IMMUNE AND ALLERGIC REACTIONS TO DIPHTHERIA TOXIN AND TOXOID IN GUINEA-PIGS*

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(With 9 Figures in the Text)

Alum-precipitated diphtheria toxoid or aluminium-phosphate-precipitated diphtheria toxoid injected into guinea-pigs induce hypersensitivity to formol toxoid; this hypersensitive state consists of a mixture of early and delayed reactions (Ben-Efraim & Long, 1957). In this paper an analysis of these reactions is made and their relationship to antitoxic immunity studied.

MATERIALS AND METHODS

Materials

Highly purified diphtheria toxin and toxoid, provided by Dr C. G. Pope (1957), was 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, 1957). 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. Either interpretation is possible. All that can be said with certainty is that the experiments described in this paper were carried out using one or more substances and the evidence obtained should be considered with this reservation in mind.

However, the actual degree of purity of the preparation of diphtheria toxin used was not, apparently, of major importance in the experiments described: the international standard for diphtheria (Schick) toxin, a comparatively crude preparation, behaved in a manner that did not differ significantly from that of the highly purified preparations of toxin. Similarly, the current laboratory standard for formol toxoid behaved, in these comparatively crude experiments, in a manner similar to that of the toxoid prepared from highly purified crystalline toxin-protein provided by Dr C. G. Pope. The explanation may be that contaminating materials have physical and chemical properties so similar to pure toxin and toxoid that their immunological actions, as measured in these experiments, are similar to those of toxin or toxoid. The immunological and toxic potency of these batches of diphtheria toxin or toxoid were assayed in terms of the appropriate international standards and are expressed in international units (Humphrey, Long & Perry, 1957).

Alum-precipitated diphtheria toxoid. 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 this was injected into the adductor muscles of the right leg. The frequency of injections is stated in each experiment.

Tuberculin and tuberculo-protein. The international standards for old tuberculin and for PPD were used as test allergens. These preparations, like all such materials, have been purified by drastic means, and therefore have undergone some denaturation. Tuberculo-protein prepared by the method of Boyden & Sorkin (1955), and provided by Dr E. Sorkin, was included in the experiment because it is probable that this material has not been denatured and resembles that liberated by the tubercle bacillus under natural conditions. The analogy between this extract and diphtheria toxoid is almost certainly closer than the analogy between this substance and the international standards for old tuberculin and for PPD.

BCG. Two mg. wet weight, suspended in physiological saline, were injected in a volume of 1 ml. into the adductor muscles of the right leg of guinea-pigs in order to render them hypersensitive to the various test allergens. Tuberculin testing was carried out not less than 28 days later.

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.

Methods

Passive transfer of immunity and/or hypersensitivity

(a) *With serum*. Sera were injected intraperitoneally 48 hr. before the skin tests.

(b) *With cells*. The technique for obtaining cells is essentially that of Suter (1952). Ten ml. of glycogen (0·1 mg./ml.) in physiological saline were injected intraperitoneally into the donor guinea-pigs. Four days later the animals were killed with chloroform and, at once, 25 ml. of ice-cold Tyrode's solution was injected intraperitoneally. The peritoneal cavity was opened and the free fluid transferred to a centrifuge tube and spun in the cold at 1000 revolutions per minute for 10 min. The cells were twice resuspended in fresh cold Tyrode's solution and then injected intraperitoneally into the recipient animal. Each animal received approximately 50 million cells—mainly mononuclear cells—suspended in 2 ml. of Tyrode's solution.

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 0·2 ml. and the diluent was physiological saline. Reactions were measured at the time intervals shown in each experiment. Two methods were used. The first is essentially that of Long & Miles (1950). The diameters of round lesions were read with a plastic ruler to the nearest 0·5 mm.; the long and short diameters of elliptical lesions were measured and the square root of their product recorded as their

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diameter. This method provides the basis for the accurate assay of substances that produce in the skin defined areas of inflammation, i.e. diphtheria toxin (Gerwing, Long & Mussett, 1957) or tuberculin (Long, Miles & Perry, 1954). It is less satisfactory for measuring ill-defined reactions with much oedema (i.e. Arthus reactions).

A second method, well suited to the latter purpose, was therefore employed. 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. These values were plotted against time (see, for example, Fig. 1).
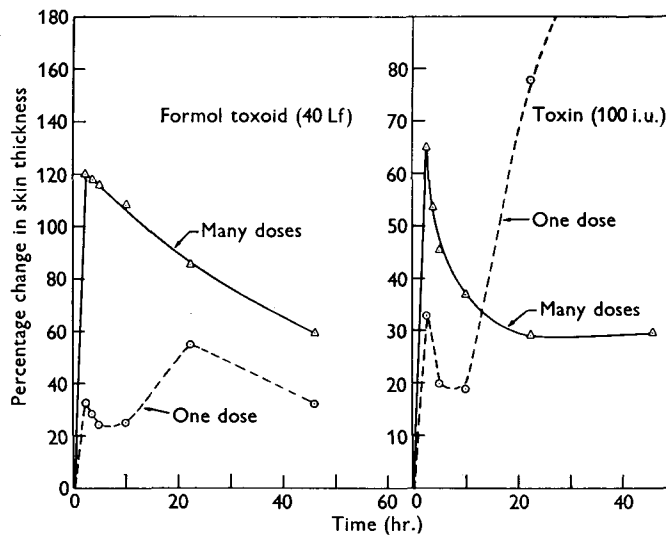

Fig. 1. Guinea-pigs immunized with one or many doses of APT.
Response to intradermal toxoid and toxin.

RESULTS

Experiment I. 100 international Schick units of diphtheria toxin injected into the skin of a normal guinea-pig produce an area of inflammation which takes many hours to develop and leads eventually to necrosis of the skin. If toxin is injected into a guinea-pig, which has been immunized with alum-precipitated diphtheria toxoid, an observable reaction occurs within a few minutes and persists for many hours (Fig. 1). This early reaction of the immune animals is not due to toxic damage, for it occurs also following the injection of toxoid (Fig. 1). A group of partially immune guinea-pigs, which had received a single dose of alum-precipitated toxoid 28 days earlier, produced in response to toxoid a small response followed by a marked delayed reaction. A delayed response also occurred to diphtheria toxin, but in this case, in these relatively non-immune animals, the prolonged delayed reaction is not primarily allergic and can be attributed to the direct toxic action of toxin. No circulating antitoxin could be detected in the sera

of these animals by the classic Römer & Sames (1909) intradermal technique. But it is seldom appreciated that small quantities of non-avid guinea-pig diphtheria antitoxin cannot be detected with accuracy by this method. However, these animals produced in response to toxin smaller skin lesions than the controls, showing that circulating antitoxin was present in slight but significant amounts (Long, 1950).
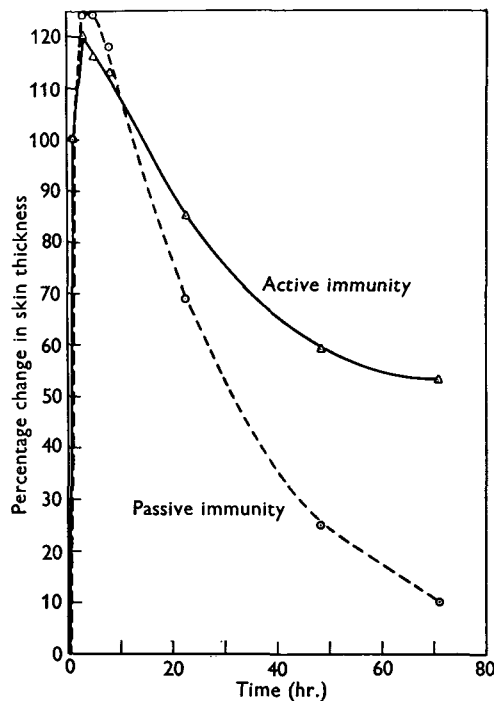

Fig. 2. Response of hyperimmune guinea-pigs to intradermal toxoid.

In contrast to the partially immune animals, a group of hyperimmune guinea-pigs produced an early allergic reaction to both toxoid and toxin—in each case the response curve rose steeply and declined gradually. After 24 hr. the response curves to formol toxoid of partially immune, and of hyperimmune, guinea-pigs ran parallel to each other (Fig. 1) suggesting that at this stage they were analogous.

Experiment II. In Expt. II (Fig. 2), the response curves to formol toxoid of two groups of guinea-pigs are superimposed. One group was actively, and the other passively, hyperimmunized so that the level of circulating antitoxin was similar in both groups. The quality of the antitoxin was presumably also similar in both groups, because the serum used for passive transfer was obtained from additional actively immunized guinea-pigs of the same group as those included in the experiment. The assumption is made that there were no quantitative or qualitative differences in the circulating antitoxin in the actively and passively immunized groups (i.e. that they did not differ in amount, in avidity or in the ratio of precipitating to non-precipitating antitoxin). The response curves, however, differed. They rose together, but that of the passively immunized guinea-pigs returned to

the base line relatively quickly. Clearly, the major part, *but not all*, of this predominantly early (Arthus-type) reaction was passively transferred with serum.

Experiment III. A similar experiment (Fig. 3) with partially immunized guinea-pigs produced an early, transient and rapidly declining response to formol toxoid in passively immunized guinea-pigs, whereas the actively immunized animals showed a significant early, followed by a large delayed, response. This experiment
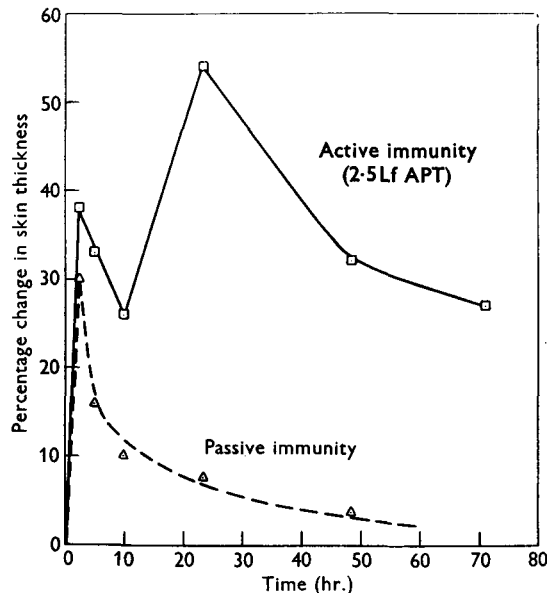

Fig. 3. Response of partially immune guinea-pigs to intradermal toxoid (40 Lf).

is imperfect. Undoubtedly there were marked qualitative differences in the circulating antitoxin in the two groups. It was not possible to obtain sufficient circulating antitoxin, from these partially immune guinea-pigs, to produce the degree of passive immunity required in this experiment and instead a small quantity of relatively high titre, high avidity guinea-pig antitoxin, with a high ratio of precipitating to non-precipitating antitoxin was used.

Nevertheless, in both Expts. II (Fig. 2) and III (Fig. 3) much, but not all, of the hypersensitivity response can be *transferred with serum*, but the response curve of the passively immunized animal declines more rapidly than that of the actively immunized animal. The hypothesis was advanced that the difference was due to the presence of fixed antibody in the cells of actively immunized animals, so that they were capable of producing both an early (Arthus-type) and a delayed (tuberculin-type) reaction (Rich, 1950), the former increasing as the level of circulating antitoxin rose and tending to mask and perhaps alter the response curve of the latter.

Experiment IV. In Expt. IV (Fig. 4) one group of guinea-pigs was passively immunized with serum, the other with cells. Both serum and cells were pooled samples from one group of actively immunized guinea-pigs. Unfortunately, there is a considerable quantitative difference between the two responses. It had been hoped that there would have been a marked qualitative difference, but apparently

the donated cells produced so much circulating antitoxin that an early allergic response was passively transferred. This early response was not observed with the naked eye and was detected only by the sensitive caliper technique. If the method of Uhr and his colleages (Uhr, Salvin & Pappenheimer, 1957) had been the sole criterion of hypersensitivity, a dogmatic claim for passive transfer of delayed hypersensitivity might have been made. Such a claim would possibly have been valid because the response curve of the guinea-pigs receiving cells declines rather
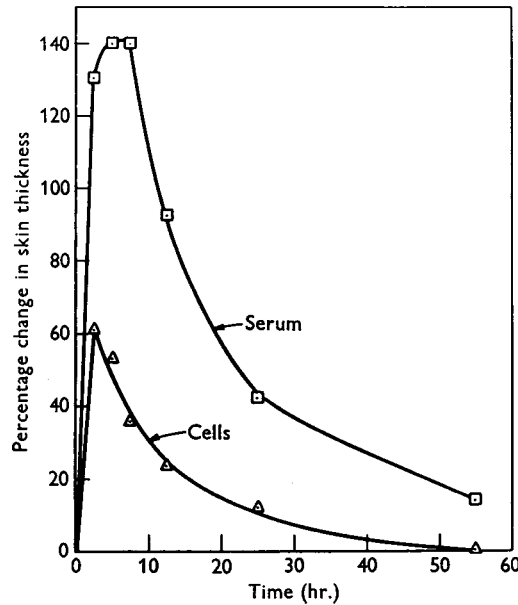

Fig. 4. Response of guinea-pigs passively immunized with cells or serum
to intradermal toxoid.

slowly and resembles that of the actively hyperimmunized animals in Expt. I (Fig. 1), but it could not have been made with confidence. It appears that the correlation between the response to formol toxoid of actively immunized guinea-pigs, on the one hand, with that of passively immunized guinea-pigs on the other, is closer when cells are used to transfer immunity than when serum is used. However, the experiment is inconclusive. It illustrates the difficulty encountered in interpreting claims of passive transfer of delayed (tuberculin-type) sensitivity with cells which are actively producing precipitating antibody and are therefore capable of transferring hypersensitivity of the early (Arthus) type. Invariably such claims should be treated with reserve and with scepticism, if the cells were injected into the recipient by the subcutaneous, as opposed to the intraperitoneal or intravenous route.

Experiment V. In Expt. V (Fig. 5) the effect on the reaction of the skin of hyperimmune guinea-pigs of different doses of formol toxoid was studied. It was postulated that a vigorous early (Arthus-type) reaction to a large dose of toxoid might mask a delayed response, and that the distinction might be made apparent by using small doses of toxoid. This hypothesis proved to be incorrect (Fig. 5).

Experiment VI. In Expt. VI (Fig. 6) tuberculo-protein, a suspension of BCG, and the international standard for PPD were assayed by the method of Long *et al.* (1954) in terms of the international standard for old tuberculin. The development of these lesions, as measured with the caliper technique, is shown in Fig. 7. The
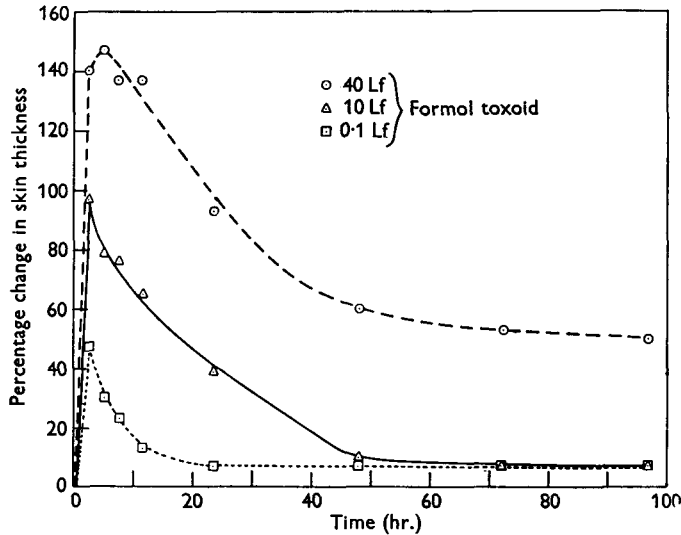

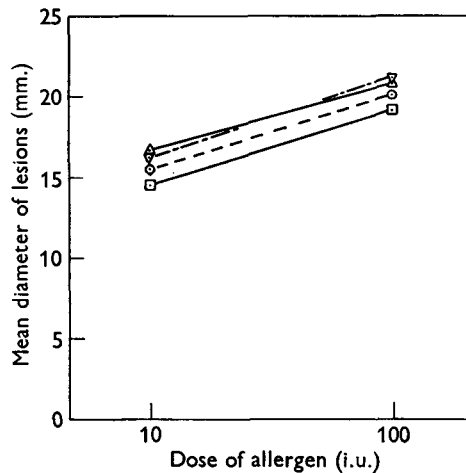Fig. 5. Dose response of hyperimmune guinea-pigs to intradermal toxoid.

Fig. 6. △, BCG; ⊙, international standard old tuberculin; ⊡, international standard PPD; ▽, tuberculo-protein.

significant, though slight, early response cannot be transferred with serum and has not yet been transferred with cells, although failure of the latter may be a question of quantity. The progress of the delayed responses to the various preparations of tuberculin show marked differences. They are all 'delayed' (tuberculin-type) reactions but the response to BCG might be more properly termed a 'Koch phenomenon'. The curve obtained with tuberculo-protein resembles that obtained with formol toxoid in actively partially immune guinea-pigs (Figs. 1, 3). This

experiment shows very clearly that the time of reading an assay of different preparations of tuberculin is important. The percentage 'take' following testing with a particular type of tuberculo-protein might well be influenced by the interval between injection and reading.

Experiment VII. In Expt. VII the effect of an early (Arthus-type) reaction to formol toxoid on the development of a delayed reaction to international standard PPD is studied. The resulting artificially combined 'early' and 'delayed' reactions produce two humps in the response curve (Fig. 8) analogous to those obtained
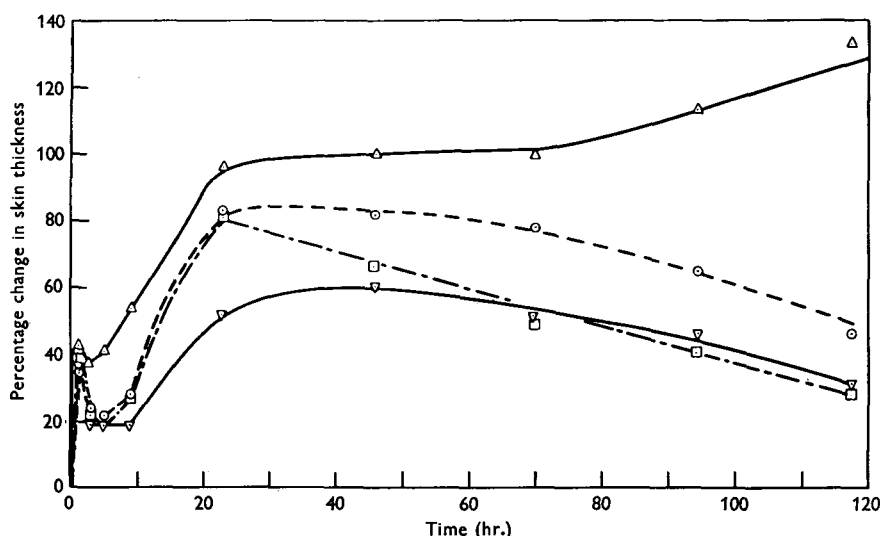

Fig. 7. Response of BCG-infected guinea-pigs to intradermal allergens. △, BCG (0·1 mg.); ⊙, international standard old tuberculin (100 i.u.); ⊡, tuberculo-protein (100 i.u.); ▽, international standard PPD (100 i.u.).

naturally with formol toxoid in actively partially immunized guinea-pigs (Fig. 3). This supports the hypothesis that hypersensitivity curves to diphtheria toxoid are due to a mixture of early (Arthus-type) and delayed (tuberculin-type) reactions.

Experiment VIII. In Expt. VIII the response of actively hyperimmunized guinea-pigs to formol toxoid is compared with that to alum-precipitated toxoid (Fig. 9). The response curve to the latter is similar to that obtained with BCG-infected guinea-pigs (Fig. 7). In this respect the BCG vaccine and the diphtheria antigen, in this particulate and insoluble form, might both be said to produce a 'Koch phenomenon', a fact that might be important in view of the association between adsorbed antigens and an increased incidence of paralysis in patients with poliomyelitis (Ben-Efraim & Long, 1957).

DISCUSSION

The allergic response curve to formol toxoid of guinea-pigs actively immunized with a single dose of APT (2·5 Lf) consists of two humps. These indicate an 'early' and a 'late' allergic response. This 'two-humped' response does not occur in hyperimmune guinea-pigs (Figs. 1, 2) even when the severity of the reactions is reduced

by decreasing the dose of the test allergen (Fig. 5). On the other hand, the response of such hyperimmune guinea-pigs cannot be transferred completely with serum (Fig. 2). An early response can be transferred with serum, but this does not
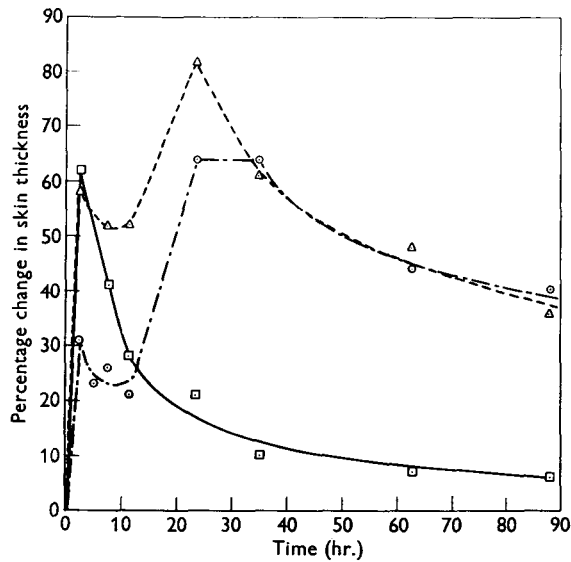

Fig. 8. Response of guinea-pigs infected with BCG and/or immunized with diphtheria anti-toxin to intradermal injection of a mixture containing formol toxoid (40 Lf) and PPD (100 i.u.). △, BCG + antitoxin; ⊙, BCG; ⊡, antitoxin.
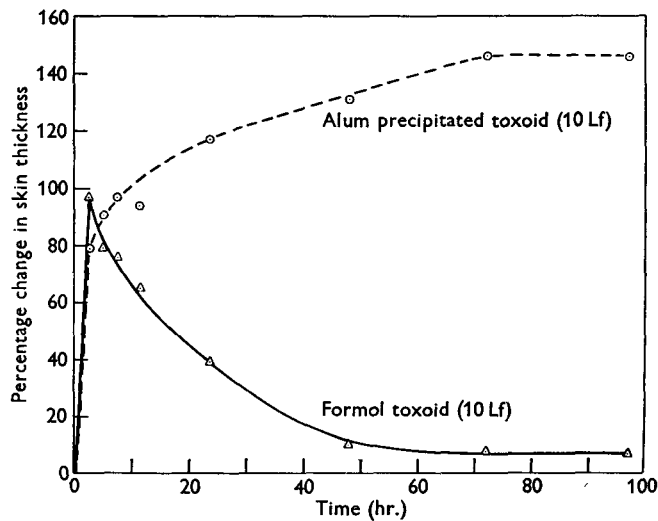

Fig. 9. Response of hyperimmune guinea-pigs to intradermal toxoids.

persist for as long as an analogous response in actively immunized animals. The obvious hypothesis that hypersensitivity to formol toxoid in guinea-pigs immunized with APT consists of a mixture of immediate (Arthus-type) reactions and delayed (tuberculin-type) reactions is probably true but difficult to prove.

The logical next step is to attempt to passively transfer a pure 'delayed' response with cells, and to prove that sera will transfer the 'early response' and cells the 'delayed response', and that a combination of the two produces a replica of the curves shown in Figs. 1 and 2. But it had not been appreciated that peritoneal cells, mostly lymphocytes, produced circulating antitoxin in such large amounts in the recipient, that an 'early response' was passively transferred and the 'delayed response', if it indeed existed, was masked. The response curves passively transferred with cells (Fig. 4) and that produced by 0·1 Lf of formol toxoid in actively immunized animals (Fig. 5) are almost identical. It is therefore probable that mononuclear cells transfer both elements of hypersensitivity whereas serum does not. But these experiments do not prove the presence of a 'delayed response'.

The artificial blending of an 'early response' to formol toxoid and a 'delayed response' to tuberculin (Fig. 8) produced a two-humped response curve analogous to those produced naturally in Figs. 2 and 3. This synthetic evidence supports the hypothesis that hypersensitivity to diphtheria toxoid is a mixture of early and late reactions, but again it does not provide proof.

Indeed, it is difficult to see how proof can be obtained. Histological evidence is, in the author's hands, unreliable as an index of the pathogenesis of allergic reactions. It is possible that a histological examination of lesions of comparative size in actively and passively immunized (see Fig. 2) guinea-pigs might show a difference, and that by carrying out quantitative immunological methods and histological studies in parallel, a real contribution might be made. Although some histological studies have been reported (see the stimulating review by Ovary (1958)), for the most part they lack precision and can be excluded from serious consideration on technical grounds.

Attempts have been made to decrease the early response with antihistamines and the delayed response with corticosteroids (unpublished observations). This work is being continued and with the corticosteroids of increasing potency now available this may prove to be a more fruitful approach. But although such methods strengthen the evidence, they do not provide proof of the hypothesis.

The response of actively immunized guinea-pigs to the intradermal injections of diphtheria toxin is similar to the response to toxoid. It differs only in the fact that damage, directly due to the toxic action of toxin, is superimposed on damage due to hypersensitivity to toxin. The degree of toxic damage depends on the titre of circulating antitoxin (Long, 1950).

How far this work is related to that of Uhr and his colleagues (1957) and of Salvin (1958) is uncertain, because the antigen they used was not APT. In addition, their methods for detecting an early (Arthus-type) reaction and excluding the presence of circulating antitoxin were less sensitive than those described in this paper. The relative immunizing and sensitizing properties of alum-precipitated diphtheria toxoid and of the antigen-antibody complex used by these workers (Uhr *et al.* 1957) are being investigated.

It is noteworthy that a peritoneal exudate rich in lymphocytes but containing few, if any, plasma cells produced much diphtheria antitoxin.

The passive transfer of an 'early' (Arthus-type) reaction by this means would

suggest that precipitating antitoxin in large amounts is produced by such cells in guinea-pigs.

The different response curves recorded in Fig. 7 underline the heterogeneity of different preparations of tuberculo-protein (see Long *et al.* 1954) and those in Fig. 9 show the importance of the physical nature of test allergens in determining the shape of such curves.

SUMMARY

Guinea-pigs immunized 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 delayed (tuberculin-type) reactions, the former increasing as the level of circulating precipitating antitoxin rose and tending to mask the latter. In a hyperimmune guinea-pig the residual damage following the intradermal injection of toxin is due, at least in part, to hypersensitivity to toxin; toxicity probably contributes relatively little to the extent of the lesion in animals with a high titre of antitoxin.

The technical difficulties of proving the presence of a delayed allergic reaction in animals with an early allergic reaction are discussed.

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