Immunology, 1962, 5, 153.

The Prevention of Delayed Hypersensitivity to Homologous Serum and Transplantation Antigens in Guinea Pigs

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(*Received 12th April* 1961)

Summary. The injection of normal guinea-pig serum in Freund's complete adjuvant into guinea pigs other than the serum donor led to the development of a long-lasting, delayed hypersensitivity. Serum alone, without adjuvant, had no sensitizing capacity. Circulating antibodies to the allotypic antigens could not be detected.

Injection of as much as 8 ml. of serum into sensitized animals did not achieve desensitization. However, the intravenous injection of the same amount of serum prevented normal guinea pigs from becoming sensitized to the same antigen for over 100 days. This unresponsiveness was interpreted to be due to an interference by the serum with the process of sensitization.

This method of producing unresponsiveness was applied to the homograft reaction: guinea pigs, given a series of intravenous injections of spleen extracts containing transplantation antigens, could not be subsequently sensitized by the injection of spleen cells from the same donors. However, immunization provided by skin grafting could probably break through this unresponsiveness: guinea pigs, judged to be unresponsive by the intradermal injection of spleen cells before skin grafting, all developed an intense cutaneous hypersensitivity after they had rejected the graft.

INTRODUCTION

Delayed hypersensitivity reactions have been demonstrated in bacterial (Rich, 1941) and fungal (Conant, 1952) infections, in sensitivity to simple chemicals (Chase, 1959), and in some instances, in reactions to purified protein antigens (Dienes and Schoenheit, 1929; Uhr, Salvin and Pappenheimer, 1957). Recently it has been suggested that this type of immune reaction might also play a role in the rejection of skin homografts (Medawar, 1946; Mitchison and Dube, 1955; Lawrence, 1960). The analogy between delayed hypersensitivity and the homograft reaction has been strengthened by the demonstration of Brent, Brown and Medawar (1958) that guinea pigs sensitized by skin grafting can also respond to an intradermal injection of the appropriate antigens with a characteristic delayed skin reaction.

The study of delayed hypersensitivity has been greatly hindered by the difficulty of inducing it without stimulating the production of circulating antibodies. The relationship

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between these two types of responses is not clear. Uhr *et al.* (1957), using antibody antigen conjugates for immunization could induce delayed hypersensitivity to a given antigen, but succeeded only in retarding the appearance of circulating antibodies. Recently, while the present investigation was in progress, Benacerraf and Gell (1961) reported that delayed hypersensitivity in guinea pigs to homologous serum proteins could be induced while circulating antibodies to these antigens could not be detected. Uhr and Pappenheimer (1958) have found that intravenous injection of the antigen into sensitized animals will desensitize the delayed reaction temporarily, but will accelerate the appearance of circulating antibodies. The only instance of a successful and long lasting 'blocking'of delayed hypersensitivity has been that reported by Chase (1946): guinea pigs fed with picryl chloride before sensitization could not be sensitized subsequently to the same chemical.

Results obtained in the present investigation confirming and extending those of Benacerraf and Gell (1961) will be presented. In addition a method of rendering adult guinea pigs specifically unresponsive to sensitization with homologous serum and the application of this method to the homograft reaction will be described.

MATERIALS AND METHODS

Two groups of guinea pigs were employed in this investigation: guinea pigs belonging to an inbred strain (Heston) were used as a source of transplantation antigens and skin; outbred albinos of both sexes, weighing approximately 400 g., were used as recipients for sensitization and skin grafting. Both groups were supplied by The Research Institute, Animal Virus Diseases, Pirbright, Surrey.

ANTIGENS. The antigens used were guinea-pig serum, serum protein fractions, a red blood cell lysate, spleen cells and extracts of transplantation antigens.

In order to have a constant supply of guinea-pig serum containing the same number and distribution of allotypic antigens, two preparations were used throughout: serum from guinea pigs of the Heston strain (I serum) and a serum pool (P serum) prepared from the blood of 150 donors selected for maximum disparity according to coat colour and origin. The former was freshly drawn as required, while the latter was kept frozen in small aliquots. Freezing and thawing three times, heating at 56° for 30 minutes and, storage at −20° for as long as 6 months did not impair the antigenicity of the sera.

The two serum protein fractions used were prepared from P serum by fractionation with ammonium sulphate: they were the supernatant (albumin) and precipitate (globulins) obtained at 60 per cent saturation.

The red blood cell lysate was prepared by adding nineteen volumes of distilled water to one volume of thoroughly washed, packed red cells. The solution was clarified by centrifugation and solid sodium chloride was added to a concentration of 0.9 per cent. The final solution was obtained after a second centrifugation.

Cell suspensions and extracts of transplantation antigens were freshly prepared from spleens according to the method of Billingham, Brent and Medawar (1958). The last step in the preparation of the extract, i.e. the ultracentrifugation, was omitted. The concentration of the extracts was adjusted so that 0.1 ml. of the solution should correspond to not less than 25 mg. of the spleen.

SENSITIZATION. Sensitization of the guinea pigs consisted in injecting into the hind footpads 0.1 ml. of the antigen in an equal volume of Freund's complete adjuvant (Difco).

DETECTION of SENSITIVITY. Guinea pigs were usually skin tested 6 days after sensitiza-

tion; they were clipped and their skin depilated with a barium-sulphide suspension. All test solutions were injected intradermally in a volume of 0.1 ml. The reactions were inspected 24 hours after skin testing and they were traced on a sheet of polythene.

DETECTION OF CIRCULATING ANTIBODIES. Tests for circulating antibodies were carried out by anaphylaxis: sensitized animals were injected with 1 ml. undiluted guinea-pig serum intravenously. All intravenous injections were given by the femoral vein.

SKIN GRAFTING. Skin-grafting experiments were performed as described by Billingham and Medawar (1951). Grafted guinea pigs were injected with 1 mg. tetracycline hydrochloride* on the first 4 post-operative days; they were also supplied with terramycin* (approximately 1.5 mg. per cent) in their drinking water. The skin grafts were usually inspected 6, 9, 12 and 14 days after grafting.

RESULTS

(A) REACTION TO THE ALLOTYPIC ANTIGENS IN GUINEA-PIG SERUM

Approximately 80 per cent of guinea pigs sensitized with a guinea-pig serum other than its own responded with a characteristic delayed skin reaction. The degree and distribution of skin reactions among twenty-four guinea pigs sensitized with P serum, shown in

TABLE 1

DEGREE AND DISTRIBUTION OF SKIN REACTIONS AMONG TWENTY-FOUR GUINEA PIGS SENSITIZED WITH A GUINEA-PIG SERUM POOL

Reaction	*Very strong*	*Strong*	*Weak*	*No reaction*
Diameter	12–20 mm.	10–20 mm.	5–15 mm.	
Colour	deep purple	red	faint red	
Number 24	7	9	4	4
Per cent	29	37	17	17

Table 1, was typical of the pattern obtained in over a hundred guinea pigs tested. The evidence for the reaction being of the delayed, cellular variety is two-fold: (1) The reaction first appeared at about 8 hours and was maximum 24 hours after skin testing. (2) Circulating antibodies could not be detected at any time during 6 months after sensitization in guinea pigs showing a high degree of skin sensitivity. As much as 10 ml. of serum of a highly sensitive donor could not transfer the reaction to a normal recipient.

Reactivity to serum was fully developed 5 days after sensitization and was of long duration. Of twenty-seven guinea pigs sensitized with P serum, three groups of five tested on the first, second and third day after sensitization, respectively, were negative. Two out of six guinea pigs challenged at 4 days gave positive reactions while six guinea pigs tested at 5 days all reacted. Of the six guinea pigs, first tested at 4 days, four gave positive reactions when tested again a week later indicating that, at least four in this group were

* Pfizer, Folkestone, Kent.

capable of acquiring sensitivity to P serum. In an experiment designed to find out the duration of sensitivity twenty-four guinea pigs were sensitized: twenty-one were found reactive 7 days after sensitization; thirteen of these were tested again a week later when five gave reactions of the same intensity as before, four gave stronger and four gave weaker reactions. Six guinea pigs tested 3 and 5½ months later were just as positive as they had been 7 days after sensitization.

The reaction was found to be highly specific. Guinea pigs injected with Freund's adjuvant or with serum alone did not respond to challenge with guinea-pig serum. Single or repeated injections of serum given intravenously, subcutaneously or intradermally, in amounts up to 10 ml., had no sensitizing capacity. Serum of inbred guinea pigs did not sensitize animals from within the same inbred strain: six inbred (Heston) guinea pigs injected with I serum and Freund's complete adjuvant did not develop sensitivity to the serum.

TABLE 2

THE SPECIFICITY OF THE DELAYED CUTANEOUS RESPONSE IN GUINEA PIGS SENSITIZED TO VARIOUS GUINEA-PIG TISSUES*

Number sensitized	*Tissues used for sensitization*	*Tissues used for skin testing*		
		Spleen extract	*Serum or serum globulins*	*Red blood cell lysate*
14	Spleen cells or spleen extract	14/14†	No reaction	No reaction
5	Serum	No reaction	5/5†	No reaction
2	Red blood cell lysate	Not tested	No reaction	No reaction

* Red blood cell lysate was included in this experiment as a control, because several of the sera used were haemolysed.

† The numerator refers to the number of positive reactions obtained; the denominator gives the number of animals tested.

The allotypic antigens of the serum were distinct from the individual specific transplantation antigens (Table 2). Five guinea pigs sensitized with I serum gave no reaction to spleen extracts, also derived from inbred guinea pigs; the fourteen guinea pigs sensitized with spleen cells and antigenic extracts did not give a reaction to I serum. The antigens in serum were not derived from constituents of red blood cells: thus red blood cell lysates could neither sensitize nor evoke a reaction in guinea pigs sensitized to serum and spleen antigens. Serum globulin preparations were as efficient in producing and detecting sensitivity as whole, unfractionated, sera; serum albumin was devoid of activity.

Desensitization and the Production of Immunological Unresponsiveness

Desensitization was attempted by injecting sensitized animals with high doses of serum.* Twelve guinea pigs were given a series of five injections during the 10 days following sensitization; six were injected intravenously and six subcutaneously and intraderm-

* That small amounts of serum, injected intradermally, did not interfere with the reaction was indicated by the observation that guinea pigs could be skin tested several times in succession with no apparent diminution in the degree of their sensitivity.

ally, each guinea pig receiving a total of 5 ml. of serum. Skin testing on the eleventh day did not reveal any effect ascribable to the injections: three reactions were very strong, five strong and four essentially negative, as might have been expected in sensitized but otherwise untreated animals (see Table 1). If, however, sensitization was preceded, rather than followed, by a series of serum injections, the animals did not develop either delayed or anaphylactic sensitivity to the serum. Fifteen guinea pigs were injected with 1 ml. aliquots of P serum before sensitization (i.e. injection of 0.1 ml. of P serum in an equal volume of Freund's adjuvant): eight were given five injections intravenously and seven received seven injections subcutaneously and intradermally in the course of 2 weeks. Seven recipients gave negative reactions when skin tested 1 day before and 9, 17, 25, 43, 74 and 104 days after sensitization. Sensitization at 144 days elicited a definite positive response in all seven guinea pigs. Six other recipients were sensitized at day 1, 37 and 119 and were skin tested 9, 17, 25, 43, 74, 118 and 125 days after the first sensitization. Positive reactions appeared only after the third sensitization.

In subsequent experiments two groups of eight guinea pigs were rendered unresponsive to I serum and fourteen animals to P serum, all having been injected intravenously; intradermal and subcutaneous injections did not induce unresponsiveness regularly.

This refractoriness to sensitization was found to be specific; although guinea pigs unresponsive to P serum could not be sensitized to I serum, or vice versa, seven guinea pigs unresponsive to P serum, could all acquire sensitivity to I spleen cells.

(B) REACTION TO TRANSPLANTATION ANTIGENS

Skin reactions to transplantation antigens could be produced regularly by the injection of either intact spleen cells or antigenic extracts in adjuvant, 6 days before testing. Antigenic extracts alone, without adjuvants, could not elicit a reaction. This reaction was also specific; six inbred guinea pigs could not be sensitized with antigenic extracts (and adjuvant) prepared from the spleens of guinea pigs of the same strain although the same preparation elicited very strong reactions in eleven outbred, sensitized, animals (see also Table 2).

Since antigenic extracts alone could not render guinea pigs sensitive, it was possible to apply to this system the method used to produce unresponsiveness to the allotypic antigens of the serum. In a preliminary experiment eight guinea pigs were given a series of five intravenous injections of antigenic extract; extract from one spleen was divided among the eight recipients for each injection. Although the reactivity of these guinea pigs was not completely supressed, the results were sufficiently different from those given by uninjected, but sensitized, controls to warrant further experiments: two of the eight guinea pigs gave strong and and six gave weak reactions, while five reactions in the control group were extremely strong, two strong and one weak (Fig. 1). In subsequent experiments comprising a total of thirteen animals, eight pre-injections were given, raising the total amount of extract injected into each guinea pig to that derived from a whole spleen. Of the thirteen animals sensitized and skin tested only one gave a positive skin reaction, indicating that unresponsiveness, as judged by the skin reaction, was achieved in twelve out of thirteen guinea pigs. Nevertheless ten of these guinea pigs subsequently skin grafted rejected the graft with a tempo indistinguishable from that of six grafted, but otherwise untreated, controls. These guinea pigs skin tested again 2 weeks after skin grafting responded with extremely strong reactions.

DISCUSSION

The allotypic reaction in guinea pigs takes the form of delayed hypersensitivity. This reaction was chosen for a study of delayed hypersensitivity mainly for three reasons: (1) Sensitivity to serum could be easily induced and once acquired was long lasting; (2) guinea pigs could be skin tested repeatedly without the danger of desensitization and (3) this sytem was free from the usual complications imposed by the presence of circulating antibodies and Arthus reactions.

Allotypy of serum proteins has been described in man (Cumley and Irwin, 1943) and in rabbits (Oudin, 1956; Dray and Young, 1958; Dubiski, Dudziak and Skalba, 1959). In

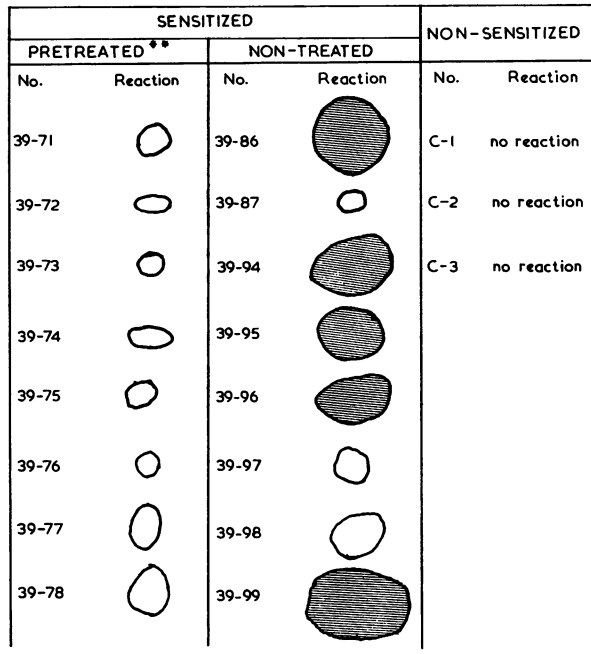

FIG. 1. Skin reaction of guinea pigs to transplantation antigens.*

* Drawings represent tracings of skin reactions, taken 24 hours after skin testing. Cross hatching denotes intense, purple-coloured reactions.

** Given a series of injections of spleen extracts containing transplantation antigens before sensitization.

rabbit serum the number of allotypes has been shown to be as high as 7 (Oudin, 1960). It is reasonable to assume that there are also several allotypes in guinea-pig serum (Benacerraf and Gell, 1961). Since the aim of this investigation was not to elucidate the number of allotypes in guinea-pig sera but to study the delayed hypersensitivity reaction induced by these antigens, it was essential to use antigenic preparations of the same allotypic composition, capable of sensitizing the maximum number of recipients. Both these requirements were met by the antigenic preparations used: the inbred guinea pigs could be relied upon to supply sera of the same composition and the guinea-pig serum pool could regularly sensitize 80 per cent of the recipients.

Sensitivity to the allotypic antigens was demonstrable for as long as 6 months after sensitization. Since all the guinea pigs observed were skin tested repeatedly, the possibility

cannot be excluded that the repeated antigenic stimulation was responsible for the long maintenance of the sensitized state. This possibility may, however, be remote as it would demand that the test antigen, which could not by itself induce sensitivity, be capable of perpetuating sensitivity once acquired. On the other hand it can be stated with certainty that the repeated injections of serum did not achieve the opposite effect, i.e. desensitization.

Circulating antibodies to the allotypic antigens could not be revealed either by direct anaphylaxis or by Arthus (early type) skin reactions in guinea pigs displaying a high degree of delayed sensitivity or unresponsiveness to these antigens. The failure to detect circulating antibodies in guinea pigs given single or multiple injections of serum, over a wide range of concentrations with or without adjuvants, might be relevant to a consideration of the hypothesis postulating that delayed hypersensitivity is an intermediary step leading to the production of circulating antibodies (Salvin and Smith, 1960).

Uhr and Pappenheimer (1958) succeeded in curtailing delayed hypersensitivity and accelerating the appearance of circulating antibodies in sensitized animals by the intravenous inoculation of the antigen. Injection of guinea-pig serum by any route, in amounts up to 8 ml., did not desensitize guinea pigs in the present investigation. However, the intravenous injection of the same amount of serum, before sensitization, prevented the recipients from becoming sensitized to the serum antigens. The process, by which unresponsiveness is acquired, cannot be desensitization, as the injections must precede sensitization, nor can it be attributed to an inhibition of the reaction by an 'antigen excess' as the same amount of serum could not interfere with the manifestation of a sensitivity already acquired; it has to be viewed rather as an interference by the serum with the process of sensitization. It is difficult to envisage this interference without assuming that the antigens reach the antibody-producing centres. One explanation of subsequent events may be that the antigen which reaches these centres is in a form which cannot stimulate but can only block the subsequent immune response. The serum antigens which were injected to produce unresponsiveness could not in fact, by themselves, without Freund's adjuvant, induce sensitivity. A similar phenomenon has been described by Chase (1946): guinea pigs, fed with picryl chloride became resistant to subsequent sensitization by the same chemical. Ingestion of picryl chloride, analogous to the intravenous injection of serum in the present experiments, introduces the antigen in a form which cannot produce hypersensitivity. In order to produce unresponsiveness, this step must precede sensitization, i.e. the administration of the antigen in an active form. This is achieved when the chemical is painted on the skin in one case, and when the serum is injected together with Freund's adjuvant, in the other. Chase believes that the unresponsiveness comes about as a result of blocking of the antibody-producing sites by the hapten.

Further elucidation of this reaction will depend upon a better understanding of the mode of action of adjuvants. If one accepts the view that the allotypic antigens can get to the antibody-producing centres in a concentration sufficient to block the antibody response, then clearly, the role of adjuvant in the process of sensitization must be more than simply promoting the transport of antigen.

Brent *et al.* (1958) have shown that transplantation hypersensitivity in guinea pigs can manifest itself as a dermal hypersensitivity of the delayed type. Antigens, which can induce transplantation hypersensitivity, can be obtained in cell-free extracts in a soluble form (Billingham *et al.*, 1958). This investigation has shown that such extracts, injected intravenously, like the allotypic antigens of the serum, could not induce delayed cutaneous

hypersensitivity and that a series of such injections rendered guinea pigs resistant to sensitization by intact spleen cells or by extracts in Freund's complete adjuvant. However, all the guinea pigs judged to be unresponsive by the intradermal injection of antigenic extracts or cells, rejected the corresponding skin grafts with a tempo indistinguishable from that of the controls. These results could be interpreted to mean either that delayed cutaneous reactions and the rejection of skin grafts are not manifestations of the same sensitivity or that skin grafting provides a stronger stimulus to the immune system than the injection of cells and can thus break through the unresponsiveness produced by the previous treatment. The fact that all the 'unresponsive' guinea pigs gave a very strong skin reaction after they had rejected the skin graft would favour the second interpretation.

ACKNOWLEDGMENTS

I wish to thank Professor Haddow for providing facilities to carry out this investigation and for his support and interest throughout this work. I gratefully acknowledge the financial support by the Jane Coffin Childs Memorial Fund for Medical Research. I am indebted to Dr. L. Brent, of University College, London, for a demonstration of the technique of skin grafting and to my colleague Dr. J. A. F. P. Miller and to Dr. J. L. Turk of the National Institute of Medical Research, London, for reading the manuscript. The technical assistance of Miss Janice Hayes is gratefully acknowledged. This investigation has been supported by grants to the Chester Beatty Research Institute (Institute of Cancer Research: Royal Cancer Hospital) from the Medical Research Council, the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service.

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