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Inhibition of Delayed Hypersensitivity by Pre-immunization without Complete Adjuvant

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Summary. Guinea-pigs were immunized by injections of blood group substance with incomplete adjuvant, followed after an interval of approximately 2 weeks, by intracutaneous immunization with the same antigen and Freund's adjuvant containing *M. tuberculosis*. This treatment inhibited the appearance of delayed skin reactions, while circulating antibody production took place as in controls which had received complete adjuvant only with blood group substance, and had delayed skin reactions. The inhibition of the skin reaction was found to be antigen-specific with regard to unrelated antigens, but showed cross-inhibition for serologically different human blood group substances. The first immunization had to be given more than 2 days before the immunization with complete adjuvant. A similar phenomenon was seen with ovalbumin as antigen. In addition to inhibition of the delayed skin reaction, there appeared to be less γ_2-antibody to ovalbumin than in ovalbumin plus complete adjuvant-only controls. Passive administration of antibody did not affect the development of a delayed hypersensitivity state in complete adjuvant-immunized animals with blood group substance or ovalbumin as antigen. Present evidence favours an explanation of the phenomenon in terms of temporary paralysis on the part of some of the antibody-producing cells—viz. those concerned with delayed hypersensitivity and γ_2-antibody production.

INTRODUCTION

The mechanism of delayed hypersensitivity to antigen is usually regarded as a cellular one, but its relationship to accompanying or subsequent antibody production, and the role of adjuvant in producing or enhancing it, are by no means well understood.

Guinea-pigs injected with a water-in-oil emulsion of a protein antigen show both increased levels of circulating antibody, and increased skin hypersensitivity of delayed type if killed mycobacteria are added to the injection mixture (Freund's complete adjuvant). With mucopolysaccharide antigens such as the blood group substances, the addition of mycobacteria to the adjuvant has the effect not only of enhancing the circulating antibody response, but also of initiating delayed hypersensitivity which, in our hands, does not otherwise develop in immunized animals. Our earlier experiments (Holborow and Loewi, 1962) indicated that, while circulating antibody produced by guinea-pigs in response to inoculation of blood group mucopolysaccharide is reactive with antigenic determinants carried on the polysaccharide components of the macromolecule (Morgan, 1960), delayed hypersensitivity is apparently mediated by a different part of the molecule, probably the peptide moiety. This apparent dissociation of immunological reactivities

within the antigen molecule led us to investigate the possibility of dissociating the two responses by sequential immunization with incomplete, followed by complete Freund's adjuvant. 'Incomplete' adjuvant differs from 'complete' adjuvant in containing no mycobacteria.

METHODS

Immunization and testing

Albino guinea-pigs, 250–350 g were used. In most experiments two injections at 1 week's interval of 100 μg antigen, either blood group substance or ovalbumin, were given with incomplete adjuvant into two footpads and intracutaneously or subcutaneously in a volume of 0·5 ml. One week after the second injection the animals were tested for both immediate and delayed hypersensitivity by an intracutaneous injection of 10 μg antigen in 0·1 ml of saline. Immediate hypersensitivity due to circulating antibody was demonstrated by giving 2 per cent Coomassie Blue solution intravenously and noting development of blue staining at the skin injection site (Holborow and Loewi, 1962); the delayed hypersensitivity reaction at the same site was looked for 24 hours later. Following this, 100 μg of antigen emulsified in complete Freund's adjuvant, containing 2 mg heat-killed *M. tuberculosis*/ml, in a volume of 0·5 ml was injected intradermally in several sites, in the nuchal fold. Ten days later, the animals were tested for circulating antibody and delayed hypersensitivity as before. Control animals were given complete instead of incomplete adjuvant at the earlier immunizations, and tested in the same way.

Antigens

Human blood group substances A and Lewis^a (Le^a) were generously provided by Professor W. T. J. Morgan of the Lister Institute, London. The immunological characteristics of these substances have been discussed in an earlier publication (Holborow and Loewi, 1962). Ovalbumin, twice crystallized, was obtained commercially.

Sera

Blood was obtained by heart puncture. Sera were stored at −20°. Tanned-cell haemagglutination was performed according to the method of Stavitsky (1954) using formalin-treated sheep red cells. Complement fixation was carried out by the method of Donnelley (1951), and passive cutaneous anaphylaxis by the method of Ovary and Bier (1953), allowing 18 hours for antibody fixation in the skin. Serum globulins were fractionated by DEAE-cellulose chromatography by the method of LoSpalluto, Chegoriansky, Lewis and Ziff (1960). Immuno-electrophoresis in agar was done in barbiturate HCl buffer at pH 8·2.

RESULTS

A. BLOOD GROUP SUBSTANCES AS ANTIGEN

Thirty guinea-pigs were given blood group A substance in incomplete adjuvant, followed by A substance with complete adjuvant. On testing, none of these animals showed a significant delayed reaction at 24 hours, i.e. erythema of more than 5 mm diameter or induration of the skin, at the site of challenge with 10 μg A substance. Twelve guinea-pigs served as controls in these experiments and were given complete Freund's adjuvant only

with blood group substance; all these showed delayed reactions, with an arithmetic mean diameter of 15 mm, with induration. Eight guinea-pigs, similarly given incomplete adjuvant with Lewis^a blood group substance, also failed to show delayed skin reactions following complete adjuvant. Although the delayed hypersensitivity response was inhibited in animals that had been pretreated with incomplete adjuvant, circulating antibody was regularly demonstrated, both before and after complete Freund's adjuvant by the intravenous Coomassie Blue technique, and also, in many animals, by haemagglutination. Precipitating antibody was not produced by any of the animals injected, and no Arthus reactions were seen at skin test sites in immune animals.

The specificity of inhibition of delayed hypersensitivity was tested as follows: six guinea-pigs were immunized with A substance and incomplete adjuvant. All showed evidence of circulating antibody by direct cutaneous anaphylaxis, but no delayed reaction at 24 hours. They were then re-immunized with 100 μg ovalbumin and complete Freund's adjuvant; 10 days later all showed delayed hypersensitivity reactions to a 10 μg ovalbumin challenge. However, cross-inhibition was shown between various human blood group substances. Guinea-pigs preimmunized with A substance and incomplete adjuvant were given a second injection of Le^a or another blood group substance with complete adjuvant; they showed no delayed skin responses when subsequently tested with A, Le^a or other blood group substances.

Time relations of inhibition

Twelve guinea-pigs were immunized with A substance and incomplete Freund's adjuvant. Following a further immunization with A substance in complete adjuvant, no delayed hypersensitivity responses were obtained to a test dose of 10 μg A substance.

TABLE 1

INFLUENCE OF TIME INTERVAL BETWEEN INCOMPLETE AND COMPLETE ADJUVANT IMMUNIZATION

Experimental group	Days following complete adjuvant	No. of animals showing circulating antibody	No. of animals showing 24-hour skin reaction of >6 mm diameter
A	0	6/8	0/8
	11	8/8	0/8
B	0	0/8	0/8
	11	8/8	3/8
C	0	0/8	0/8
	11	8/8	5/8
D	0	0/8	0/8
	11	8/8	8/8

Group A received A substance with incomplete adjuvant 14 days before A substance with complete adjuvant. For group B, the interval was 9 days, for group C 5 days, and for group D 2 days. All animals tested with 10 μg A substance immediately preceding complete adjuvant immunization and again 11 days later.

However, when tested again with a similar dose 1 month later, all animals showed 24-hour skin reactions, of average diameter 13 mm, with a range of 9–16 mm. This experiment demonstrated that the state of specific inhibition was not permanent.

In order to test the influence of the duration of the immune state on the subsequent failure to develop a delayed skin reaction, an experiment was set up in which different intervals occurred between immunization with antigen in incomplete adjuvant and antigen in complete adjuvant. A total of thirty-two guinea-pigs was divided into four

equal groups, designated A, B, C and D in Table 1. All were given A substance, four in each group 10 μg, the other four 100 μg, with incomplete adjuvant. Fourteen, 9, 5 and 2 days later, respective groups were given a second dose of A substance, but with complete adjuvant. Eleven days later all were tested for circulating antibody and delayed skin reactions to 1 and 10 μg test doses. The results are shown in Table 1. It is apparent that delayed cutaneous reactions were regularly inhibited with a 14-day interval after the first dose of antigen, inhibited in some animals with a 5 or 9 day interval, and not at all inhibited when only 2 days intervened.

Transfer experiments

To test whether inhibition of the delayed reaction might be due to the presence of a serum antibody elicited by immunization with incomplete adjuvant, immune serum was administered to guinea-pigs undergoing active immunization with complete adjuvant. The sera were obtained from donor animals that had been immunized with blood group substance and incomplete adjuvant (pool haemagglutination titre 1:1280), with blood group substance and complete adjuvant (pool haemagglutination titre 1:5120) or from normal guinea-pigs. Recipients were given 1 ml serum intravenously and injected with 100 μg blood group substance and complete adjuvant some hours later. Subsequently, 1 ml serum was given daily i.v. until the animals were challenged with 10 μg blood group substance on the tenth day after immunization. Results are shown in Table 2.

TABLE 2

DELAYED HYPERSENSITIVITY RESPONSES OF GUINEA-PIGS RECEIVING IMMUNE SERUM PASSIVELY AND UNDERGOING IMMUNIZATION WITH Le^a SUBSTANCE AND COMPLETE FREUND'S ADJUVANT

Serum donors' immunization regime	No. of animals in test groups	24-hour skin test with 10 μg Le^a substance (average diameter mm)
Incomplete adjuvant + Le^a	6	9 (range 8–11)
Complete adjuvant + Le^a	7	11 (range 6–14)
Unimmunized guinea-pigs	8	11 (range 8–14)

It was concluded that circulating antibody played no direct role in suppressing the delayed skin reaction.

An attempt was made to discover whether macrophages from animals that had been immunized with blood group substance and complete adjuvant could transfer delayed hypersensitivity to animals that had been made refractory by prior immunization with antigen and incomplete adjuvant. Donor guinea-pigs were immunized with A substance and complete Freund's adjuvant, and gave strong delayed skin reactions. Peritoneal macrophages were obtained after injection of paraffin. Each recipient was given $1{\cdot}5 \times 10^8$ cells intravenously and skin-tested with 50 μg A substance immediately. Some recipients had been prepared by immunization with A substance with incomplete followed by complete adjuvant with the same antigen. Delayed responses were poor in all recipients, whether they had been pretreated or not, and it was therefore not possible to draw any definite conclusion.

Circulating antibody response of inhibited animals

In several experiments, involving immunization with A substance, antibody was estimated by tanned-cell haemagglutination. Titres of sera of animals that had only complete adjuvant with antigen were usually higher than those of animals that had incomplete instead of complete adjuvant. When complete adjuvant was given following incomplete adjuvant, antibody titres rose, although no delayed skin response occurred.

TABLE 3

ANTI-A SUBSTANCE TANNED-CELL HAEMAGGLUTINATION TITRES (RECIPROCAL) OF GUINEA-PIGS IMMUNIZED WITH INCOMPLETE, FOLLOWED BY COMPLETE ADJUVANT WITH 100 μg A

Titre following incomplete adjuvant immunization	Titre following further immunization with complete adjuvant
320	1280
90	2430
810	7290
270	810
640	1280

Table 3 shows this increase of titre in sera from a group of guinea-pigs treated in this way. Separation of the globulins of these sera on DEAE-cellulose showed haemagglutinating activity in three peaks, eluted respectively at 0·01 M, pH 7·0; 0·1 M, pH 6·0; and 0·3 M, pH 5·0. The distribution was similar whether delayed hypersensitivity was inhibited or not.

B. OVALBUMIN AS ANTIGEN

When ovalbumin was used as antigen in experiments involving twenty guinea-pigs, delayed responses to antigen were again found to be inhibited in animals pretreated with incomplete adjuvant, and subsequently receiving complete adjuvant. Whether pretreated or not, guinea-pigs immunized with ovalbumin produced precipitating antibody and on skin testing exhibited reactions of the Arthus type.

Sera of guinea-pigs immunized with ovalbumin and complete adjuvant showed precipitation as well as complement fixation with ovalbumin. On the other hand, guinea-pigs receiving three injections of ovalbumin with incomplete adjuvant gave no complement-fixing antibody, even after two further doses of complete adjuvant. After a third dose of complete adjuvant, only one animal out of four tested produced complement-fixing antibody. Complement fixation was exclusively associated with the γ_2-globulin fraction of immune sera, as obtained by DEAE-cellulose chromatography. Sera that gave no complement fixation failed to show complement fixation by γ_2-globulin isolated from them. A further serological difference between animals given complete adjuvant and others that had been pre-treated with incomplete adjuvant was observed by immuno-electrophoresis in agar. Fig. 1 shows that animals pretreated with incomplete adjuvant formed less of the slow or γ_2-globulin fraction following subsequent complete adjuvant immunization. Thus, inhibition of the delayed cutaneous reaction appeared to be associated with reduced formation of γ_2-globulin in response to antigen with complete adjuvant, at least in the case of ovalbumin. As with blood group substance, we tested the

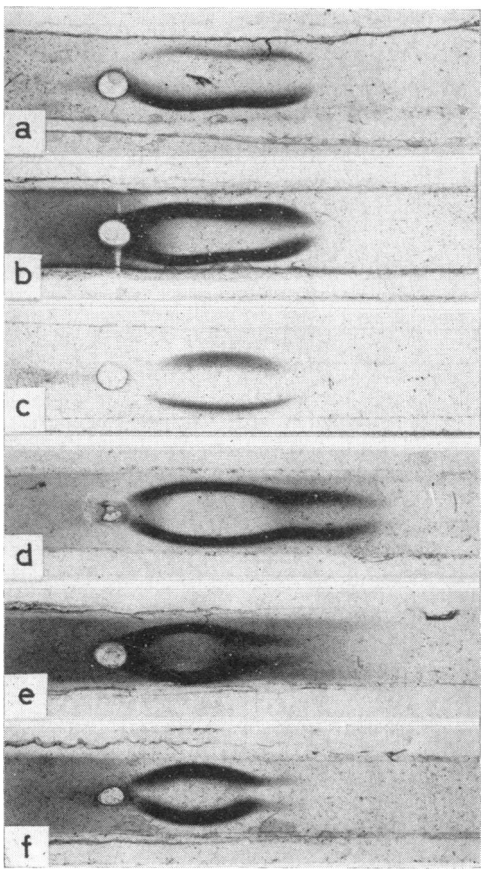

FIG. 1. Immuno-electrophoresis in agar of sera from guinea-pigs immunized with ovalbumin. (a) Three immunizations with complete adjuvant. Upper line developed with 10 μg ovalbumin/ml, lower with 100 μg ovalbumin/ml. γ_1 and γ_2 arcs present. (b) Four immunizations with complete adjuvant. γ_1 and γ_2 arcs. (c) Three immunizations with incomplete adjuvant. γ_1 line only. (d) Three doses of incomplete adjuvant followed by one dose complete adjuvant. γ_2 arc is much weaker than γ_1. (e) Three doses incomplete, followed by two of complete adjuvant. Very little γ_2. (f) Immunized as (d). Only trace of γ_2 present. (b–f) developed with 100 μg ovalbumin/ml.

effect of passively administered antibody on the state of delayed hypersensitivity produced by injection of ovalbumin and complete adjuvant. Serum containing complement-fixing antibody was given to guinea-pigs intravenously for the first 5 days following immunization. An unmodified delayed response was demonstrated on subsequent testing showing that no inhibition had been brought about by this regime.

DISCUSSION

Inhibition of development of delayed skin hypersensitivity by pretreatment of adult guinea-pigs with antigen has been previously noted. Boyden (1957) found that guinea-pigs given tuberculoprotein before BCG vaccination were subsequently tuberculin-negative, and Gordon (1962) found that guinea-pigs given allotypic serum alone could not subsequently be sensitized to the same serum given with complete adjuvant. Recently Asherson and Stone (1965) reported a similar phenomenon (which they term 'immune

deviation'), using several different protein antigens and hapten–protein conjugates. Chase (1946) originally observed that feeding picryl chloride to adult guinea-pigs prevented subsequent development of contact hypersensitivity to this hapten, and further investigations (for example, Battisto and Chase, 1965) show that in this system the delayed response can be completely circumvented, or can be directed to occur either before or after the appearance of circulating antibody.

The results reported here show that a similar inhibition of delayed hypersensitivity can be obtained with human blood group mucopolysaccharide. This antigen is appropriate for study in this connection not only because of the structural dissociation within the molecule of immunological reactivities already referred to, but also because it does not produce Arthus reactions at skin test sites in immune guinea-pigs, thus facilitating the reading of delayed skin reactions.

In addition we used ovalbumin in the present experiments, since more serological information could be obtained with the precipitating antibody against this antigen than with the non-precipitating antibody against blood group substance. In all our experiments we found that pre-injection of antigen with incomplete adjuvant, or without adjuvant, produced a state of temporary unresponsiveness as far as development of delayed skin reactivity to the same antigen was concerned. This state of inhibition was antigen-specific and lasted for at least 1 month.

Pre-immunization had to take place more than 2 days before immunization with complete adjuvant, for such inhibition to be demonstrable. This delay might simply reflect the time taken by antigen to reach all cellular sites of immune stimulation or blocking, or might represent the time taken to produce enough antibody of a type capable of blocking the mechanism of delayed hypersensitivity. The latter seems unlikely, unless a labile antibody is postulated, since we were unable to inhibit the delayed response by the passive administration of antiserum throughout the period of immunization. It therefore appears preferable to account for the observed inhibition in cellular terms, and two possible explanations come to mind. The first is that there are at least two populations of immunologically competent cells capable of responding to the mucopolysaccharide blood group substance antigen, one dealing with production of antibody to the polysaccharide determinants, and one with immune response to the peptide fraction. In such circumstances the inhibition observed might represent a selective immune paralysis of the second population of cells.

A second explanation would postulate that the type of response made by a single population of immunologically competent cells is pre-empted by the mode of presentation of the antigenic stimulus. Either of these explanations would allow the appearance of delayed skin reactivity to the same antigen at a later stage, due to emergence of new cells not thus paralysed or pre-empted. On a basis of immune paralysis the explanation of the phenomenon in the case of ovalbumin would be that cells capable of γ_1-antibody production are stimulated, while potential γ_2-producing cells, and cells mediating the delayed skin reaction, are paralysed rather than stimulated when the antigen is presented without mycobacterial adjuvant. This paralysis is not of long duration; either these cells recover, or new ones emerge. When mycobacterial adjuvant is given in the first immunization, however, all cell lines are activated.

The results are more difficult to explain by the pre-emptive theory, for it would have to be postulated that all cells capable of responding to the antigen are each capable either of forming γ_1-antibody or alternatively of forming γ_2-antibody or mounting a delayed skin

response. Pre-immunization without complete adjuvant would pre-empt them all to make γ_1-antibody. The diverse nature of these three different immune responses makes it seem unlikely that one cell is capable of them all, and the pre-emptive theory seems less likely on present evidence to offer the correct explanation.

The delayed skin response to blood group substances is primarily directed against the peptide part of the molecule, and it is this response that is blocked by pre-immunization without complete adjuvant. An animal so treated gives no delayed response to another serologically different human blood group substance administered in the second immunization with complete adjuvant, and this is explicable in terms of the similarity of the peptides associated with the various human blood group substances. Our evidence does not however allow us to deduce definitely whether antibody is produced against the polysaccharide of the second blood group substance—a matter of obvious interest since the peptide can be regarded as a carrier in this context, and lack of response to it might be expected to influence response to the polysaccharide functioning as hapten. If specific antibody were formed to the second blood group substance—a question we are currently investigating with this and other systems—it would suggest that although the cells capable of giving a delayed hypersensitivity response have temporarily been paralysed, there are others capable of responding by producing circulating antibody when challenged by the same carrier bearing different polysaccharide determinants. The increase in antibody titres, obtained in animals after a second immunization with adjuvant following a preliminary non-adjuvant injection, would also suggest such a state of affairs. It seems therefore, from these considerations, that the peptide of blood group substance, although in these circumstances not antigenic in itself, is still capable of playing a 'silent' role.

Our inability at present to distinguish between γ_1- and γ_2-antibody to blood group substance owing to the non-precipitating nature of the antibody renders further analysis difficult. Stewart-Tull, Wilkinson and White (1965) have shown that in guinea-pigs an effect of using mycobacterial adjuvant with ovalbumin as antigen is to evoke production, of γ_2-antibody (as distinct from the mainly γ_1 response obtained without mycobacteria) in addition to delayed hypersensitivity. Our finding that pre-immunization of the animals with ovalbumin with incomplete adjuvant modifies the subsequent production of γ_2-antibody, as well as delayed hypersensitivity, provides additional evidence that with this protein as antigen at any rate, delayed hypersensitivity and γ_2-antibody production may be related.

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