DELAYED HYPERSENSITIVITY

I. Induction of Hypersensitivity to Diphtheria Toxin in Guinea Pigs by Infection with Corynebacterium diphtheriae*

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Infections in animals and in man, caused by a wide variety of bacteria, commonly lead to a state of hypersensitivity in the recovered host, specifically directed against the infecting organism or its products. While the most extensively studied example of bacterial allergy is tuberculosis (1), many other infectious diseases, including those caused by viruses, fungi, protozoa, and other bacteria such as streptococci, *Brucella*, and *Corynebacterium diphtheria* induce the hypersensitive state. Small doses of specific antigens cause delayed inflammatory reactions when injected intradermally into sensitized animals. Larger doses may give rise to severe delayed systemic reactions which may terminate in fatal shock.

Only two methods have been available for experimental induction of the delayed hypersensitive state to protein antigens. The first method is the classical one of infection, usually with an attenuated organism such as BCG, in which the animal becomes sensitized to a number of the proteins and other components elaborated by the infecting organism. The second method for induction of the hypersensitive state consists in injecting an antigen, such as ovalbumin, in adjuvant containing either killed *Mycobacteria* (Dienes (2) and Freund and McDermott (3)) or certain lipides contained in the "hard wax" fraction of *Mycobacteria* (Raffel (4)). Although incorporation of an antigen into oil-water emulsion results in enhanced antibody formation, delayed hypersensitivity cannot usually be demonstrated unless *Mycobacteria* themselves or the mycobacterial wax fraction is added.

Neither of these methods has proved entirely satisfactory for experimental

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studies on the mechanisms underlying the hypersensitive state. The use of adjuvants results in a high circulating antibody titer against the antigen used. Consequently, skin reactions of the Arthus type are encountered which may be confused readily with reactions of the tuberculin type (Gell and Hinde (5)). The differentiation can only be definitely achieved by the tedious method of transfer of delayed sensitivity to normal hosts using washed leucocytes from sensitized donors (Metaxas and Metaxas-Bühler (6)). Thus Pound (7) in attempting to repeat the work of Raffel (4) on the effect of tubercle bacillus wax on induction, in guinea pigs, of delayed sensitivity to tuberculoprotein, obtained reactions which he concluded to be predominantly of the Arthus type. Pound apparently did not attempt to transfer sensitivity to normal animals by means of leucocyte suspensions from his sensitized guinea pigs.

A few years ago it was observed in classroom experiments that guinea pigs, infected with toxigenic diphtheria bacilli and protected with horse antitoxin a few hours later according to the routine test for toxigenicity, showed delayed inflammatory skin reactions of the tuberculin type when challenged with small amounts of purified toxoid 2 to 3 weeks after infection. Serum drawn from the sensitized animals contained no detectable antitoxin. These preliminary experiments suggested that guinea pigs infected with toxigenic diphtheria bacilli might prove useful experimental animals for further studies on the factors underlying the delayed hypersensitive state, particularly if it could be shown that sensitivity was specifically directed against diphtheria toxin. For reasons discussed elsewhere (8) the toxin-antitoxin system possesses many advantages for study of experimental hypersensitivity. In this paper the early experiments have been confirmed and extended and the specificity of the sensitivity towards diphtheria toxin itself has been established.

Materials and Methods

Cultures.—One toxigenic and four non-toxigenic strains of *C. diphtheriae* were used. The toxigenic strain was C7 (β) (mitis) derived from C7 by infection with β-phage. The non-toxigenic strains included the C7 strain, the lysogenic but non-toxigenic strains C7 (f) and C7 B and Halifax (Hf), a non-toxigenic, non-lysogenic strain of the gravis type (9). The last three strains were isolated and kindly supplied to us by Dr. W. Lane Barksdale of this department. All of the non-toxigenic strains gave rise to pyogenic lesions when injected into the skin of rabbits. The lesions in guinea pig skin were smaller and more localized. In both animals, the skin lesions produced were readily distinguishable from the necrotic areas caused by the toxigenic C7 (β) strain.

Cutaneous Infection of Guinea Pigs.—The organisms were grown on a modified Mueller and Miller medium (10) at 32°C. with continuous shaking. Bacteria were harvested during the exponential growth phase and diluted to an optical density at 590 mμ equivalent to *circa* 10^8 cells per ml. Albino guinea pigs (300 to 400 gm.) were injected intradermally at several abdominal sites with a total of 0.2 ml. diluted suspension (*i.e.*, 2×10^7 organisms). In the case of the toxigenic strain, the animals were protected with either rabbit antitoxic serum or horse antitoxic globulin administered, as in the usual "virulence" test, 3 hours after infection. In certain experiments antitoxin was given before cutaneous infection or simultaneously by suspending the culture in antitoxin.

Antitoxins.—Horse antitoxic γ-globulin 5353 AD was used. This was a rapidly flocculating antitoxin showing a single broad zone of flocculation. It contained 430 units per ml.

Rabbit antitoxin 379–380 was whole serum from rabbits immunized with purified toxoid according to the method of Cohn (11). It contained 40 units per ml. and traces of antibody against non-toxic diphtherial proteins.

The human antitoxin used was a pooled preparation containing 140 units per ml. by rabbit intracutaneous titration (12). It was obtained from Schick negative cirrhotic patients, 12 to 14 days following a "booster" injection of 50 Lf purified toxoid[1] (13). The pooled serum was fractionated with alcohol at low temperature according to the method of Lever *et al.* (14). The final preparation of human antitoxic gamma globulin contained 12 mg. protein per ml. and 80 units per ml. by rabbit skin test. A quantitative precipitin reaction with purified diphtheria toxin gave a curve characteristic of a single antigen-antibody system (15). The solution contained 900 μg. antitoxin globulin per ml. equivalent to 60 *in vitro* units per ml.

Gamma globulin was similarly prepared from serum taken from Schick-positive individuals containing no detectable antitoxin.

Toxin and Toxoid.—Schick test materials and purified toxoid were obtained through the courtesy of Dr. James A. McComb, Biologic Laboratories, Massachusetts Department of Health. The crude toxoid was a formalinized culture filtrate containing 40 Lf toxoid per ml. About 25 per cent of the total protein nitrogen was specifically precipitable by horse antitoxin. Toxin KP28 was a highly purified preparation, previously shown to be 95 per cent specifically precipitable (16).

Dilutions used in skin tests were made in saline containing 1 per cent normal guinea pig serum in order to prevent surface denaturation.

Atoxic Proteins from Non-Toxigenic Strains.—Atoxic proteins were prepared from the non-toxigenic C7, C7 (f), and Hf strains according to methods to be described in detail elsewhere (17). Heavy suspensions of washed organisms were suspended in iron-free medium and grown with continuous shaking for 10 to 16 hours at 34°C. under conditions favorable for toxin production by toxigenic strains. The culture filtrates were dialyzed against saturated ammonium sulfate in the cold and the precipitates dissolved in buffered saline, pH 7. The solutions were further fractionated with ammonium sulfate. The fraction soluble in ⅓ saturated but precipitated by ⅔ saturated ammonium sulfate was collected and dialyzed against 0.1 M phosphate buffer, pH 7, until free of ammonium sulfate. Solutions for skin tests were diluted with saline so as to contain 30 μg per ml. protein as determined by the method of Lowry *et al.* (18).

Antitoxin-Absorbed Toxoid.—To a 1 ml. sample of antitoxic human γ-globulin (60 *in vitro* units per ml.) was added 20 Lf purified toxoid and to another 1 ml. sample, 20 Lf crude toxoid, diluted in saline so as to bring the volume to 2 ml. in each case. The mixtures were incubated at 37°C. for 1 hour and then left overnight in the cold. The specific precipitates were removed by centrifugation leaving atoxic diphtherial proteins (P fraction) in the supernate (19). To similar 1 ml. samples of γ-globulin from Schick-positive human serum were added 20 Lf purified and 20 Lf crude toxoid in saline so as to bring the volumes to 2 ml. These solutions were used in experiments designed to test the specificity of sensitization to toxoid.

RESULTS

Sensitization of Guinea Pigs by Infection with C7 (β).—Table I shows that of 13 guinea pigs infected intracutaneously with 2×10^7 toxigenic diphtheria bacilli and protected 3 hours later by intraperitoneal injection of 25 units rabbit or 500 units horse antitoxin, all showed delayed inflammatory reactions following intradermal challenge 2 to 3 weeks later with highly purified diph-

[1] We are indebted to Dr. Arthur Prensky for the collection and assay of this serum.

theria toxoid. The skin reactions were first apparent 4 to 5 hours after injection and were maximal at about 24 hours when they appeared as red, indurated areas. In several instances as little as 0.003 μg. purified toxoid sufficed to elicit a definite reaction.

Effect of Time of Antitoxin Administration on Sensitization to Toxin.—Table I shows that even when guinea pigs are given antitoxin 24 hours *prior* to infection with a toxigenic strain of *C. diphtheriae*, sensitization to toxin is induced. Two animals were infected with the C7 (β) strain suspended in 25 units of rabbit antitoxin. Eight animals received 20 units horse antitoxin 1 hour before and 6 received antitoxin 24 hours before cutaneous infection. All but one of

TABLE I

Delayed Cutaneous Reactions in Guinea Pigs Challenged with Purified Diphtheria Toxoid 2 to 3 Weeks after Infection with C7 (β) Strain

No. of guinea pigs	Units	Antitoxin species	Time*	No. of animals showing reactions at 24 hrs. to 3 μg (1 Lf) toxoid			No. of Schick-negative
				Diameter			
				>20 mm.	>10 <20 mm.	None	
			hrs.				
4	25	Rabbit	+3	3	1	0	4
9	500	Horse	+3	4	5	0	0
2	25	Rabbit	0	0	2	0	2
8	20	Horse	−1	8	0	0	Not tested
2	25	Rabbit	−24	2	0	0	2
4	500	Horse	−24	1	2	1	0

* (+) denotes antitoxin given *after* infection, (−) *before* infection, and (0) organisms suspended in antitoxin.

these animals showed marked delayed reactions when challenged intracutaneously with 3μg. purified toxoid protein 2 to 3 weeks after infection. In most animals, pronounced reactions were obtained even with 1/1000 this amount of toxoid.

Administration of antitoxin before infection prevents the necrotic lesions caused by toxin that characterize the usual positive "virulence" test. It is of interest, however, that 2 of the 4 animals protected with only 20 units antitoxin given 1 hour before infection, developed sharply demarcated, slow healing ulcers at the site of injection of organisms. These "punched-out" ulcers resemble those seen in cutaneous diphtheria in man.

Antitoxin Production.—Glenny and Sudmersen (20), Hartley (21), and others have shown that passive administration of excess horse antitoxin to guinea pigs blocks active immunization by toxoid given simultaneously or shortly afterwards. Horse gamma globulin is an excellent antigen in guinea pigs and is

rapidly eliminated soon after its antibody appears in the circulation. Thirteen of the animals listed in Table I that were given horse antitoxin were Schick-tested at the time of skin testing 2 to 3 weeks after infection and all proved to be Shick-positive. Serum from 9 of the 13 were tested for antitoxin, 7 contained less than 0.001 unit per ml. and the remaining two contained 0.001 to 0.002 units per ml. On the other hand, all 8 animals that received whole rabbit antitoxic serum remained Schick-negative. Moreover, an uninfected control guinea pig that received the same amount of rabbit antitoxin was also found Schick-negative when tested 2 weeks later. These observations confirm those of Adler (22) who has recently shown that guinea pigs injected with normal rabbit serum (in contrast to horse serum) fail to produce significant amounts of antibody against immune globulin. On the other hand, Adler found that washed specific precipitates containing rabbit immune globulin caused the production of potent anti-rabbit immune globulin in guinea pigs.

Specificity of Sensitization to Toxoid.—Table II shows that skin reactions to purified toxoid were far more severe in the guinea pigs sensitized with the toxigenic C7 (β) strain than in the two animals infected with the closely related, though non-toxigenic C7 (f) strain. Moreover, while specific removal of the toxoid component caused a striking diminution in the intensity of the delayed reactions observed in the C7 (β)-sensitized animals, its removal had no effect on the size of the reactions in guinea pigs infected with the non-toxigenic strain.

It will be noted that injection of crude toxoid gave rise to somewhat larger reactions than those elicited by the same amount of purified toxoid. Moreover, even after specific removal of the toxoid component from the crude preparation, the atoxic P proteins remaining in the supernate gave rise to marked though diminished inflammatory reactions. In the case of purified toxoid, the specificity is even more striking and the titer dropped almost 1000-fold following specific precipitation of toxoid. It would appear, from the delayed reactions observed with crude toxoid, following absorption of toxoid with antitoxic globulin, that infection with C7(β) induced sensitivity to other diphtherial proteins as well as to toxin. However, it is clear that the most pronounced sensitivity was directed against the toxin component itself.

Cell Transfer.—Cells were teased into Tyrode's solution from axillary and popliteal lymph nodes removed from 7 sensitized animals. The cells were washed with Tyrode's solution and 1.2×10^8 and 1.8×10^8 cells injected intraperitoneally into normal guinea pigs. 72 hours later, the two recipients were skin-tested wiht 3 μg. purified toxoid. Delayed reactions measuring 10 x 7 and 7 x 5 mm. were observed at 24 hours.

Sensitization by Infection with Non-Toxigenic Strains.—Table III summarizes the results of skin tests carried out on guinea pigs infected 2 to 3 weeks previously with 4 closely related *mitis* strains all derived from C7, and one non-toxigenic, non-lysogenic strain (Hf) of the gravis type. Intradermal injection

of 1 Lf (3μg.) purified toxoid elicited severe delayed reactions exceeding 20 mm. in diameter in animals sensitized with the toxigenic strain. The same amount of toxoid caused smaller, though definite, reactions in animals sensitized by infection with non-toxigenic strains. Comparison with Table II shows that

TABLE II

Specificity of Delayed Reactions to Toxoid in Guinea Pigs 16 to 20 Days after Infection with C7 (β) and C7 (f) Strains

Guinea pig No.	Infecting strain	Test solution	Skin reactions in mm. at 24 hrs. to 0.1 ml. test solution			
			Undiluted*	1:10	1:100	1:1000
1	C7 (β)	Crude toxoid	30 × 28	18 × 16	17 × 15	9 × 8
		Antitoxin-absorbed supernate	20 × 16	8 × 7	±	±
2	C7 (β)	Crude toxoid	35 × 25	22 × 17	14 × 12	9 × 8
		Antitoxin-absorbed supernate	19 × 18	11 × 10	8 × 5	±
3	C7 (β)	Purified toxoid	29 × 22	17 × 15	16 × 12	9 × 6
		Antitoxin-absorbed supernate	10 × 9	7 × 6	±	±
4	C7 (β)	Purified toxoid	25 × 21	19 × 13	12 × 9	±
		Antitoxin-absorbed supernate	9 × 7	±	±	±
5	C7 (f)	Purified toxoid	9 × 7	4 × 3	0	0
		Antitoxin-absorbed supernate	7 × 7	4 × 4	0	0
6	C7 (f)	Purified toxoid	8 × 6	±	0	0
		Antitoxin-absorbed supernate	6 × 5	±	0	0

* Undiluted toxoid, whether crude or purified, contained 1 Lf (3 μg.) toxoid protein per 0.1 ml.

Antitoxin-absorbed supernates were prepared as outlined under Materials and Methods.

only 0.01 Lf toxoid sufficed to cause reactions in the C7 (β)-infected guinea pigs which were equivalent in size to those elicited by 100 times as much (1 Lf) toxoid in animals sensitized by infection with any one of the non-toxigenic strains. Moreover, in the latter case, specific removal of the toxoid component by precipitation with antitoxin failed to reduce further the size of the reactions.

When non-toxigenic strains of *C. diphtheriae* are grown under conditions of

iron deficiency that are optimal for toxin production by toxigenic strains, they release into the culture medium an atoxic protein with electrophoretic mobility and solubility similar to toxin (17). Table III shows that the reactions elicited by atoxic proteins, prepared from three different non-toxigenic strains, were much smaller than those caused by toxoid in guinea pigs sensitized by infection with the C7(β) strain. Animals sensitized by infection with lysogenic but non-toxigenic C7(f) and C7(B) strains showed slightly larger reactions to atoxic proteins from C7 and C7(f) than to the same amount of highly purified toxoid.

TABLE III

Induction of Hypersensitivity in Guinea Pigs by Infection with Toxigenic and Non-Toxigenic Strains of C. diphtheriae

Infecting strain	No. of animals	Average diameter in mm. of skin reactions at 24 hrs. to 3 μg. of*				
		Purified toxoid	Antitoxin-absorbed supernate	C7 protein	Hf protein	C7 (f) protein
C7 (β)	4	24 × 21	—	13 × 12	13 × 11	12 × 11
	2	27 × 22	10 × 8	—	—	—
C7 (f)	4	7 × 7‡	7 × 7	—	—	11 × 10
C7 (B)§	2	10 × 8	—	14 × 12	—	—
C7	4	13 × 11	14 × 11	—	—	—
Hf	4	11 × 10	9 × 8	—	—	—

* (—) indicates not tested.

‡ 10 × 10 reaction to 1 Lf crude toxoid.

§ Non-toxigenic, lysogenic strain supplied by Dr. W. L. Barksdale. These animals received horse antitoxin; other animals infected with non-toxigenic strains did not.

The experiments with non-toxigenic strains suggest that there is no cross-reactivity between toxin and its atoxic counterpart, although cross-sensitization to other diphtherial proteins common to both toxigenic and non-toxigenic variants does occur. These results are in agreement with *in vitro* tests (17) which have failed to reveal interaction between the atoxic protein released by non-toxic diphtheria bacilli and diphtheria antitoxin.

SUMMARY AND CONCLUSIONS

Guinea pigs infected by intradermal injection of living toxigenic diphtheria bacilli and protected by horse antitoxic globulin, given either before or after infection, develop delayed hypersensitivity of the tuberculin type to diphtherial proteins. The highest degree of hypersensitivity is specifically directed against diphtheria toxin (or toxoid) itself, although smaller delayed skin reac-

tions may be evoked in sensitized animals by other diphtherial proteins common to both toxigenic and non-toxigenic strains.

Animals sensitized to diphtheria toxin by infection with a toxigenic strain in this way react positively to the Schick test and their serum usually contains no detectable antitoxin 2 to 3 weeks after the initial infection.

Animals infected with living non-toxigenic diphtheria bacilli become sensitized to proteins common to both toxigenic and non-toxigenic strains but do not show sensitivity to toxin.

The observations suggest that a minute amount of toxoid, or of toxin comparable to that which might be liberated during infection, might induce the hypersensitive state if injected in the form of a complex with excess antitoxin. This prediction is verified by the results reported in the following paper (23).

BIBLIOGRAPHY

1. Rich, A. R., Hypersensitivity in disease, *Harvey Lectures*, 1946–47, **42**, 106.
2. Dienes, L., Technic of producing tuberculin type of sensitization with egg white in tuberculous guinea pigs, *J. Immunol.*, 1929, **17**, 531.
3. Freund, J., and McDermott, K., Sensitization to horse serum by means of adjuvants, *Proc. Soc. Exp. Biol. and Med.*, 1942, **49**, 548.
4. Raffel S., The components of the tubercle bacillus responsible for the delayed type of "infectious" allergy, *J. Infect. Dis.*, 1948, **82**, 267.
5. Gell, P. G. H., and Hinde, I. T., Observations on the histology of the Arthus reaction and its relation to other known types of hypersensitivity, *Internat. Arch. Allergy*, 1954, **5**, 23.
6. Metaxas, M. N., and Metaxas-Bühler, M., Frühreaktion and Spätreaktion bei der Serum Allergie des Meerschweinchens und ihr Trennung durch passive Übertragung, *Z. Allg. Path. u. Bakt.*, 1954, **17**, 128.
7. Pound, A. W., The effect of a tubercle bacillary "wax" on the response of guinea pigs to injection of tubercle bacillary protein, *J. Path. Bact.*, 1955, **70**, 119.
8. Pappenheimer, A. M., Jr., Use of diphtheria toxin and toxoid in the study of immediate and delayed hypersensitivity in man, *J. Immunol.*, 1955, **75**, 259.
9. Barksdale, W. L., Sur quelques bactériophages de *C. diphtheriae* et leur hôtes, *Compt. rend. Acad. sc.*, 1955, **240**, 1831.
10. Mueller, J. H., and Miller, P., Production of diphtheria toxin of high potency on a reproducible medium, *J. Immunol.*, 1941, **40**, 21.
11. Cohn, M., Immunochemical methods for determining homogeneity of proteins and polysaccharides, *Methods Med. Research*, **5**, sec. 3, 1952.
12. 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.
13. Havens, W. P., Shaffer, J. M., and Hopke, C. J., The production of antibody by patients with chronic hepatic disease, *J. Immunol.*, 1951, **67**, 347.
14. Lever, W. F., Gurd, F. R. N., Uroma, E., Brown, R. K., Barnes, B. A., Schmid, K., and Schulz, E., Chemical, clinical and immunological studies on the products of human plasma fractionation XL. Quantitative separation and determi-

nation of the protein components in small amounts of normal human plasma, *J. Clin. Inv.*, 1952, **30**, 99.

15. Cohn, M., and Pappenheimer, A. M., Jr., A quantitative study of the diphtheria toxin-antitoxin reaction in the sera of various species including man, *J. Immunol.*, 1949, **63**, 291.
16. Pappenheimer, A. M., Jr., Edsall, G., Lawrence, H. S., and Banton, H. J., A study of reactions following administration of crude and purified toxoid in an adult population, *Am. J. Hyg.*, 1950, **52**, 323.
17. Yoneda, M., and Pappenheimer, A. M. Jr., data in preparation.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., Protein measurement with the Folin phenol reagent, *J. Biol. Chem.*, 1951, **193**, 265.
19. Lawrence, H. S., and Pappenheimer, A. M., Jr., Immunization of adults with diphtheria toxoid I. Immunological properties of formalinized diphtherial protein fractions from culture filtrates, *Am. J. Hyg.*, 1948, **47**, 226.
20. Glenny, A. T., and Sudmersen, H. J., Notes on the production of immunity to diphtheria toxin, *J. Hyg.*, 1921, **20**, 176.
21. Hartley, P., The antigenic properties of precipitates produced by the interaction of diphtheria toxin and antitoxin, *Brit. J. Exp. Path.*, 1925, **6**, 112.
22. Adler, F. L., Antibody formation after injection of heterologous immune globulin, *J. Immunol.*, 1956, **76**, 217.
23. Uhr, J. W., Salvin, S. B., and Pappenheimer, A. M. Jr., Delayed hypersensitivity. II. Induction of hypersensitivity in guinea pigs by means of antigen-antibody complexes, *J. Exp. Med.*, 1957, **105**, 11.