OCCURRENCE OF DELAYED HYPERSENSITIVITY DURING THE DEVELOPMENT OF ARTHUS TYPE HYPERSENSITIVITY

By S. B. SALVIN, Ph.D.

With the Technical Assistance of Jane Nishio

(From the United States Department of Health, Education, and Welfare, Public Health Service, National Institutes of Health, National Institute of Allergy and Infectious Diseases, Rocky Mountain Laboratory, Hamilton, Montana)

(Received for publication, July 30, 1957)

Small amounts of diphtheria toxoid or of ovalbumin in the form of complexes with excess homologous antibody are capable of inducing delayed hypersensitivity in guinea pigs (1). Sensitivity became manifest about the 4th day after a primary sensitizing injection of toxoid-antitoxin complex, and persisted for at least 6 weeks in the absence of detectable antibody. This sensitivity could be transferred to normal guinea pigs by introduction of lymph node cells.

In studies on the mechanism by which antigen-antibody complexes induce tuberculin type sensitivity, guinea pigs were injected intradermally with small amounts of soluble antigen. The present paper describes experiments which show that such administration of soluble antigen does produce a characteristic delayed hypersensitivity over a period of time which varies with the quantity and nature of the sensitizing antigen. This delayed hypersensitivity is usually followed by the Arthus type of sensitivity and the concomitant appearance of circulating antibody.

Materials and Methods

Animals.—Guinea pigs were white or albino and weighed 400 to 500 gm.

Antigen.—Purified diphtheria toxoid (KP-50) was obtained through the courtesy of Dr. James A. McComb, Biologic Laboratories, Massachusetts Department of Health. Crude concentrated toxoid (1200 Lf/ml.) was obtained through the courtesy of Dr. George Brigham of Parke, Davis & Company, Detroit, and toxin from Eli Lilly and Company, Indianapolis. Thrice recrystallized ovalbumin was also used for sensitization of guinea pigs. The purified crystals were dissolved in saline and filtered. The concentration of protein was ascertained by determination of the specific absorption in 0.25 N acetic acid at 277 mμ in a Beckman spectrophotometer (2). To reduce surface denaturation, antigens for skin testing were diluted in physiologic saline containing 1 per cent normal guinea pig serum.

Antiserum.—Rabbit antitoxin was whole serum from rabbits that had been injected in the footpads with 2 doses, a week apart, of 1 Lf purified toxoid in Freund adjuvant without mycobacteria (Difco, incomplete adjuvant). The rabbits were bled 3 to 4 weeks after the 2nd injection, and serum obtained after centrifugation of the clot was stored at −10°C.

Technique of Sensitization.—The antigen was dissolved in physiologic saline containing 1 per cent guinea pig serum and emulsified in a syringe with an equal volume of Freund

adjuvant, without mycobacteria. Except when specified, guinea pigs were sensitized with 0.5 ml. of this oil-water emulsion, distributed among the digits of the feet.

Skin Tests.—Guinea pigs were tested intradermally with 0.1 ml. of serial 10-fold dilutions of either purified toxoid (10 Lf/ml.) or ovalbumin (30 μg./ml.). Reactions were observed at intervals for the first 6 to 8 hours after challenge, at 18 to 24 hours, and usually at 48 hours. The diameters of the areas of induration were measured with a millimeter ruler.

Passive Transfer of Sensitivity.—Popliteal, inguinal, and axillary lymph nodes from 4 to 10 sensitized guinea pigs were excised and suspended in cold Tyrode's solution. The nodes were minced, squeezed through a small garlic press, and the resulting cell suspensions washed by centrifugation 2 to 3 times in 10 to 12 ml. cold Tyrode's solution. The cells were then resuspended, counted with the aid of a Levy hemocytometer, and injected intraperitoneally into normal guinea pigs. At an appropriate time thereafter, usually 48 hours, the recipient guinea pigs were tested intradermally with 3 μg. of homologous antigen.

RESULTS

Response of Guinea Pigs to 1 Lf (3 μg.) Diphtheria Toxoid.—

Each of 98 guinea pigs in a total of 5 experiments was injected in the footpads with 1 Lf purified diphtheria toxoid in an oil emulsion. Subsequent to the 2nd day after inoculation, individual guinea pigs were skin-tested with 1.0, 0.1, and 0.01 Lf purified toxoid and Schick toxin. Each guinea pig was tested intradermally only once, and carefully examined for the presence of Arthus and delayed types of hypersensitivity.

None of 6 animals tested on the 3rd day after intradermal injection showed evidence of hypersensitivity, either of the delayed or Arthus type (Table I). On the 4th day, however, of 9 animals challenged intradermally with 1 Lf toxoid, 3 showed significant responses. These reactions were inapparent during the first 4 hours after testing, but became manifest shortly thereafter and reached a peak diameter in about 24 hours. These reactions, therefore, resembled the delayed type rather than the Arthus type of hypersensitivity in their time of appearance. On the 5th, 6th, and 7th days, 17 of 26 guinea pigs had reactions at 24 hours with a diameter of induration 10 mm. or more, while 6 others had reactions 5 to 9 mm. in diameter. Three animals did not show inflammatory response to intradermal testing. All these reactions were of the delayed type in that they were not apparent at 4 hours after intradermal challenge, but developed rapidly thereafter to a maximum at about 24 hours.

None of 8 animals tested on the 8th day after intradermal injection of toxoid showed any sign of Arthus type sensitivity. All reactions were of the delayed type in their time of onset, although they were smaller than those occurring in animals tested on the 3 previous days. That the animals were in a period of transition was emphasized on the following day (the 9th) when 1 of 4 animals developed a marked Arthus type response to 1 Lf toxoid. A second animal showed some erythema and edema at 2 to 4 hours, but antibody was not detected by the rabbit intracutaneous test. This guinea pig was, therefore, classified as one with delayed sensitivity (7 × 8 mm. at 24 hours). During the 10th and 11th day, the delayed reactions were small, while 2 animals developed Arthus

type responses. On the 12th and 13th day, almost all reactions to 1 Lf toxoid were of the combined type. Thereafter, delayed responses did not occur without having been preceded by the Arthus type of hypersensitivity.

In summary, after intradermal injection of 1 Lf diptheria toxoid in adjuvant, a latent period follows during which the guinea pig fails to show an overt response to an intradermal challenge of 1 Lf toxoid. From the 5th to the 8th day, only a delayed type reaction is manifest. About the 9th day, an Arthus type of response begins to be superimposed on the delayed reaction and remains pre-

TABLE I

Development of Delayed and Arthus Types of Hypersensitivity after Intradermal Injection of 1 Lf Diptheria Toxoid in Freund Adjuvant without Mycobacteria

Skin reactions to 1 Lf (3 μg.) toxoid in 0.1 ml. volume												
Time in days (after intradermal sensitization)												
3	4	5	6	7	8	9	10	11	12	13	15	16–21
mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
0	12 × 10	16 × 17	16 × 20	20 × 15	10 × 10	22 × 22	10 × 10	*19 × 19*	5 × 5	10 × 12	22 × 19	*30 × 35*
0	8 × 8	11 × 13	19 × 19	16 × 18	9 × 9	*12 × 12*	8 × 9	*15 × 14*	0	8 × 9	20 × 20	*24 × 22*
0	6 × 6	10 × 12	18 × 19	12 × 12	5 × 5	7 × 8	4 × 5	8 × 10	20 × 20	*21 × 21*	*19 × 18*	*22 × 22*
0	4 × 4	11 × 11	16 × 14	10 × 10	4 × 4	0	4 × 4	8 × 9	*20 × 20*	*19 × 20*	*18 × 18*	*17 × 15*
0	4 × 4	10 × 10	15 × 14	8 × 8	2 × 2		0	8 × 8	*18 × 18*	*18 × 17*	*12 × 13*	*15 × 13*
0	4 × 4	9 × 10	12 × 12	7 × 7	2 × 2		0	3 × 4	*18 × 17*	*4 × 4*		*12 × 10*
	2 × 2	7 × 7	12 × 12		0			0	*16 × 16*			*12 × 10*
	0	0	10 × 8		0				*15 × 14*			*12 × 12*
	0		9 × 9									*12 × 12*
			8 × 8									*10 × 10*
			4 × 4									*8 × 8*
			0									*6 × 6*
												5 × 5

Italicized figures indicate Arthus type reaction, read at 4 hours. Others negative at 4 hours, and indicate delayed type hypersensitivity.

dominant. Since there is marked decrease in the diameters of the delayed responses on the 7th and 8th days, this response may be suppressed somewhat before the Arthus type of sensitivity becomes dominant.

Responses to 3 μg. Ovalbumin.—A similar series of responses also develops in guinea pigs inoculated in the footpads with 3 μg. ovalbumin in adjuvant (Table II). Twenty-four of 25 animals challenged intradermally with 3 μg. of the homologous antigen on the 2nd, 3rd, or 4th day after injection did not show either delayed or Arthus type sensitivity. The one exception was a mild 10 × 11 mm. reaction on the 4th day. On the 5th day, however, striking reactions of the delayed type developed to intradermal challenge and prevailed in the guinea pigs until the 8th day postinoculation. From then on, the Arthus type of sensitivity predominated.

In the guinea pig, ovalbumin seems to behave as a stronger antigen than diphtheria toxoid. The latent period is the same as with an equivalent dose of

toxoid, but the delayed type of reaction persists for a shorter period. In addition, the amount of induration developing after intradermal challenge of 3 μg. ovalbumin is greater than that developing after a similar challenge of toxoid.

Characteristics of the Delayed Type of Hypersensitivity That Follows Injection of Soluble Protein Antigen.—The delayed or tuberculin type of hypersensitivity

TABLE II

Development of Delayed and Arthus Types of Hypersensitivity after Intradermal Injection of 3 μg. Ovalbumin in Freund Adjuvant without Mycobacteria

Skin reactions to 3 μg. ovalbumin						
Time in days after ID sensitization						
3	4	5	6	7	8	9
	mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
0	10 × 11	26 × 23	23 × 22	23 × 20	22 × 14	*30 × 30*
0	0	22 × 22	22 × 20	18 × 20	*16 × 16*	*25 × 20*
0	0	20 × 19	22 × 18	17 × 20	*14 × 13*	*20 × 20*
0	0	20 × 19	24 × 18	20 × 19	*7 × 8*	
0	0	20 × 18	20 × 20	20 × 18	*4 × 4*‡	
0	0	18 × 18	20 × 18	20 × 16?		
0	0	18 × 15	20 × 17	18 × 18		
0	0	16 × 17	18 × 18	12 × 11		
	0	16 × 16	18 × 17	11 × 11*		
	0	16 × 16	17 × 17	*20 × 25*		
	0	16 × 16	15 × 15	*10 × 10*		
	0	11 × 12	12 × 11			
		9 × 10				

Italicized figures indicate Arthus type reaction read at 4 hours. Others negative at 4 hours.

?, This animal had an area of erythema (no edema) at 4 hours of 6 × 8 mm., but showed no signs of anaphylactic shock when injected with 1 mg. ovalbumin. None of the animals that had delayed reactions showed signs of anaphylactic shock. Italicized figures indicate Arthus type reaction read at 4 hours. These animals died of anaphylactic shock after intravenous injection of 1 mg. homologous antigen. Others negative at 4 hours.

* Had a 5 × 5 mm. edematous reaction at 3 to 4 hours, but did not show signs of anaphylaxis after intravenous injection.

‡ At 4 hours this animal had an area of edema 4 × 4 mm., but died of anaphylactic shock after intravenous injection of 1 mg. ovalbumin.

is characterized not only by the delayed development of the inflammatory response after intradermal introduction of the specific antigen, but by other important properties. The hypersensitive state can be transferred to normal animals by leucocytes from sensitive donors but not by serum (3–5). The hypersensitive state can develop in the absence of detectable circulating antibody, which, in the diphtheria toxoid-antitoxin system, is less than 0.0025 μg. antitoxin N/ml.

All guinea pigs in the foregoing experiments were bled either just before the skin test or just after the 24 hour reading of the test.

Sera from guinea pigs injected with toxoid were assayed for antibody by means of the rabbit intracutaneous test (6), which test can detect, as stated above, 0.001 units antitoxin per ml., equivalent to 0.0025 μg. antitoxin N/ml. Animals that had been sensitized with ovalbumin were examined for circulating antibody by the intravenous injection of a shocking dose (1 mg.) of specific antigen. This test is believed capable of detecting 0.01 mg. antibody N/ml. (7).

None of the sera obtained from animals within 8 days after injection of the sensitizing dose of 1 Lf toxoid contained as much as 0.001 units of antitoxin. These sera were from injected animals that failed to respond to skin testing or showed a response delayed "in time."

Similarly, the animals that had been originally injected in the footpads with 3 μg. ovalbumin and failed to respond or showed a delayed response after skin testing did not develop anaphylaxis following intravenous challenge. All guinea pigs that produced an Arthus reaction on the 8th and 9th day after administration of the sensitizing dose succumbed to anaphylactic shock. Three guinea pigs did not strictly follow this pattern (Table II): two animals with questionable Arthus reactions did not show anaphylactic response; one animal with only slight erythema at 4 hours died of anaphylaxis.

Cell transfer studies also were performed on the guinea pigs inoculated in the footpads with purified toxoid.

These animals were used as donors 1 day after skin test. The washed cells from the inguinal, popliteal, and axillary lymph nodes of groups of 4 or 5 guinea pigs were transferred intraperitoneally to a normal recipient and the latter tested intradermally 48 hours after the cell transfer (Table III).

Of the 9 guinea pigs tested intracutaneously 48 hours after having received lymph node cells from positive donors, 6 showed positive reactions (areas of induration 10 mm. or more in diameter), 2 had reactions 5 to 8 mm. in diameter and 1 failed to react.

The 3 recipients of lymph node cells from 15 guinea pigs sensitized with 3 μg. egg albumin were more reactive than the recipients of toxoid-sensitive cells (Table III). The 3 animals, each of which received 330×10^6 cells intraperitoneally, showed delayed reactions about 15 mm. in diameter to 3 μg. homologous antigen injected intradermally 48 hours after introduction of lymph node cells.

Sera drawn from 6 toxoid-sensitive recipients the day after skin testing had less than 0.001 units of antibody (0.0025 μg. antibody N) per ml. Since lymph node cells from immunized animals continue to produce antibody after transfer to a normal animal, recipient guinea pigs possibly may develop a delayed sensitivity because of the introduction of antibody-forming cells and the ultimate

formation of enough antibody to produce delayed type of hypersensitivity. Also, if delayed sensitivity is primarily a result of antibody production by the transplanted cells, then the longer the cells remain in the recipient's body, the greater should be the potentiality to produce the delayed reaction. But Chase (4) and Metaxas and Metaxas-Buehler (8) agree that the ability to respond to the specific antigen reaches its peak in 24 to 48 hours after the intraperitoneal injection of the sensitive cells and then wanes rapidly. This latter decline in reactivity would not occur if antibody production alone is directly associated with the development of the delayed type of hypersensitivity.

TABLE III

Cell Transfer of Delayed Sensitivity

Lymph node cells collected 1 day after skin test

No. of donors	Sensitivity of donors, average diameter, to 3 μg. antigen	Time of lymph node removal after ID sensitization	No. of cells transferred × 10^6	Recipient's skin reaction to 3 μg. antigen	Sensitizing antigen
	mm.	*days*		*mm.*	
4	0	3	130	0	Toxoid 1 Lf
4	5	4	230	8 × 10	"
4	10	5	390	5 × 5	"
4	13	6	350	10 × 10	"
4	3	8	550	10 × 10	"
4	9	9	370	12 × 13	"
4	?	10	250	8 × 8	"
4	11	4	380	10 × 10	" 25 Lf
4	?	6	270	15 × 14	" 25 Lf
5	17	5	330	15 × 16	EA* 3 μg.
5	18.5	6	330	12 × 15	"
5	18	6	330	15 × 15	"

* EA refers to ovalbumin.

Sensitivity Responses after Injection of Higher Doses of Antigen.—If sensitivity is a stage in antibody formation, the amount of antigen administered may affect the rate of antibody production and as a result the nature of the sensitivity response.

Accordingly, 25 Lf (75 μg.) toxoid in adjuvant were injected into the footpads of each of 25 guinea pigs and the animals tested for sensitivity at irregular intervals thereafter (Table IV).

None of the 4 animals tested on the 1st day after the sensitizing dose showed any reaction. However, of 3 animals tested on the 2nd day with an intradermal challenge of 1 Lf, all showed delayed reactions of 6 to 8 mm. Similar delayed responses to 1 Lf toxoid, with induration up to 15 mm. in diameter,

occurred during the 3rd and 4th day. By the 6th day, the animals were beginning to show circulating antibodies and the associated Arthus reaction.

Thus, increasing the sensitizing dose from 1 to 25 Lf reduces the latent period from 3 days to 1 day. Also, the time during which the animals show a

TABLE IV

Development of Delayed and Arthus Types of Hypersensitivity after Intradermal Injection of 25 Lf Diphtheria Toxoid in Freund Adjuvant without Mycobacteria

Skin reactions to 1 Lf (3 μg.) toxoid in 0.1 ml.							
Time in days (after intradermal injection)							
1	2	3	4	6	7	8	11
	mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
0	8 × 8	3 × 3	9 × 9	*12 × 15*	*7 × 7*	*8 × 8*	*12 × 12*
0	6 × 6	5 × 6	12 × 12	9 × 9	10 × 10		*22 × 22*
0	6 × 6	6 × 6	9 × 9	*10 × 10*	8 × 9		
0		6 × 7	14 × 15	9 × 9			

Italicized figures indicate Arthus type reaction read at 4 hours. Others negative at 4 hours.

TABLE V

Development of the Delayed and Arthus Types of Hypersensitivity after Intradermal Injection of 300 Lf Diphtheria Toxoid in Freund Adjuvant without Mycobacteria

Skin reactions to 1 Lf (3 μg.) toxoid in 0.1 ml.							
Time in days (after intradermal injection)							
2	3	4	6	7	8	9	13
mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
3 × 3	5 × 5	10 × 12	6 × 6	13 × 15	*8 × 6*	*25 × 28*	*13 × 14*
9 × 9	7 × 8	10 × 10	10 × 10	15 × 13	*12 × 10*	*11 × 10*	*14 × 14*
8 × 8	7 × 7	8 × 8	8 × 8	17 × 15	*16 × 18*	*23 × 23*	*16 × 16*
8 × 7	6 × 6	8 × 8	*10 × 9*	8 × 8	*20 × 20*	*18 × 18*	
9 × 9							

Italicized figures indicate Arthus type reaction read at 4 hours. Others negative at 4 hours.

delayed response does not persist from the 4th to the 9th or 10th day, as with a sensitizing dose of 1 Lf, but from the 2nd to the 5th or 7th day. At this latter point, circulating antibody is detected, which markedly alters the nature of the sensitivity reaction.

When a sensitizing dose of 300 Lf was injected into each of 32 guinea pigs, the response again was different from that following a sensitizing dose of 1 Lf (Table V). Four of 5 animals tested intradermally with 1 Lf toxoid 2 days

after injection of the sensitizing dose developed weakly positive delayed reactions of 8 to 9 mm. The delayed reaction persisted until the 5th to 7th day, with areas of induration attaining diameters up to 17 mm. By the 8th day, all animals had circulating antibodies and developed combined reactions to an intradermal challenge of 1 Lf toxoid. Again, hastening of antibody formation is noted, when compared to the rate of development of sensitivity and antibody in animals sensitized with 1 Lf toxoid.

Sensitivity Responses after Injection of Lower Doses of Antigen.—If a larger dose of antigen hastens development of delayed sensitivity and the subsequent appearance of circulating antibody, then conversely a smaller amount of sensitizing antigen may delay the process.

TABLE VI

Development of Delayed and Arthus Types of Hypersensitivity after Intradermal Injection of 0.01 Lf Diphtheria Toxoid in Freund Adjuvant without Mycobacteria

Skin reactions to 1 Lf (3 μg.) toxoid in 0.1 ml.									
Time in days (after intradermal injection)									
3	4	6	8	10	11	13	17	20	25
mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
0	0	5 × 5	9 × 9	0	4 × 4	12 × 12	0	16 × 17	10 × 12
6 × 6	0	5 × 5	7 × 8	0	8 × 8	8 × 8	5 × 5	11 × 12	8 × 8
0	5 × 5	4 × 6	0	0	0	8 × 8	8 × 8	13 × 13	8 × 8
	0	6 × 5		8 × 8	6 × 7	0	7 × 7	13 × 12	7 × 8

All reactions were of the delayed type. No Arthus type reactions were observed.

A dose of 0.01 Lf toxoid was inoculated into each of 38 guinea pigs which then were tested for sensitivity at irregular intervals (Table VI).

By the 6th day after sensitization, weak delayed responses to 1 Lf toxoid (5 to 6 mm. in diameter) were generally observed. These delayed reactions persisted in the absence of detectable circulating antibody through the 25th day, at which time the experiment was concluded because all the guinea pigs had been tested. None of 38 animals bled the day after intradermal challenge with 1 Lf toxoid had detectable circulating antitoxin, *i.e.*, <0.001 units (0.0025 μg. antibody N/ml.), by the rabbit intracutaneous test (Fig. 1). Thus, reduction of the amount of antigen may lengthen the latent period, as well as the duration of delayed sensitivity.

When guinea pigs were sensitized with smaller quantities of ovalbumin, the time also was extended during which the delayed response prevailed.

Forty-four guinea pigs were injected in the footpads with 0.03 μg. ovalbumin in Freund adjuvant without mycobacteria, and individual guinea pigs were tested at intervals for sensitivity (Table VII, Fig. 1).

From the 5th to 7th day these animals developed extensive delayed reactions to intracutaneous challenge with no signs of an Arthus response or circulating antibody (as detected by intravenous injection of 1 mg. ovalbumin). From about the 8th to the 11th day, the delayed reaction was either minimal or absent, and the Arthus type gradually appeared. The absence of sensitivity of both types in most animals during this period is noteworthy. From about the 12th day on, Arthus type sensitivity prevailed.

Influence of the Inclusion of Mycobacteria in the Sensitizing Inoculum.— Mycobacteria have the property of enhancing the delayed response of an ani-

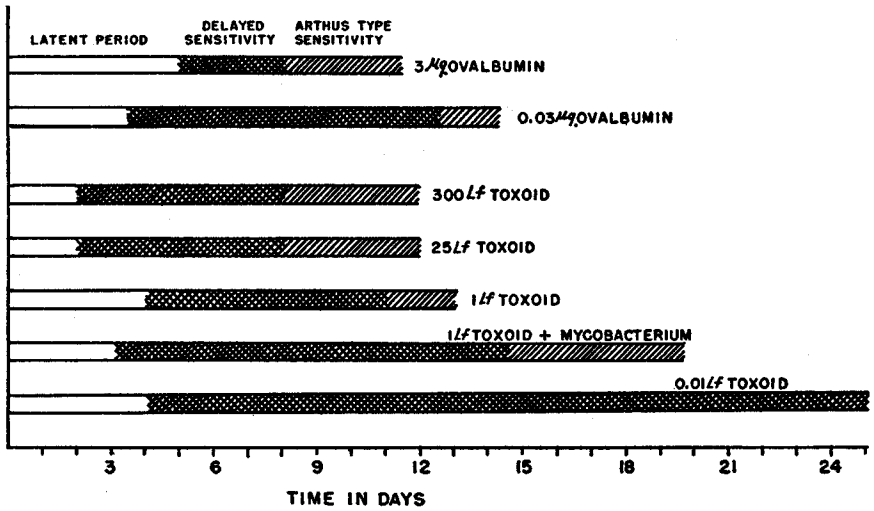

FIG. 1. Development of delayed and Arthus type hypersensitivities with different sensitizing doses of diptheria toxoid or egg albumin.

mal. When 2 mg. of dried mycobacteria (Jamaica strain of *Mycobacterium tuberculosis*) were included in the inoculum of 1 Lf toxoid per guinea pig, delayed hypersensitivity, with responses 10 to 15 mm. in diameter, was discernible in 5 of 7 animals on the 3rd day postinjection. From the 4th to 13th day, striking reactions of the delayed type were elicited to 1 Lf toxoid. During these days, all 26 guinea pigs showed highly indurated responses, mostly from 20 to 40 mm. in diameter, without any evidence of an Arthus reaction. From the 13th to 18th day, Arthus type sensitivity developed. The appearance of the Arthus type response was therefore markedly delayed in comparison to its time of appearance in animals sensitized with 1 Lf toxoid without mycobacteria.

Thus, the inclusion of mycobacteria in the inoculum causes in guinea pigs an early development of the delayed type of allergy. Also, the resulting sensitivity is more extensive and persists longer than it does in animals sensitized with inocula free of mycobacteria.

The Latent Period.—A period exists during which neither delayed sensitivity nor circulating antibody can be demonstrated. This period has varied from about 2 to 6 days, depending on the size and nature of the sensitizing dose. Exactly what is happening in the lymph nodes during this interval is not known.

Lymph nodes were removed from 4 guinea pigs on each of the 4 days following the administration of 1 Lf toxoid. The nodes were teased apart, ground in sterile sand, and suspended in a volume of Tyrode's solution equal to the weight of the nodes. When tested for antitoxin by the rabbit intracutaneous test, such node suspensions did not contain any trace of circulating antibody.

TABLE VII

Development of Delayed and Arthus Types of Hypersensitivity after Intradermal Injection of 0.03 μg. Ovalbumin in Freund Adjuvant without Mycobacteria

Skin reaction to 3 μg. ovalbumin											
Time in days after sensitizing injection											
4	5	6	7	8	9	10	11	12	13	14	16
mm.	*mm.*	*mm.*	*mm.*			*mm.*	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
12 × 11	20 × 23	20 × 20	21 × 21	0	0	4 × 4	14 × 15	*30 × 32*	*18 × 22*	4 × 5	*23 × 19*
6 × 8	11 × 14	0	20 × 18	0	0	9 × 11	2 × 2	*33 × 27*	*17 × 24*	0	*22 × 29*
0	6 × 7	17 × 19	20 × 18	0	0		0	*27 × 25*	*5 × 7*	4 × 5	
	0	17 × 20	12 × 13				0	*4 × 5*			
			12 × 13				0				
			12 × 12				0				
			10 × 8								

Italicized figures indicate Arthus type reaction read at 4 hours. Others negative at 4 hours.

Thus, it can be assumed that less than 0.002 units circulating antitoxin existed in nodes during the latent period.

Nevertheless, when these nodes from animals that had been inoculated with antigen but which had not yet formed circulating antibody in detectable amounts were injected into normal animals, most recipient guinea pigs developed a delayed sensitivity. The donor-recipient ratio, however, had to be high. Lymph node cells from as many as 10 to 20 donors were needed to produce passive sensitization in a single recipient, and even then some recipients developed little or no response to intradermal challenge. Some recipients of lymph-node cells from guinea pigs sacrificed on the 2nd to 4th day after sensitization to toxoid developed induration as large as 12 x 12 mm. in diameter on intradermal injection of 1 Lf toxoid (Table VIII). One recipient of cells from 20 guinea pigs sensitized to ovalbumin had a reaction as large as 24 x 26 mm. in diameter. It is to be emphasized that the donors, at this interval after the administration of the sensitizing dose, do not show positive reactions to intradermal injection of the specific antigen.

The Effect of Toxoid-Antitoxin Precipitate on the Arthus Phenomenon or

Antibody Production.—Previous work (1) had shown that injection of minute amounts of washed precipitates, formed in the region of antibody excess, produced delayed sensitivity in guinea pigs, but failed to evoke Arthus reactions or circulating antibody (<0.0025 μg. antitoxin N/ml.). The theory has therefore been advanced that if delayed sensitivity is a step in the development of the Arthus type, then injection of the precipitate plus the homologous antigen should produce an earlier or stronger Arthus reaction than injection of an equivalent amount of antigen alone.

TABLE VIII

Cell Transfer of Delayed Sensitivity during First 4 Days after Sensitization

No. of donors	No. of lymph node cells injected ($\times 10^6$)	Recipient's reaction	Time after sensitization when nodes removed	Time between receipt of cells and challenge	Antigen
			days	*days*	
8	110	10 × 11	2	2	Toxoid
8	190	8 × 8	2	3	"
8	110	8 × 8	2	5	"
8	110	9 × 10	2	5	"
8	240	10 × 12	3	3	"
10	410	9 × 10	4	2	"
10	410	10 × 11	4	2	"
9	440	9 × 10	4	2	"
10	610	12 × 12	4	3	"
4	330	13 × 13	6	2	"
4	330	13 × 12	6	2	"
4	330	20 × 19	6	3	"
4	330	18 × 20	6	3	"
20	450	9 × 10	4	3	EA
20	510	24 × 26	4	2	EA

EA refers to ovalbumin.

This hypothesis was tested by 2 types of experiments.

In one type, one set of animals was first injected with 1 Lf toxoid-antitoxin precipitate and 3 or 4 days later with 1 Lf toxoid. Their reactions were compared with others injected with one dose of 2 Lf toxoid at the time the experimental group received toxoid.

On the 2nd and 3rd day after injection of the soluble antigen, neither group showed Arthus reactions, although recipients of the combination of precipitate and toxoid showed striking, delayed reactions (up to 38 mm. in diameter) which were greater than those in the animals receiving precipitate alone (Table IX). On the 4th day, recipients of precipitate toxoid showed pronounced Arthus reactions (up to 15 mm. in diameter), and very large delayed reactions (up to 40 mm. in diameter). Animals that had received 2 Lf toxoid alone showed only

weak to moderate delayed reactions (up to 10 mm. in diameter) while those that received precipitate alone developed delayed reactions about 22 mm. in diameter. On the 5th day, results were similar. By the 7th and 8th days, recipients of toxoid alone began to show Arthus reactions as well. Guinea pigs

TABLE IX

The Effect of Toxoid-Antitoxin Precipitate (in Antibody Excess) on the Arthus Type Reaction

Time of intradermal challenge after sensitization	Diameter					
	Precipitate (1 Lf) + toxoid (1 Lf)		Toxoid (2 Lf)		Precipitate alone (1 Lf)	
days	at 4 hrs.	at 24 hrs.	at 4 hrs.	at 24 hrs.	at 4 hrs.	at 24 hrs.
	mm.	*mm.*	*mm.*	*mm.*	*mm.*	*mm.*
2	0	20 × 25	0	0		
	0	22 × 22	0	0		
	0	20 × 20	0	0		
3	0	32 × 35	0	0		
	0	30 × 35	0	8 × 8		
	0	33 × 38	0	9 × 9		
4	9 × 9	38 × 40	0	0		
	10 × 15	33 × 33	0	7 × 7		
	12 × 15	33 × 35	0	10 × 10	0	18 × 18
5	0	30 × 30	0	20 × 13		
	8 × 8	22 × 25	0	20 × 26		
	15 × 17	22 × 22	0	8 × 13	0	23 × 25
6	15 × 17	24 × 24	0	21 × 21		
	20 × 23	22 × 20	0	24 × 30		
	20 × 20	24 × 24	7 × 8	15 × 14	0	16 × 16
7	17 × 18	20 × 23	0	23 × 24		
	17 × 20	26 × 30	7 × 8	20 × 23		
	19 × 20	23 × 25	8 × 8	20 × 24	0	20 × 20
8	17 × 20	28 × 32	4 × 8	18 × 20		
	13 × 15	27 × 28	12 × 12	20 × 22	0	18 × 22

that received precipitate alone continued to show only the delayed-type reaction (1).

In the second type of experiment, one set of animals was injected with precipitate and 3 days later with antigen. At the same times, a second set was given similar quantities of antigen alone and a third group received similar amounts of precipitate alone. The results were essentially the same as those above.

Thus, injection of precipitate plus an antigen and the subsequent enhancement of delayed sensitivity was followed by an earlier and stronger Arthus reaction than appeared in animals injected with antigen alone.

DISCUSSION

It is generally recognized that the parenteral introduction of a soluble foreign protein into a guinea pig is followed by development of hypersensitivity of the early inflammatory or Arthus type.

Dienes (9), however, working with tuberculous guinea pigs, observed that crystalline egg albumin produced an Arthus reaction, whereas under similar experimental conditions, egg globulin produced a delayed or tuberculin type reaction. The animals received moderately large doses of antigens, namely, single or multiple doses up to a total of about 0.3 to 2.0 mg. of antigen. However, normal pigs were not used, and the time of development of sensitization with antigen was not followed. Later, Jones and Mote (10) noted that human patients, on repeated intradermal testing with rabbit peritoneal fluid, showed changes in the type of response. When first tested, few patients reacted, but after several doses many of the same patients showed a 24 hour tuberculin type reaction. Still later, after continuation of the intradermal injections, immediate wheal and flare reactions began to appear. Simon and Rackemann (11) obtained similar results in man with repeated intracutaneous injections of guinea pig serum.

The Arthus and delayed types of local reactions can thus be different phases of the same immune process. The delayed type of response represents an earlier stage; the Arthus type, together with the formation of antibodies, a later stage in the development of the sensitization process. The rate at which the three main phases (*i.e.* latent period, delayed phase, and Arthus phase) progress depends upon the particular antigen being studied, as well as its dose level. An increase in the amount of antigen increases the rate of transition from one phase to the next, possibly because of an increased stimulation of the sensitivity-inducing cells. At comparable doses, egg albumin also tends to produce a shorter period of delayed sensitivity than diphtheria toxoid and the Arthus type of sensitivity develops earlier after sensitization.

The delayed sensitivity that precedes the Arthus type has reactions that are considered characteristic. Thus, antibody cannot be detected in serum of highly sensitive guinea pigs by the rabbit intracutaneous test, which is capable of indicating antibody in quantities as low as 0.0025 μg. antitoxin N/ml. Also, injection of this serum into recipient guinea pigs did not induce sensitivity of any type (12). Finally, cells from the lymph nodes of guinea pigs with the delayed type of sensitivity transfer the sensitivity to normal recipients.

Animals were sensitized *via* the intradermal route in the presence of Freund adjuvant (without mycobacteria). The importance of this route has been further emphasized by studies on sensitization to simple chemicals (13, 14), wherein topical application in oil of such substances as picryl chloride induces a delayed

type of allergy. Also, when antigen-antibody complex is injected *via* the intradermal route, greater delayed sensitivity is induced than when it is injected *via* the intraperitoneal, subcutaneous, or intramuscular route (1). The oil-water emulsion, used as a vehicle for the antigen, apparently acts to increase the local inflammatory response (15).

Of great interest is the action of dried mycobacteria when included in the inoculum with 1 Lf toxoid in oil-water emulsion. Guinea pigs develop the delayed type of allergy earlier than animals similarly sensitized but without mycobacteria in the inoculum. The delayed type reaction produces a more severe inflammatory response. This enhancement of the delayed response can be due to an actual stimulation of the cellular processes involved. However, inhibition of a competitive simultaneous process, such as the Arthus type of allergy, can be equally responsible. That this inhibition actually may occur is indicated by the observation that guinea pigs sensitized with toxoid and mycobacteria do not develop the Arthus response as early as animals sensitized with toxoid without mycobacteria. Formation of detectable amounts of circulating antibody is thereby prolonged in the absence of mycobacteria.

The extent of this three phase process of sensitization after the injection of a soluble antigen cannot be estimated from data presented in this paper. The assumption is made that each protein is homogeneous to the extent that it does not consist of two components, one of which causes delayed sensitivity and the other the Arthus type. The two protein antigens studied induced the three phase sensitization. Injection of guinea pigs with either 3 μg. or 0.3 μg. purified Type I pneumococcal polysaccharide[1] failed to produce any indication of delayed sensitivity (12). It is possible, however, that the process of sensitization to soluble proteins in guinea pigs consists generally of three phases: a latent period, a period of delayed sensitivity, and a period of superimposed Arthus type sensitivity, with the latter ultimately accompanied by a detectable amount of circulating antibody. It is to be expected that each individual protein has its own characteristics, at comparable dose levels, with regard to the duration of these phases.

These experiments also emphasize the role delayed hypersensitivity may play in an actual infection. The amount of protein that has been injected into the skin of the guinea pig is comparatively small. The smaller this amount, the longer the tendency has been to extend the period of delayed allergy, and the more the appearance of circulating antibody has been postponed. If this period of sensitivity is sufficiently long and severe, then much damage can be done to the host animal before the protective antibody mechanism starts to function. Recently, studies involving transfer of lymph node cells from mice sensitized with *Histoplasma capsulatum* have indicated that delayed sensitivity by itself

[1] Obtained through the courtesy of Dr. C. M. MacLeod.

can be injurious to an animal (12). Thus, in slowly progressing chronic diseases such as tuberculosis and histoplasmosis, the antigen may stimulate the formation of enough antibody to produce delayed sensitivity but not enough to incite the protective mechanism to function.

SUMMARY

Guinea pigs were injected in the footpads with either purified diphtheria toxoid or recrystallized egg albumin in Freund adjuvant without mycobacteria. Each guinea pig was then skin-tested only once with the specific antigen and bled for antibody determination. After injection of the sensitizing antigen, a latent period occurred during which neither sensitivity nor circulating antibody could be detected. A period of delayed sensitivity followed wherein circulating antibody could not be discerned and which could be transferred by lymph node cells. Ultimately, the Arthus type sensitivity developed, accompanied by circulating antibody. The duration and severity of reactions to homologous antigens during the last 2 phases varied with the antigen and with the dose. An increase in the sensitizing dose decreased the duration of the delayed type of allergy, a decrease in the dose prolonged the delayed type. Inclusion of mycobacterium in the sensitizing inoculum tended to introduce delayed sensitivity earlier and delay the onset of Arthus type sensitivity. When specific precipitate in antibody excess was included with the toxoid in the sensitizing dose, the onset of the Arthus phase was hastened. When lymph nodes from a large number of sensitized donors were removed during the latter part of the latent period, recipients of the cells showed a delayed type sensitivity.

BIBLIOGRAPHY

1. Uhr, J. W., Salvin, S. B., and Pappenheimer, A. M., Delayed hypersensitivity. II. Induction of hypersensitivity in guinea pigs by means of antigen-antibody complexes, *J. Exp. Med.*, 1957, **105,** 11.
2. Gitlin, D., Use of ultraviolet absorption spectroscopy in the quantitative precipitin reaction, *J. Immunol.*, 1949, **62,** 437.
3. Landsteiner, K., and Chase, M. W., Experiments on transfers of cutaneous sensitivity to simple compounds, *Proc. Soc. Exp. Biol. and Med.*, 1942, **49,** 688.
4. Chase, M. W., The cellular transfer of cutaneous hypersensitivity to tuberculin, *Proc. Soc. Exp. Biol. and Med.*, 1945, **59,** 134.
5. Lawrence, H. S., The cellular transfer of cutaneous hypersensitivity to tuberculin in man, *Proc. Soc. Exp. Biol. and Med.*, 1949, **71,** 516.
6. Fraser, D. T., The technic of a method for quantitative determination of diphtheria antitoxin by a skin test in rabbits, *Tr. Roy. Soc. Canada*, Section V, 1931, **25,** 175.
7. Kabat, E. A., and Landow, H., A quantitative study of passive anaphylaxis in the guinea pig, *J. Immunol.*, 1942, **44,** 69.
8. Metaxas, M. N., and Metaxas-Buehler, M., Studies on the cellular transfer of tuberculin sensitivity in the guinea pig, *J. Immunol.*, 1955, **75,** 333.

9. Dienes, L., Comparative study of the anaphylactic and tuberculin types of hypersensitiveness. II. The influence exerted by the nature of the antigen on the development of the different types of hypersensitiveness, *J. Immunol.*, 1931, **20,** 333.
10. Jones, T. D., and Mote, J. R., The phases of foreign protein sensitization in human beings, *New England J. Med.*, 1934, **210,** 120.
11. Simon, F. A., and Rackemann, F. M., The development of hypersensitiveness in man following intradermal injection of antigen, *J. Allergy*, 1934, **5,** 439.
12. Salvin, S. B., unpublished results.
13. 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.
14. 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.
15. Freund, J., The mode of action of immunologic adjuvants. *Advances Tuberc. Research*, 1956, **7,** 130.