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Transfer of Delayed and Arthus Sensitivity with Blood Plasma from X-Irradiated Guinea-Pigs

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Summary. Lethal X-irradiation of sensitized guinea-pigs failed to release a mediator of delayed-type hypersensitivity into plasma in sufficient concentration to effect passive transfer of reactivity to tuberculoprotein or hen ovalbumin. But plasma from such animals did sensitize recipients for an Arthus reaction; and some of this early inflammation, both in actively and passively sensitized subjects, was still apparent at 24 hours. The significance of these late reactions is discussed in relation to the problem they pose when attempting to demonstrate a mediator of delayed-type hypersensitivity.

INTRODUCTION

Delayed-type hypersensitivity (DTH) has long been regarded as an immunological response mediated by intact lymphoid cells and not by circulating antibody (Landsteiner and Chase, 1942; Bloom and Chase, 1967). Failure of serum to effect passive transfer of sensitivity suggests that if antibody were involved, it is associated with intact cells and is effectively absent from serum. It has been reported, however, that a mediator of DTH is released following treatment of donors or their cells with the eliciting antigen *in vivo* (Kochan and Bendel, 1966) or *in vitro* (Kessel, Braun and Plescia, 1966). More recently, Dupuy, Perey and Good (1969) reported that a mediator of tuberculin sensitivity is released by X-irradiation into the plasma of sensitized guinea-pigs. A portion of the animals receiving plasma from irradiated donors displayed a 'positive DTH reaction' after repeated skin tests with PPD.

The possibility that the elusive vector of DTH is released from X-irradiated cells is of such theoretical importance that it prompted the present investigation; but in numerous attempts we have failed to duplicate the experimental findings of Dupuy *et al.* (1969) with even larger volumes of plasma taken from exquisitely sensitive guinea-pigs. Sporadic reactions to PPD were sometimes found at 24 hours; but they were trivial, and could be demonstrated to result in part, if not entirely, from incomplete resolution of an antecedent Arthus reaction.

EXPERIMENTAL METHODS

Animals

The guinea-pigs used were males and females of a randomly bred albino strain which has been maintained as a closed colony at the Trudeau Institute for more than 40 years. Plasma donors weighed approximately 500 g; and recipients, approximately 250 g.

Sensitization

Guinea-pigs were sensitized to ovalbumin, tuberculin or both. Immunization with ovalbumin (Pentex Inc., Kankakee, Illinois) made use of complete or Freund's incomplete adjuvant. In the former, *Mycobacterium tuberculosis* $H_{37}R_V$ (Trudeau Mycobacterial Collection, TMC No. 102) was added at a concentration of 1 mg/ml. The organism was grown on Proskauer and Beck medium for 10 days before the pellicle was removed, heated to 100°C for 10 minutes and lyophilized.

Guinea-pigs which were sensitized with 10 μg of ovalbumin in the *complete* adjuvant received a second injection of the same material, 14 days later. Those immunized with *incomplete* adjuvant were initially injected with 50 μg of ovalbumin. Intradermal injections of ovalbumin in 0·85 per cent sodium chloride were continued at weekly intervals until intense Arthus (3-hour) reactions were observed at the injection sites.

A third group of guinea-pigs were sensitized with living *M. bovis* (BCG, Phipps; TMC No. 1008). The organism was grown on Sauton's medium for 10 days before the pellicle was removed and homogenized in 1 per cent buffered gelatin (Collins, Mackaness, Montalbine and Smith, 1968). The inoculum was standardized photometrically and diluted to contain approximately 10^8 BCG/ml. At an interval of 14 days, guinea-pigs received two footpad injections containing $9{\cdot}3\times 10^6$ and $8{\cdot}8\times 10^6$ viable units, respectively.

X-Irradiation

Forty-eight hours after they were shown to have a satisfactory level of skin reactivity, the immunized guinea-pigs were exposed to a nominal mid-plane dose of 900 r of whole body X-irradiation. Pairs of guinea-pigs were placed on a masonite backscatter platform of 10 cm thickness. A Westinghouse single tube unit, 250 kV, 15 mA, with added filtration of 0·5 mm Cu and 1·0 mm Al, generated X-rays with a half-value layer of 1·5 mm Cu. The source to target distance was 50 cm, and the dose rate was 105 r/min.

Blood plasma

Panels of X-irradiated guinea-pigs were bled out by cardiac puncture 3, 4 and 5 days after irradiation. The blood was drawn into heparinized syringes, transferred to 50 ml polycarbonate tubes and centrifuged at 4° for 30 minutes at 1000 ***g***. Plasma obtained in this way was pooled and injected intraperitoneally into normal recipients. An aliquot of 120 ml of plasma taken from animals sensitized with BCG was centrifuged in the cold for 90 minutes at 100,000 ***g***. Cultivation of the sediment on Middlebrook 7H10 agar for 6 weeks failed to reveal any viable BCG.

Skin testing

Skin reactivity in plasma donors was measured by the intradermal injection of 2·5 μg of PPD in buffered diluent (Parke-Davis, Detroit, Michigan) or 10–50 μg of ovalbumin in saline. A 1 per cent (w/v) solution of ovalbumin was sterilized by passage through a sintered glass filter and diluted immediately before injection. Plasma recipients and normal guinea-pigs were skin tested with 10 μg of PPD, 50 μg of ovalbumin or both. In each case, an equal volume (0·1 ml) of buffered diluent or saline was injected into skin of the opposite flank.

Test injection sites were inspected at 3 hours and again at 24 hours for erythema and local thickening. In many cases, additional observations were made at 6, 48 and 72 hours. Skin thickening was measured by folding the skin about the injection site and gently

embracing it with strain gauge calipers (Schnelltaster-Kröplin, Frankfurt, Germany). Calculation of the difference in skin thickness before and after testing, less that present at the diluent or saline injection site, provided an estimate of the increase caused by antigen alone.

RESULTS

PASSIVE TRANSFER OF HYPERSENSITIVITY WITH PLASMA OF BCG-IMMUNIZED DONORS

A group of sixty guinea-pigs were infected twice with living BCG and shown to be exquisitely sensitive to 2·5 μg of PPD (Fig. 1). The circumscribed zones of erythema present at 24 hours persisted for at least 3 days, and a central area of necrosis occurred at many of the test sites.

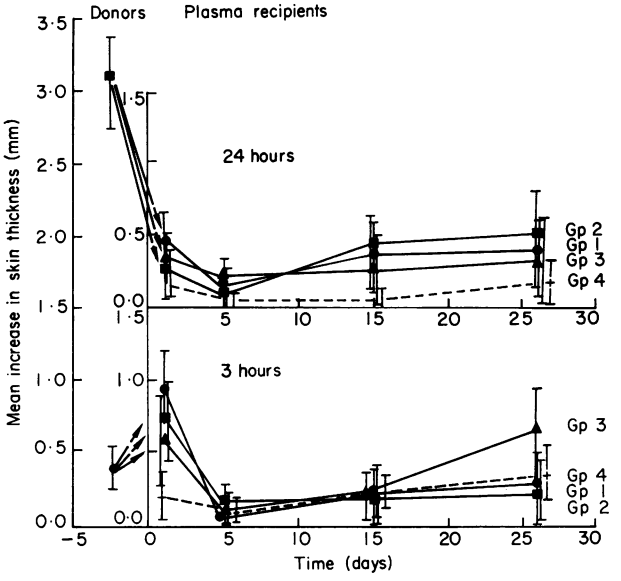

FIG. 1. Graphic representations of the results of skin tests in recipients of plasma from sensitized X-irradiated donors. The mean increase in skin thickness at 3 and 24 hours is indicated on the ordinate and the intervals chosen for repeated skin testing with respect to the time of plasma infusion, are shown on the abscissa. Donor reactivity immediately before 900 r whole body X-irradiation is shown in relation to the extreme left hand scale. Each donor and recipient panel consisted of ten guinea-pigs. Confidence intervals are ±2 SD. The respective differences in the treatment of donor and recipient panels are indicated for each figure. Donors sensitized with living BCG; recipients skin tested with 10 μg PPD. Gp 1, recipients of 16·4 ml of plasma; Gp 2, recipients of 16·4 ml of plasma; INH added to drinking water; Gp 3, recipients of 12·0 ml of centrifuged plasma; and Gp 4, controls, no plasma injected.

The entire panel of donor pigs were then irradiated and bled. The plasma was pooled and injected into three groups of normal guinea-pigs. Those of groups 1 and 2 were both given freshly-collected plasma; and the latter were provided continuously with drinking water containing 0·2 mg/ml of isonicotinic acid hydrazide (INH). This dose of INH was sufficient to inhibit the development of DTH in mice infected with 10^8 viable BCG (Lefford and Mackaness, personal communication). The guinea-pigs in group 3 were given plasma which had been centrifuged at 100,000 ***g*** for 90 minutes to remove particulate

material, including any BCG that might be present. Ten normal guinea-pigs of approximately the same age and weight served as untreated controls (group 4).

The animals of all groups were skin tested with 10 μg PPD on days 1, 5, 15 and 26 following plasma injection. Fig. 1 indicates that none of the plasma recipients exhibited tuberculin sensitivity. When examined 24 hours after injection of the skin test antigen, some of the plasma recipients, as well as several normal control guinea-pigs in group 4, had small zones of erythema (3–6 mm in diameter) and a trivial degree of local skin thickening. These feeble reactions were sporadic and could not be accepted as evidence of transferred DTH. Some animals had reactions on day 1 only, while others showed thickening (with or without erythema) on days 5, 15 or 26. The 3-hour readings indicated that the plasma recipients had some Arthus sensitivity to PPD when tested on day 1, but this was no longer present when the tests were repeated 4 days later.

From the foregoing experiment, it was concluded that X-irradiation failed to release a mediator of tuberculin sensitivity into the plasma of what were judged to be exquisitely sensitive donors. Measurement revealed some skin thickening at 24 hours; but inspection of the data suggested that this was in some manner linked to the accompanying state of Arthus sensitivity. Additional experiments were therefore undertaken to explore this relationship further. For the purpose in mind, it was expedient to immunize prospective plasma donors so as to favour the induction of either DTH or Arthus sensitivity.

PASSIVE TRANSFER OF HYPERSENSITIVITY WITH PLASMA FROM DONORS DISPLAYING MARKED DELAYED-TYPE HYPERSENSITIVITY

Plasma donors were sensitized with ovalbumin in water-in-oil emulsion containing added *Mycobacteria*. The donors displayed sensitivity toward PPD as well as ovalbumin, thus affording two opportunities to test the ability of X-irradiation to mobilize a mediator of DTH. Fig. 2 shows the skin reactions of plasma recipients tested with 10 μg of PPD.

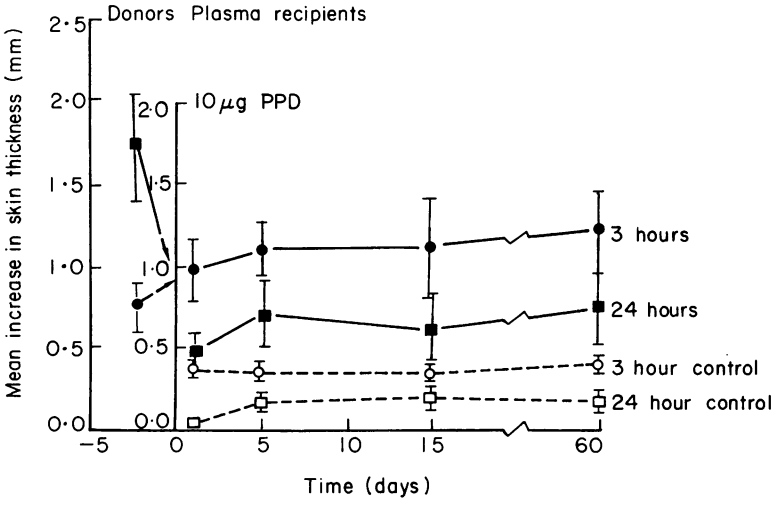

FIG. 2. Graphic representations of the results of skin tests in recipients of plasma from sensitized X-irradiated donors. Donors sensitized with ovalbumin in Freund's complete adjuvant; recipients injected with 12·0 ml plasma and skin tested with 10 μg PPD. ●, 3-hour reactions—plasma recipients; ○, 3-hour reactions—uninjected controls; ■, 24-hour reactions—plasma recipients; □, 24-hour reactions—uninjected controls.

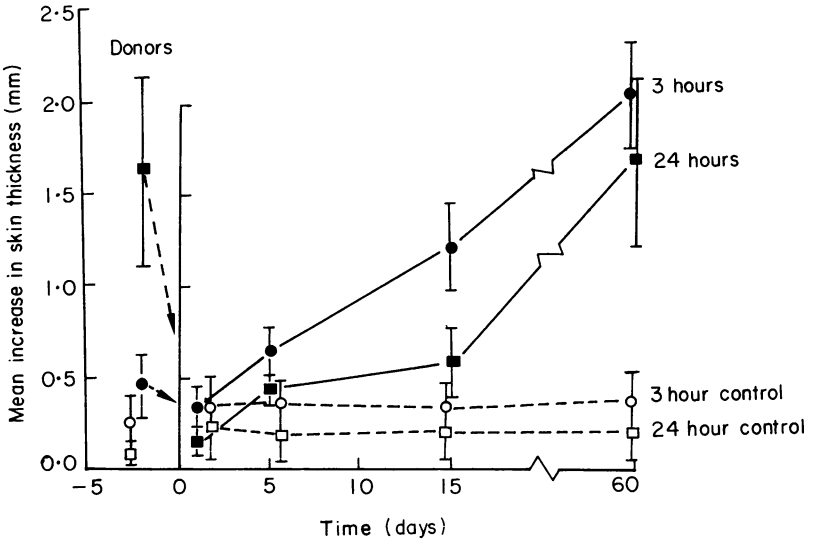

FIG. 3. Graphic representations of the results of skin tests in recipients of plasma from sensitized X-irradiated donors. Donors sensitized with ovalbumin in Freund's complete adjuvant; recipients injected with 12·0 ml plasma and skin tested with 50 μg ovalbumin. Key as in Fig. 2.

It will be noted that the plasma donors were highly sensitive to tuberculo-protein, yet 12·0 ml of their plasma failed to confer this sensitivity on normal guinea-pigs. In fact, the reactions elicited with PPD were no greater at 24 hours than those obtained in animals receiving plasma from PPD-insensitive donors (see Fig. 4).

Fig. 3 shows that guinea-pigs which were skin tested with 50 μg of ovalbumin after receiving 12 ml of plasma from donors displaying a high level of delayed hypersensitivity to this antigen, produced neither immediate nor delayed reactions at the first test. But repeated testing with the same dose of ovalbumin produced a rapidly rising level of Arthus sensitivity, and a corresponding but smaller reaction at 24 hours. This progressive increase in the 3-hour reactions suggested that the animals were becoming *actively* sensitized by the skin test antigen, but the reason for the rising reactions found at 24 hours could not be deduced from these results alone. It was clear from the mounting nature of the reactions, however, that they were not the result of *passive* sensitization.

PASSIVE TRANSFER OF HYPERSENSITIVITY WITH PLASMA FROM DONORS LACKING DTH

More compelling evidence that the 24-hour skin reactions in passively sensitized animals were unrelated to a state of delayed-type hypersensitivity emerged from an experiment in which the plasma donors were sensitized with ovalbumin in Freund's *incomplete* adjuvant. Guinea-pigs sensitized in this manner developed very little Arthus, and no delayed sensitivity to PPD; and their plasma after irradiation was almost inert so far as transfer of sensitivity to this test antigen was concerned (Fig. 4). The same donors did, however, develop very marked Arthus sensitivity to ovalbumin; and 10·8 ml of their plasma conferred mild 3-hour reactions upon each of ten recipients (Fig. 5). These reactions to 50 μg ovalbumin decreased slightly between the 1st and 5th days after plasma transfer; but increased thereafter to a level comparable with that present in the donors.

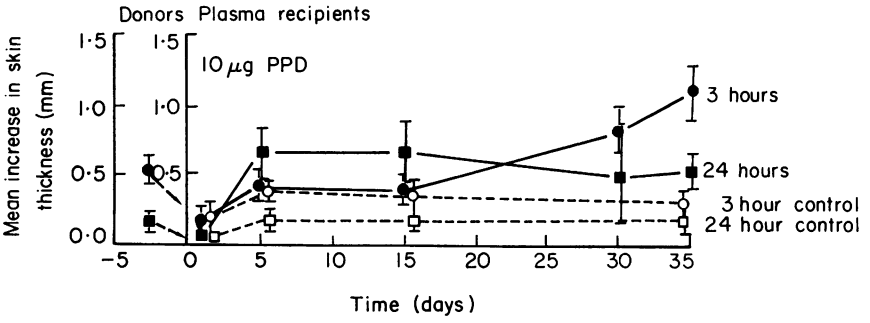

FIG. 4. Graphic representations of the results of skin tests in recipients of plasma from sensitized X-irradiated donors. Donors sensitized with ovalbumin in Freund's incomplete adjuvant; recipients injected with 12·0 ml of plasma and skin tested with 10 μg PPD. Key as in Fig. 2.

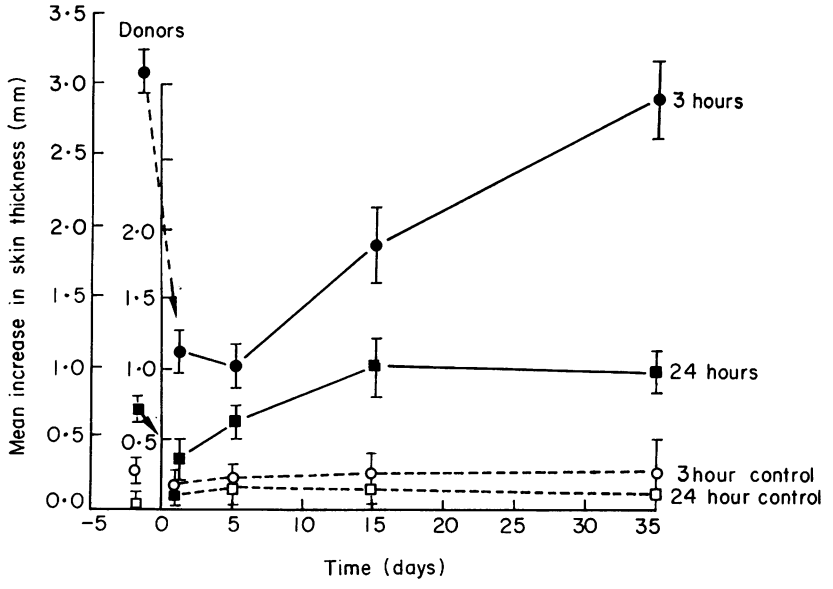

FIG. 5. Graphic representations of the results of skin tests in recipients of plasma from sensitized X-irradiated donors. Donors sensitized with ovalbumin in Freund's incomplete adjuvant; recipients injected with 10·8 ml of plasma and skin tested with 50 μg ovalbumin. Key as in Fig. 2.

There can be little doubt that the skin reactions obtained on day 1 were mediated by donor antibody because the same plasma, when mixed with ovalbumin, gave a passive Arthus reaction when injected into the skin of normal guinea-pigs (Fig. 6).

The plasma used in the foregoing experiment could not be expected to transfer DTH, because the donors themselves showed little if any delayed sensitivity toward ovalbumin. Nonetheless, the data of Fig. 5 show that ovalbumin did elicit a feeble 24-hour reaction in the skins of animals tested on the first day after plasma transfer. Again the late reactions tended to increase with repeated testing; but here, too, they were invariably less than those observed at 3 hours.

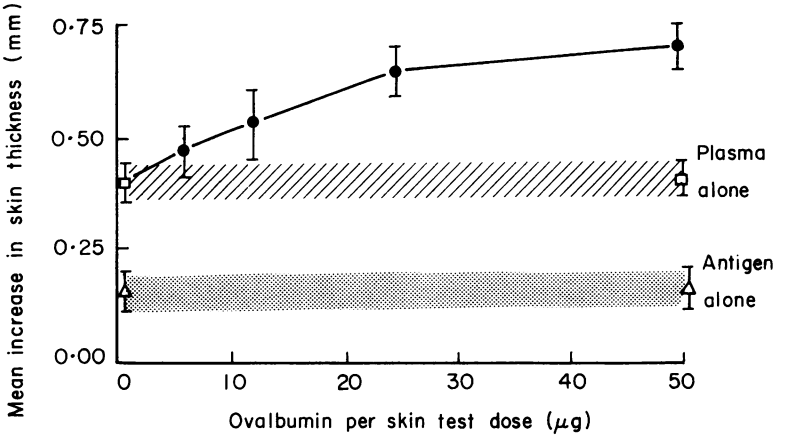

FIG. 6. Passive Arthus reactions in the skin of ten normal guinea-pigs injected intradermally with plasma premixed *in vitro* with increasing amount of ovalbumin (●). The plasma donors were sensitized by repeated injections of ovalbumin prior to X-irradiation. The coarsely hatched bar represents the 95 per cent confidence limits of the swelling observed 3 hours after injection of undiluted plasma alone into normal guinea-pigs. The stippled bar represents the corresponding reaction to 50 μg ovalbumin alone. The vertical lines represent ±2 SE.

EFFECT OF REPEATED SKIN TESTING ON DEVELOPMENT OF SENSITIVITY TO OVALBUMIN

In the foregoing experiments, repeated intradermal injections of ovalbumin disclosed an increasing level of Arthus sensitivity in plasma-inoculated guinea-pigs. The possibility that Arthus sensitivity can be induced by the testing procedure alone was examined in a panel of normal guinea-pigs. The animals employed in these experiments received no plasma; yet Fig. 7 indicates that they developed Arthus sensitivity within 2 weeks, and that the

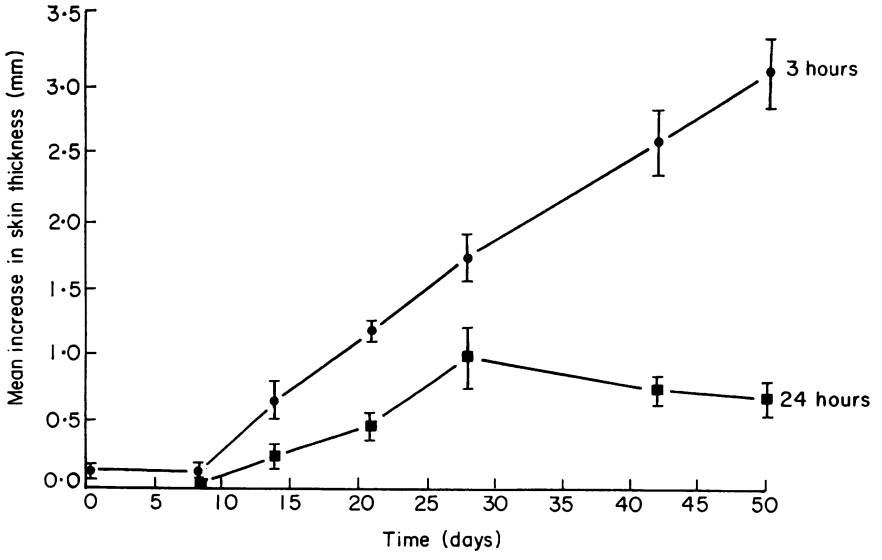

FIG. 7. The mean increase in skin thickness at 3 hours (●) and 24 hours (■) is shown in normal guinea-pigs skin tested repeatedly with 50 μg ovalbumin. The vertical lines represent ±2 SE.

level of sensitivity increased with repeated testing. Although the antigen was presented in such a manner that little or no DTH would be expected, the animals displayed some skin thickening at 24 hours in tests performed from day 14 onwards.

DISCUSSION

The recent report by Dupuy *et al.* (1969) that X-irradiation releases a mediator of tuberculin sensitivity into the plasma of tuberculin sensitive guinea-pigs was of great interest because of the important implications it holds. The present investigation was therefore undertaken in an attempt to corroborate the finding. Our experimental protocol was closely modelled on that of Dupuy *et al.* (1969); but the results obtained have been interpreted in quite a different way. The point of departure seems to lie in the evaluation of the skin reactions. In the present investigation, testing with PPD or ovalbumin sometimes resulted in local erythema and some skin thickening at 24 hours in passively sensitized animals; but these reactions could not be accepted as evidence of passively acquired DTH. Their erratic incidence, and size, and their tendency to increase with repeated testing support this contention. An even more compelling argument against the passive sensitization being of the delayed-type lies in the fact that the skin reactions bore no systematic relationship to the level of delayed-type sensitivity in the donors.

It was considered possible that the plasma of the donors might contain living BCG as a result of irradiation and that active sensitization of plasma recipients might explain the reported findings. But the recipients of uncentrifuged and unfiltered plasma failed to develop sensitivity, so that the precaution of ensuring the absence of BCG from the plasma and of treating recipients with INH gave no reward.

The question of what to regard as a 'positive' delayed reaction merits further comment. There is no difficulty in the case of strongly positive reactions, but the insensitivity of present assay methods becomes apparent when the reactions are weak, and especially when they are preceded by an Arthus reaction. The reactions obtained in adoptively sensitized animals are commonly of this type, but they are usually only a fraction of the donor's reactivity. The scoring of a marginal reaction becomes even less reliable when the method of evaluation involves subjective factors. The standards used for evaluating DTH reactions in skin are arbitrary; some workers have selected a diameter of 5 mm of erythema and induration as the lower limit of a positive reaction (Dupuy *et al.*, 1969), while others have chosen 10 mm (Lubaroff and Waksman, 1968). Obviously, the more liberal the criteria, the more difficult it becomes to discriminate between a specific DTH reaction and the inflammation caused by trauma, infection, or residual Arthus reactivity.

Uhr, Salvin and Pappenheimer (1957) first commented on the difficulty of assessing small DTH reactions in animals exhibiting intense Arthus sensitivity. Nelson and Boyden (1964) later used quantitative measurements of skin thickness to show that the acute inflammation elicited by antigen in Arthus sensitive guinea-pigs frequently fails to resolve within 24 hours. The residual skin thickening complicates, and may partially obscure, the evolution of a DTH reaction. Perhaps the feeble 24-hour reactions observed in the present study in plasma-inoculated subjects were also caused by protracted Arthus reactions. Even normal guinea-pigs developed high levels of Arthus sensitivity, together with smaller amounts of 24-hour swelling, when they were injected repeatedly with the skin test antigen (Fig. 7).

Increasing reactions at 3 hours were invariably accompanied by increased skin thickness at 24 hours. Presumably the acute inflammation elicited in Arthus-sensitive subjects failed to resolve within 24 hours (Nelson and Boyden, 1964). There is, however, a second possibility. Mills, O'Grady and Riley (1960) found that PPD can induce low levels of delayed sensitivity in putatively normal guinea-pigs. Antigen alone may be effective in this respect or the sensitivity may be evoked by immune complexes. Uhr *et al.* (1960) showed that immune complexes in antibody excess can induce DTH when the complexes are injected intradermally. It is tempting to speculate that, in the present experiments, such complexes were formed *in vivo* when circulating antibody combined locally with the skin test antigen. In this event, some of the reactions formed at 24 hours could have resulted from active sensitization (Fig. 3).

The antigenic specificity manifest in animals adoptively sensitized with living lymphoid cells implies that delayed hypersensitivity (and possibly other forms of cellular immunity) are mediated through antibody-like receptors attached to the surface of lymphocytes or monocytes (Mackaness and Blanden, 1967). The failure of the present experiments to demonstrate the release of a functionally active agent into the plasma of lethally X-irradiated guinea-pigs implies that the specific mediator is not easily dislodged from cell surfaces or cannot attach to the cells of normal recipients to render them effective vectors of delayed-type hypersensitivity.

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