172.

9. Freund, J., The mode of action of immunologic adjuvants, *Advances Tuberc. Research*, 1956, **7**, 130.
10. Treffers, H. P., and Heidelberger, M., Quantitative experiments with antibodies to a specific precipitate, *J. Exp. Med.*, 1941, **73**, 125.
11. Adler, F. L., Antibody formation after injection of heterologous immune globulin, *J. Immunol.*, 1956, **76**, 217.
12. Chase, M. W., The cellular transfer of cutaneous sensitivity to tuberculin, *Proc. Soc. Exp. Biol., and Med.*, 1945, **59**, 134.
13. Metaxas, M. N., and Metaxas-Bühler, M., Frühreaktion und spätreaktion bei der Serum Allergie des Meerschweinchens und ihre Trennung durch passive Übertragung, *Z. allg. Path. u. Bakt.*, 1954, **17**, 128.
14. Tremaine, M. M. and Jeter, W. S., Passive cellular transfer of hypersensitivity to serum antigen in rabbits, *J. Immunol.*, 1955, **74**, 96.
15. Wager, O. A., and Chase, M., Appearance of diphtheria antitoxin following transfer of cells taken from immunized rabbits, *Fed. Proc.*, 1952, **11**, 485.
16. Harris, S., Harris, T. N., and Farber, M. B., Studies on the transfer of lymph node cells 1. Appearance of antibody in recipients from donor rabbits injected with antigen, *J. Immunol.* 1954, **72**, 148.
17. Stavitsky, A. B., *In vitro* production of diphtheria antitoxin I. Procedure and evidence for general nature of phenomenon, *J. Immunol.*, 1955, **75**, 214.
18. Benacerraf, B., and Kabat, E. A., A quantitative study of the Arthus phenomenon induced passively in the guinea pig, *J. Immunol.*, 1950, **64**, 1.
19. Copeman, S. M., O'Brien, R. A., Eagleton, A. J., and Glenny, A. T., Experiences with the Schick test and active immunization against diphtheria, *Brit. J. Exp. Path.*, 1922, **3**, 42.
20. Zingher, A., and Park, W. H., Immunity results obtained in school children with diphtheria toxoid and with 0.1 L+ mixtures of toxin-antitoxin, *Proc. Soc. Exp. Biol. and Med.*, 1923–24, **21**, 383.
21. Jones, T. D., and Mote, J. R., The phases of foreign protein sensitization in human beings, *New England J. Med.*, 1934, **210**, 120.
22. Simon, F. A., and Rackemann, F. M., The development of hypersensitiveness in man I. Following intradermal injection of an antigen, *J. Allergy*, 1934, **5**, 439.
23. Landsteiner, K., and Chase, M. W., Studies on the sensitization of animals with simple chemical compounds VII. Skin sensitization by intraperitoneal injections, *J. Exp. Med.*, 1940, **71**, 237.
24. Eisen, H. N., Orris, L., and Belman, S , Elicitation of delayed allergic skin reactions with haptens: The dependence of elicitation on hapten combination with protein, *J. Exp. Med.*, 1952, **95**, 473.
25. Porter, H. M., The demonstration of delayed-type hypersensitivity in congenital agammaglobulinemia, 2nd Tissue Homotransplantation Conf., *Ann. New York Acad. Sc.*, in press.
26. Good, R. W., Homotransplantation studies in patients with agammaglobulinemia, *Ann. New York Acad. Sc.*, in press.