THE FATE OF ^{131}I-LABELLED DIPHTHERIA TOXIN AND TOXOID IN THE SKIN OF IMMUNE AND ALLERGIC GUINEA-PIGS*

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(With 8 Figures in the Text)

Guinea-pigs injected with alum-precipitated diphtheria toxoid (APT) become hypersensitive to diphtheria toxin and toxoid. This hypersensitivity is probably due to a mixture of immediate (Arthus-type) reactions and of delayed (tuberculin-type) reactions (Ben-Efraim & Long, 1957; Long, 1959). The former increases as the level of circulating antitoxin rises and tends to mask the latter (Long, 1959). In a hyperimmune guinea-pig the residual damage 24 hr. after the intradermal injection of toxin is due, at least in part, to hypersensitivity to toxin. In these circumstances, when the ratio of circulating antitoxin to intradermally injected toxin is high, toxicity of toxin probably contributes little to the severity and extent of the lesion (Long, 1959). In this paper, the fate of intradermally injected diphtheria toxin and toxoid, labelled with ^{131}I, is studied in immune and allergic guinea-pigs. The evidence suggests that hyperimmune guinea-pigs localize these antigens whereas non-immune guinea-pigs do not; localization is due to hypersensitivity of a type that cannot be transferred with serum and is probably of the delayed (tuberculin-type) of hypersensitivity.

MATERIALS AND METHODS

Diphtheria toxin and toxoid. Highly purified diphtheria toxin and toxoid, provided by Dr C. G. Pope (1957) were used in these experiments. By methods which cause partial denaturation of the proteins present in such crystalline diphtheria toxin-protein, the antigenic complexity of this material has been shown; three, and often four, antigens were detected by gel-diffusion techniques (Pope & Stevens, 1958). Whether this means that Pope's preparations contain several separate antigens or that a single protein molecule has more than one antigenic surface cannot be stated dogmatically. It is, therefore, uncertain whether the experiments described in this paper were carried out with one or more substances and the evidence should be considered with this reservation in mind. But this limitation had to be accepted for no purer preparations of diphtheria toxin or toxoid are available at the present time.

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The toxin was assayed for toxicity in terms of the international standard for Schick (diphtheria) toxin by the method of Gerwing, Long & Mussett (1957). It was also standardized in terms of the international standard for diphtheria antitoxin by means of the classic intradermal technique of Römer & Sames (1909). Flocculating potency of both toxin and toxoid was expressed in terms of the international standard for diphtheria antitoxin (flocculation). After iodination by the method of Masouredis (1957) the assays were repeated and no loss of potency was detected. The assay of toxicity by a very accurate method (Gerwing, Long & Mussett, 1957) provides a stringent test for loss of potency. The highly purified preparations produced by Dr C. G. Pope are more stable, even in unbuffered solutions, than any of the many 'Schick' toxins routinely tested in this laboratory. This inherent stability of this toxin, combined with the gentle iodination technique of Masouredis (1957), provided material with high radioactivity and full antigenic and toxic potency. Unfortunately, there is no sensitive test for denaturation of toxoid. But, in general, toxin is less stable than toxoid and a method satisfactory for iodinating toxin will probably suffice for toxoid. It might be argued that a study of the fate of ^{131}I-tagged toxoid is without value because toxoid differs from toxin only in being non-toxic and therefore, when labelled, should behave in a predictable manner comparable to that of toxin. On this basis a difference in behaviour of ^{131}I toxin and toxoid, not attributable to toxicity, would indicate denaturation of the latter. This argument may be false. The process of toxoiding almost certainly alters the antigenic surface, so that immunization with formol toxoid produces antitoxoid and immunization with toxin produces antitoxin. Unpublished observations by the present author also make it almost certain that these two antibodies have similar, but not identical, combining properties. Although proof of these statements must await the availability of pure diphtheria toxin and toxoid, differences in the behaviour of ^{131}I toxin and ^{131}I toxoid in toxoid-immunized guinea-pigs are neither attributed to denaturation of toxoid by iodination or solely to the toxic action of toxin; they are thought to be due to differences in the antigenic surface of the toxoid resulting from the process of toxoiding.

However, toxoid is more alkali-sensitive than toxin and the fact must be accepted that iodination might have caused denaturation of toxoid and that this was not detected by the crude tests available; but this is thought to be unlikely for the reasons stated.

Alum-precipitated diphtheria toxoid (*APT*). The current laboratory standard alum-precipitated toxoid (Ba 536) was used as the antigen. It was diluted with physiological saline to contain 2·5 Lf in a volume of 1 ml. and injected into the adductor muscles of the right leg. The frequency of injections is stated in the relevant places.

Animals. Groups of eight albino female guinea-pigs of not less than 450 g. in weight were used. Males were not employed because they tend to fight and the resulting scratches make accurate reading of skin reactions difficult.

Passive transfer of immunity and hypersensitivity. Immune sera were injected intraperitoneally 48 hr. before the skin tests.

Skin tests. Fur was clipped from the flanks, excluding the thin skin over the belly. Depilating paste was not used. Injections were made intradermally; the dose and nature of the test reagent is stated in each experiment. The volume injected was always 0·1 ml. and the diluent physiological saline. Reactions were measured at the time intervals shown in each experiment. The loose skin of the flank was folded across the diameter of the inflamed area, and the maximum thickness of the fold measured with calipers fitted with a magnifying dial. The utmost gentleness was used so as to interfere as little as possible with the natural development of the lesion. The results obtained were expressed as percentage change in thickness of this double layer of skin from its initial thickness before injection. The values were plotted against time (see, for example, Fig. 2).

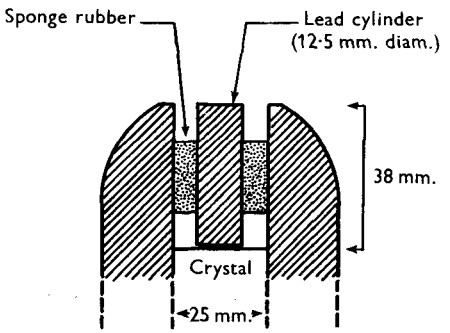

Fig. 1

The technique of measuring radioactivity. A scintillation detector with a sodium iodide, thallium activated, crystal was made with a collimator head which could be used in the conventional manner by placing the opening (25 mm. in diameter) over the skin lesion and counting the activity of the ^{131}I-labelled antigen in the area. This was called the 'total count'. The 'total count' is due largely to the high concentration of ^{131}I-labelled antigen in the centre of the bleb. This is not an important area for study because it is not yet involved in the kinetics of the immune reaction. What is important is the fate of toxin at the periphery of the lesion, where toxin or toxoid diffusing out mechanically from the relatively high tension in the bleb is involved in the reaction—immune or otherwise—of the skin. A second count was therefore carried out in which a cylinder of lead, supported by an outer cylinder of sponge rubber, filled the opening in the collimator head (Fig. 1). The end of the lead cylinder (12·5 mm. in diameter) was placed over the injection bleb. As a result, as toxin moved towards the periphery, and away from the bleb, its activity was counted and so the rate of spread of radioactive material was measured without interference from the reservoir of ^{131}I antigen in the centre of the bleb. This count was called the 'peripheral count'. In Fig. 2, the 'total' and in Fig. 3 the 'peripheral' counts carried out on the same lesions are recorded. In all experiments, 'total' and 'peripheral' counts were at least twenty times as great as the background count without the animal. Percentage changes in radioactivity of lesions are expressed in terms of the initial count. In this experiment, the 'peripheral' count is undoubtedly a better technique than the 'total' count. It

shows clearly the localization of radioactivity by hyperimmune guinea-pigs. But 'peripheral' counts have one serious disadvantage—they give lower readings than the 'total' counts. This is a handicap when small doses are essential because of the high toxicity of the antigen, neither can this problem be readily overcome by increasing the amount of ^{131}I used in tagging the antigen. Quite apart from the risk of denaturation, the more iodine used the greater the physical and chemical differences between tagged and non-tagged antigen, and presumably the greater the risk that this will lead to differences in the biological behaviour which might not be susceptible to detection by the assays of potency.

Because of this difficulty, 'total' counts were used routinely and 'peripheral' counts were used in addition whenever activity was high enough to give, with this technique, counts at least twenty times greater than that of the background. For instance, with a hyperimmune animal a large dose of toxin, resulting in a high count, can be injected.

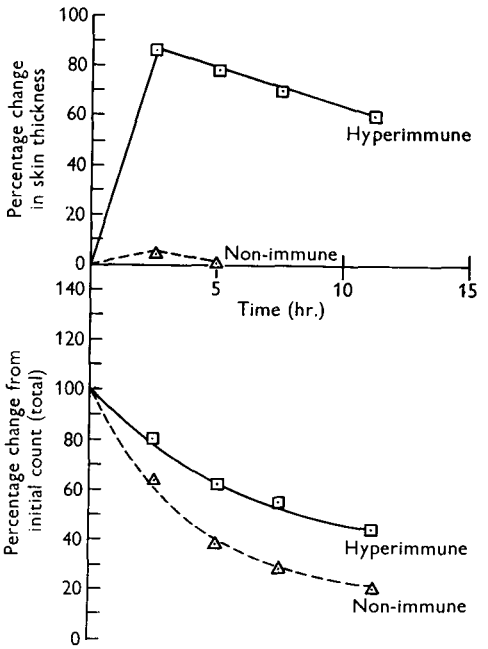

Fig. 2. Response of actively immunized guinea-pigs to intradermal toxoid ^{131}I.

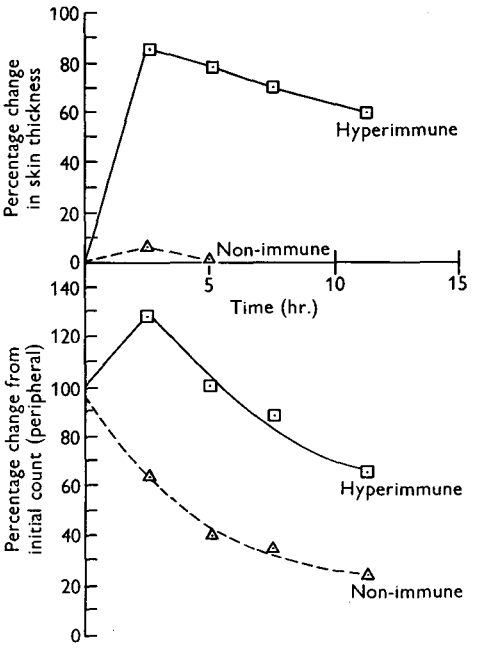

Fig. 3. Response of actively immunized guinea-pigs to intradermal toxoid ^{131}I.

RESULTS

In Expt. 1, the results of which are shown in Figs. 2 and 3, guinea-pigs, hyperimmunized over many months, with repeated doses of APT, all of whom had a high titre (more than 50 i.u./ml.) of avid precipitating antitoxin, were injected intradermally with 10 Lf of ^{131}I-tagged formol toxoid (see Table 1). A typical severe, mixed, allergic response resulted (Long, 1959). Similar material produced a slight transient area of swelling in non-immune guinea-pigs attributable to the volume of fluid injected (Fig. 2). Both the 'total' (Fig. 2) and 'peripheral' (Fig. 3)

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counts showed that radioactivity left the injection site more rapidly in non-immune than in immune guinea-pigs. The conclusion reached was that radioactivity was localized in the skin of these actively immune animals and the assumption was made that the activity denoted the position of the antigen.

Table 1

	^{131}I bound (%)	^{131}I per toxin* (moles/mole)	mg. N/ml.	Lf/ml.	μc. ^{131}I/μg. N	^{131}I T.C.A. precipitable
Toxoid	2·11	0·56	255·5	638	0·80	99·2
Toxin	2·18	0·36	288·7	865·2	0·76	99·5

* Calculations based on assumed molecular weight of 70,000 for diphtheria toxin (6).

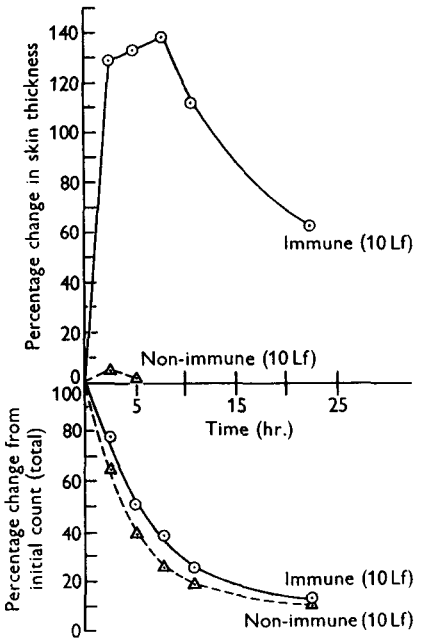

Fig. 4. Response of passively immunized guinea-pigs to intradermal toxoid ^{131}I.

In Expt. 2, the guinea-pigs passively hyperimmunized with high-titre avid precipitating antitoxin, obtained from the same group of actively immunized guinea-pigs as those used in Expt. 1, showed no localization of the radioactivity of this same antigen (Fig. 4). (A highly avid precipitating antitoxin (horse) included in this experiment did not affect the rate of loss of radioactivity in guinea-pigs. But antitoxin from this species does not passively transfer an early (Arthus-type) reaction to guinea-pigs (unpublished observations); it does, however, precipitate formol toxoid most effectively.)

The conclusion was that avid precipitating antitoxin, with or without an accompanying Arthus reaction, did not localize (or spread) the radioactivity of this antigen. In particular, the ability to localize could not be passively transferred with serum.

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This conclusion gained further support from Expt. 3, in which human precipitating diphtheria antitoxin (300 i.u. in 5 ml. physiological saline) or human non-precipitating diphtheria antitoxin (300 i.u. in 5 ml. physiological saline) did not affect the rate of loss of radioactivity. Human precipitating antitoxin transferred an early (Arthus-type) reaction to guinea-pigs (Fig. 5) (Kuhns & Pappenheimer, 1952).

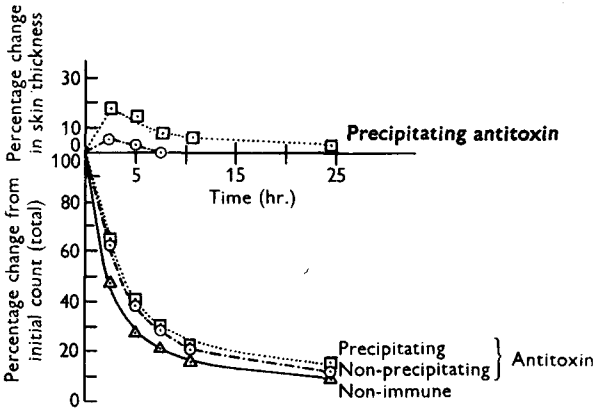

Fig. 5

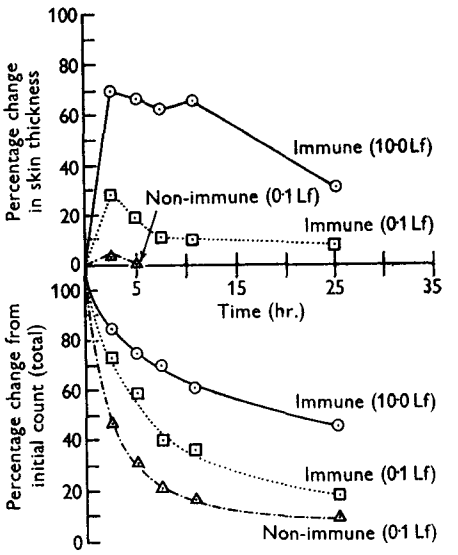

Fig. 6

Fig. 5. Response to intradermal toxoid ^{131}I of guinea-pigs passively immunized with precipitating or non-precipitating diphtheria antitoxin (man).

Fig. 6. Response of actively immunized guinea-pigs to intradermal toxoid ^{131}I.

In Expt. 4, the fate of 0·1 Lf of ^{131}I formol toxoid was compared with that of 10 Lf. The larger dose was associated with a slower rate of loss of radioactivity, and with a more severe allergic reaction, than the lower dose (Fig. 6). Both differed significantly from the controls.

In contrast, when radioactive toxin (see Table 1) was injected intradermally into a group of hyperimmune guinea-pigs comparable to those in Expt. 1, no difference between the rate of loss of activity from the skin of high and low doses of toxin was detected (Fig. 7). But the radioactivity of toxin, like that of toxoid, is retained at the site of injection in actively immunized guinea-pigs. This effect is shown by the 'total' count (Fig. 7) and even more clearly by the 'peripheral' count (Fig. 8).

DISCUSSION

Actively immunized guinea-pigs tend to localize radioactivity of toxin or toxoid. This ability to localize antigen cannot be transferred with serum taken from these animals. The obvious hypothesis is that fixed antibody, associated with a delayed (tuberculin-type) reaction in these actively immunized guinea-pigs (Long, 1959) retains antigen at the site of injection. A direct experiment to test this hypothesis

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is to inject tagged antigen into guinea-pigs so sensitized that they induce a pure delayed response. Up to the present time all attempts to produce a pure delayed response have been spoilt by the presence of a significant, though slight, early (Arthus-type) reaction (unpublished observations).

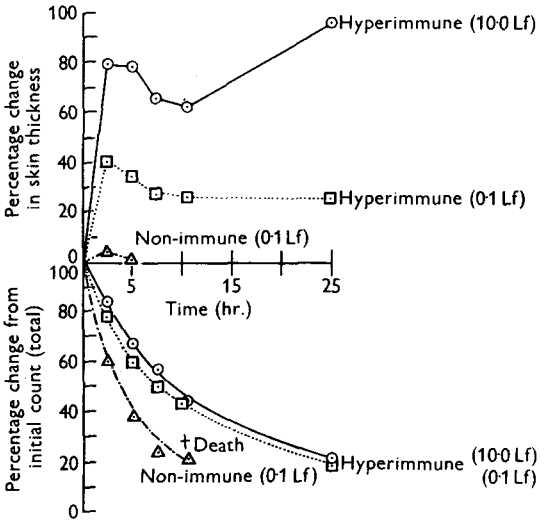

Fig. 7. Response of actively immunized guinea-pigs to intradermal toxin ^{131}I.

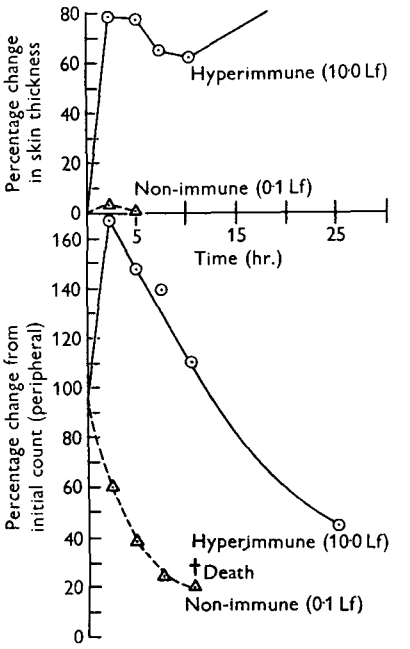

Fig. 8. Response of actively immunized guinea-pigs to intradermal toxin ^{131}I.

It is important to appreciate that certain assumptions have been made and that these may be false. It is assumed that the antigens labelled with iodine, although altered physically and chemically by the addition of iodine, are not altered in their biological behaviour. It is assumed that radioactivity denotes the position of the antigen throughout the experiment and that ^{131}I is not transferred from the antigen to some substance with a greater affinity for iodine than that possessed by the antigen itself; these assumptions apply to all work of this nature. At the present time the purity of diphtheria toxin and toxoid is unknown and it is assumed that the behaviour of the tagged material represents the behaviour of pure diphtheria toxin or toxoid.

SUMMARY

Highly purified crystalline diphtheria toxin-protein, and toxoid prepared from it, were labelled with ^{131}I, without change of potency, and injected intradermally into immune and allergic guinea-pigs. It is probable that actively immunized guinea-pigs localize these antigens and that localization is due to hypersensitivity of a type that cannot be transferred with serum; it is possibly of the delayed (tuberculin-type) of allergy.

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It is a pleasure to acknowledge the gift of highly purified (crystalline) diphtheria toxin, and toxoid prepared from it, from Dr C. G. Pope of the Wellcome Research Laboratories, England. Dr S. P. Masouredis of the Central Blood Bank of Pittsburgh kindly labelled these antigens, using the technique he devised. Dr W. J. Kuhns generously provided the human precipitating and non-precipitating diphtheria antitoxin.*

The collimator head was made by Mr J. Nechaj of the University of Pittsburgh.

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* This non-precipitating antitoxin contains small quantities of precipitating antitoxin.