

Hapten-protein conjugates as antigens for evaluation of immune competence in man

I. Reactive haptens versus conjugate

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Picryl chloride, injected into guinea pigs, produced delayed contact hypersensitivity, hemagglutinating, and anaphylactic antibodies. Repeated skin application of 0.5 per cent picryl chloride failed to produce or maintain hemagglutinating antibody in guinea pigs or in man. Picryl human serum albumin (PicHSA) injected in aqueous solution in man uniformly produced conjugate-specific delayed reactions, frequently produced (24 of 26) hemagglutinating, mercaptoethanol-sensitive antibodies, occasionally produced (4 of 26) hapten-specific reaginic antibodies, and infrequently produced (2 of 26) mercaptoethanol-resistant hemagglutinating antibodies. These antigenic properties make PicHSA a suitable antigen to study immune competence in man.

The mechanism of delayed hypersensitivity and its relationship to antibody production and immediate hypersensitivity are poorly understood. Several clinical situations exist in which one type of hypersensitivity is ablated or markedly impaired without equally affecting the other. Sarcoidosis is one example of impaired delayed hypersensitivity in which antibody production is thought to be intact.¹ Patients with Hodgkin's disease are reported to show similar immunologic defects.² In previous studies of immune responses in man, diverse antigens have been used to test delayed hypersensitivity and antibody

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Supported in part by United States Public Health Service Research Grant AI 06706 and Training Grant AI 271.

Received for publication Oct. 21, 1968.

*Research Career Development Awardee, United States Public Health Service, National Institute of Allergy and Infectious Diseases, and National Institutes of Health.

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production. In sarcoidosis, tuberculin reactivity is usually absent while production of typhoid agglutinins appeared intact³; similarly, in Hodgkin's disease, production of antipneumococcal polysaccharide antibody remains normal, while capacity for induction of contact hypersensitivity is greatly reduced.⁴

In guinea pigs, Landsteiner⁵ reported production of antihapten antibodies as well as contact reactivity after repeated application of several skin-sensitizing chemicals. The present investigation is concerned with the use of hapten antigens in guinea pigs and man to evaluate not only delayed responses but antibody production to a single antigenic determinant. The use of contact antigens, while suitable in guinea pigs for evaluation of antibody responses, was found to be unsatisfactory in man, leading to the use of hapten-human serum albumin (HSA) conjugates capable of safely eliciting both delayed and immediate immune responses. A later report is concerned with responses of patients with sarcoidosis to immunization with hapten-HSA conjugates.

MATERIALS AND METHODS

Reagents

Picryl chloride was obtained from Eastman Organic Chemicals, Rochester, New York. One fluoro-2,4-dinitrobenzene was obtained from Matheson, Coleman & Bell, Norwood, Ohio. Guinea pig albumin (GPA) was prepared by starch block electrophoresis, and purity was established by immunoelectrophoresis. Human serum albumin was kindly provided by the American National Red Cross and was processed by E. R. Squibb & Sons, New York. Human gamma globulin was obtained as Immune Serum Globulin, "Gamma Gee," from Merck Sharp & Dohme, West Point, Pennsylvania.

Preparation of conjugates

Picryl human serum albumin (PicHSA) was prepared by a modification of the method of Eisen and associates⁶ and previously used in this laboratory.⁷ Varying concentrations of hapten were dissolved in 1,4-dioxane and added dropwise to protein solutions in order to obtain preparations with different average numbers of hapten groups per molecule. Concentration of dioxane never exceeded 5 per cent by volume. Solutions were allowed to react, with constant stirring for 2 hours. Unreacted hapten was separated from the conjugate by a Sephadex G25 gel filtration. The average number of hapten groups per molecule of protein was calculated from its optical density with the use of a molar extinction coefficient of 15,400 for picryl groups at a wave length of 347 m μ . Protein concentrations were calculated from micro-Kjeldahl nitrogen determinations. Various conjugates of picryl guinea pig albumin (PicGPA) were prepared to contain 10 to 48 groups per molecule of protein. Conjugates of human serum albumin (PicHSA) and human gamma globulin (PicHGG) were prepared to contain 20 to 36 picryl groups per molecule.

Immunization

Male and female albino guinea pigs of the Hartley strain, weighing 400 to 500 grams, were immunized with picryl chloride emulsified in complete Freund's

adjuvant (Difco Labs, Incorporated, Detroit, Michigan). Each animal received 100 μ g of antigen distributed in the 4 footpads in the volume of 0.1 ml. per footpad. Animals were tested weekly with the application of 0.05 ml. of a 0.5 per cent picryl chloride solution in an acetone-olive oil vehicle (4 to 1). Control animals failed to react with an irritative lesion to this dose of antigen.

Human subjects were contact sensitized by the application of a 0.1 ml. of a 2 per cent picryl chloride solution dissolved in acetone. Subsequent weekly applications of 0.5 per cent were contained in 0.1 ml. of acetone.

Immunization of human beings with the hapten-protein conjugates was always preceded by the intradermal injection of 0.01 to 0.02 ml. of dilutions of the antigen to be injected. If no wheal and flare reaction occurred after 15 to 20 minutes, the remainder of the immunizing dose, detailed in the Results to follow, was administered subcutaneously.

Reactions

Contact reactions were read at 24 hours and were graded 1+ to 4+ on the basis of redness and induration. Intradermal skin tests for delayed sensitivity to the conjugates were read at 24 hours and recorded in the manner previously described.⁸ Areas less than 5 $\times$ 5 mm. were read as negative.

Preparation of serum

Sera obtained from donor guinea pigs at times specified as follows were inactivated at 56° C. for 30 minutes, then diluted by 4 volumes of buffer and absorbed with a 10 per cent suspension of human group O Rh positive erythrocytes for one hour at 4° C. Human serum was similarly inactivated and absorbed.

Hemagglutination

Twofold dilutions of absorbed sera starting at 1:25 were prepared with equal volumes of buffer, pH 7.5, in RB-96 Disposo trays (Linbro Chemical Company, New Haven, Connecticut); some duplicate titrations were performed with the use of an automatic diluting machine (Autotiter, Astec, Incorporated, Orange, Connecticut). To each dilution, an equal volume of 0.3 per cent suspension of hapten-coupled erythrocytes was added. Parallel titrations were carried out with absorbed sera which had been mixed with 0.133M mercaptoethanol in tris-buffered saline, pH 8.2, and incubated at room temperature for 90 minutes.⁹

Preparation of hapten-coupled erythrocytes

The detailed method of coupling 1,3-difluoro-4,5-dinitrobenzene (Aldrich Chemical Company, Milwaukee, Wisconsin) to erythrocytes in disodiummethylenediaminetetraacetate (EDTA) (Fisher Scientific Company, Fairlawn, New Jersey) buffer was previously reported and has been shown to be capable of detecting small amounts of cross-reacting antipicryl antibody.⁷

Passive cutaneous anaphylaxis (PCA)

Guinea pig and human sera tested for PCA activity were diluted in saline and tested with the use of the technique described by Ovary.¹⁰ Antigen used

to test for antibodies against the picryl group was PicGPA, 300 μ g per milliliter, diluted in equal volume of 1 per cent Evans blue dye; 1 milliliter of this solution was injected intravenously. Each serum was tested at 3 dilutions in 4 guinea pigs weighing 200 to 250 grams. An unknown serum was considered devoid of detectable antibody only when the test animal gave a positive response at a site prepared with the standard anti-picryl antiserum.

RESULTS

Immunization with picryl chloride

Guinea pigs immunized with 100 μ g of picryl chloride in complete Freund's adjuvant were challenged weekly on a shaved flank with 0.5 per cent picryl chloride, and blood was obtained concurrently for antibody determinations. As shown in Fig. 1, hemagglutinating antibody titer rose promptly, reaching a peak in 18 days, after which it fell to low levels by the forty-eighth day. Anaphylaxis was produced in 5 of 5 guinea pigs on the twenty-eighth day of immunization by intravenous injection of 300 μ g of PicGPA in saline, suggesting antihapten antibody production of the γ G1 type. Of interest was the fall in hemagglutinating activity while continuing weekly skin applications of antigen. To determine if the waning antibody titers reflected changes in responsiveness, 12 guinea pigs were immunized with 100 μ g of picryl chloride in the left fore and hind footpads in complete adjuvant. Again, weekly contact reactions were elicited, and a similar rise and fall in antibody titer was observed (Fig. 2). On Day 60, the right footpads were injected with 100 μ g of picryl chloride in complete adjuvant, and a prompt rise in antibody titer was observed. A second fall in titer with continuing weekly contact stimuli with 0.5 per cent picryl chloride was observed until the eighty-fourth day, when concentration of the contactant

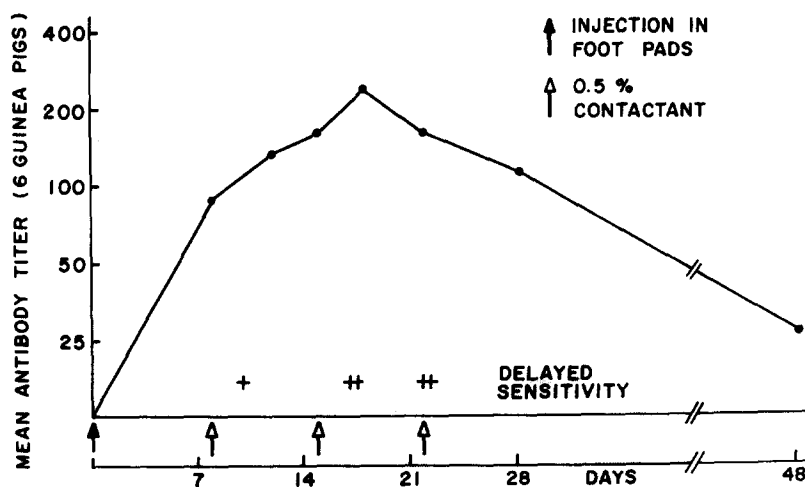


Fig. 1

Hemagglutinating antibody response to footpad injection and skin application of picryl chloride.

was increased to 3.0 per cent. A prompt rise in antibody titer again appeared. Clearly, responsiveness of the animals was unimpaired. While weekly skin applications were sufficient to produce good contact reactions, antigenic stimulation was not sufficient to maintain an existing antibody response until concentration of the reactant was increased to 3 per cent.

A total of 17 human volunteers, hospitalized for diverse diseases unrelated to the lymphoid system, was contact sensitized on one forearm with picryl chloride. None of these patients was receiving steroid medications at the time of the study. A circular glass ring was used to limit the spread of the application of 0.1 ml. of a 2 per cent picryl chloride solution in acetone. Eight of the patients developed typical contact reactions at the site of primary application 5 to 7 days after exposure without challenge. Another 8 patients reacted to a challenge of 0.1 ml. of 0.5 per cent picryl chloride in acetone on the opposite arm 2 weeks after the initial contact. The remaining patient developed contact reactivity 2 weeks after the first challenge, after an application of 0.1 ml. of 0.5 per cent picryl chloride solution. Fourteen of the 17 were subsequently challenged 4 times, and 3 were challenged 6 times; each time strong contact reactions appeared, but weekly serum determinations failed to reveal hemagglutinating or PCA antibody activity. In 3 subjects, 2 per cent contactant concentrations were used after the immunizing event, resulting in denudation of the skin and an unacceptably severe reaction. Serum antibodies were not demonstrable at any time in these patients. Eight additional patients were contact sensitized with 0.1 ml. of a 1 per cent solution of dinitrofluorobenzene (DNFB)

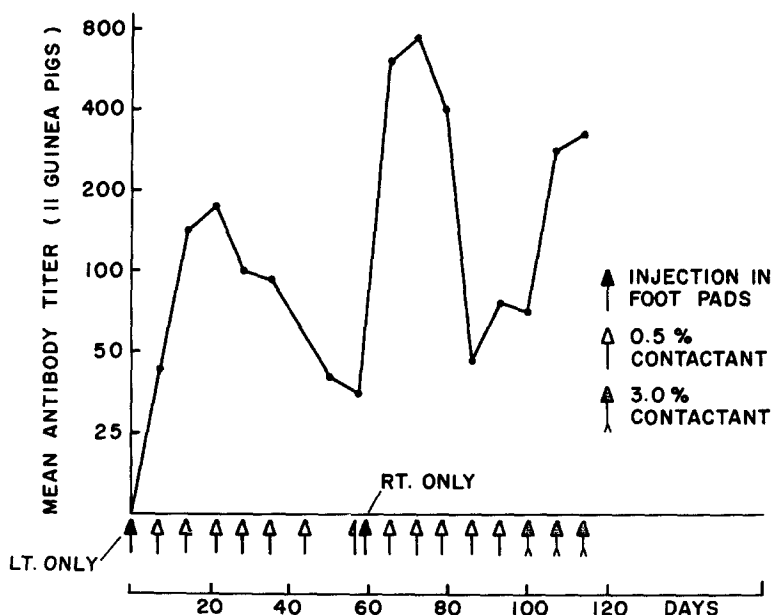


Fig. 2

Hemagglutinating antibody response to repeated footpad injection and skin application of picryl chloride.

in acetone and challenged at 2 week intervals thereafter with 0.1 ml. of a 0.25 per cent DNFB solution, with uniform production of contact sensitivity in the absence of antibody formation. In man, as in the guinea pig, moderate doses of contactants failed to induce any measurable antibody response.

Picryl groups were then coupled in vitro to a protein carrier, human serum albumin. Four normal human volunteers were immunized in the following manner: Once weekly intradermal series of test injections with 0.1 to 500 μ g of antigen were performed. If no wheal and flare reactions occurred at the test sites within 30 minutes, an immunizing dose of 15 to 25 mg. was injected subcutaneously. At the end of the first week, 6 hours after intradermal injection of 500 μ g PicHSA in 0.1 ml., erythematous, slightly tender, indurated reactions appeared in all volunteers. These reactions reached a peak in 24 hours, remained at 48 hours, and were almost gone by 72 hours. Punch biopsy of 2 of the lesions at 18 hours revealed perivascular "cuffing" and cell infiltration of the dermis with approximately 10 to 20 per cent polymorphonuclear leukocytes and 80 per cent mononuclear cells.

Serum samples were obtained on Day 0, 2, 5, 10, 14, and 21; hemagglutinating antipicryl antibodies appeared on the fourteenth day. Since HSA was the carrier protein, the half-life of HSA in immunized individuals was measured and is shown in Fig. 3. One hundred μ c of I^{131} -labeled HSA (Squibb) was injected intravenously on the twenty-first day after the onset of immunization. Samples were obtained at the times shown for counting in a Nuclear Chicago well-type scintillation counter for sufficient periods to insure a counting error of less than 3 per cent. The half-life of albumin in the immunized volunteers

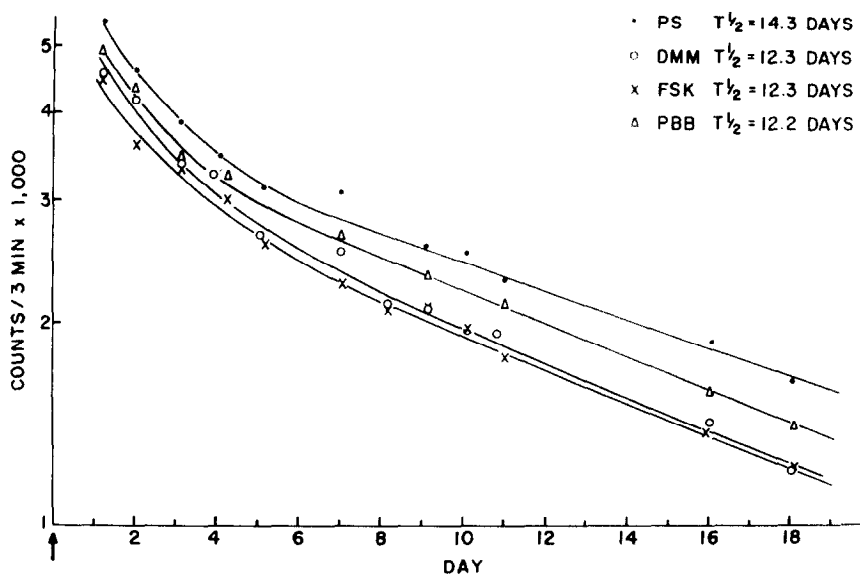


Fig. 3

Clearance of 100 μ c of human serum albumin- I^{131} in 4 volunteers immunized with PicHSA.

was 12 to 14 days, at a time when antipicryl antibodies were demonstrated in the sera. Thus, no evidence of anti-HSA antibodies was found.

In the course of other studies,^{11, 12} a total of 26 "normal" human volunteers was immunized with the same schedule. These patients were hospitalized for diseases not associated with the lymphoid system, were not taking steroid medications, and were in a good nutritional state. Five of the 26 patients developed delayed-type hypersensitivity reactions within 48 hours after the first injections of PicHSA. The remaining 21 exhibited good delayed reactions at 7 days which recurred weekly after each intradermal injection for a total of 4 weeks. Reactions were red, indurated, raised, slightly tender, and usually measured 25 to 50 mm. in diameter.

Hapten-specific hemagglutinating antibodies appeared by the second week, as shown in Fig. 4, and reached a peak during the third to fourth week. Without additional antigenic stimulation, patients were bled and sera tested on the ninetieth to one hundred tenth day after onset of immunization. No antibody activity could be measured at that time.

The nature of the antihapten antibody produced was investigated by parallel hemagglutination titrations with sera which had been mixed with 0.133M mercaptoethanol in tris-buffered saline at pH 8.2 and incubated at room temperature for 90 minutes. Of 24 sera tested with titers from 1:200 to 1:1,600, only 2 retained hemagglutinating activity after mercaptoethanol treatment. Titers without reducing agent were 1:400 and 1:800, and after treatment both fell to 1:50. Sucrose gradient ultracentrifugation of 5 sera known to have titers of hemagglutinating activity completely removed by mercaptoethanol confirmed the macroglobulin nature of the antibodies.¹³ No antibody activity was observed in the 7S portion of the gradient of the sera studied.

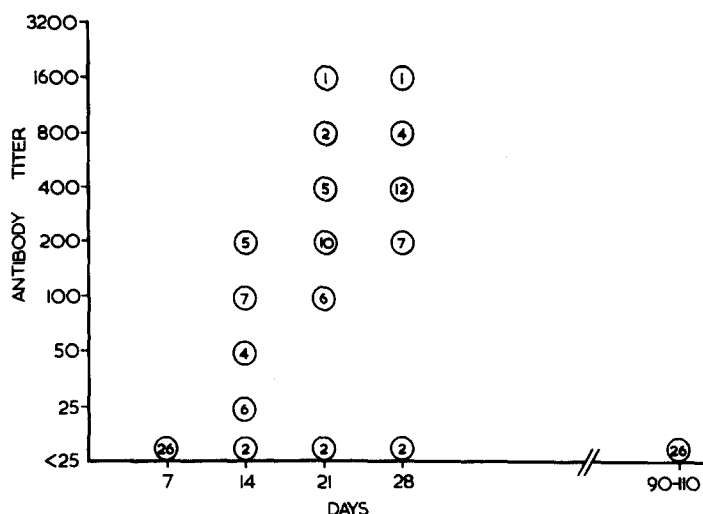


Fig. 4

Hemagglutinating antibody response in 26 normal patients to immunization with PicHSA. Numbers within circles indicate number of sera reactive at the titer indicated on the ordinate.

In 4 patients, wheal- and flare-type of immediate reactions appeared 15 minutes after intradermal injection of 100 μ g of PicHSA 3 weeks after onset of immunization. Reactions varied from 10 to 25 mm. in size and were accompanied by pseudopods and a flare of 50 mm. surrounding the injection site. Hapten specificity was determined by elicitation of the reactions with 100 μ g of PicHGG in saline. Reactions to PicHGG were of comparable size with those produced by PicHSA. No reactivity of HSA or HGG alone was observed. Delayed reactions, however, failed to appear at the site of the DNP-HGG injections, while delayed reactions measuring 20 to 40 mm. were present in the same 4 patients 24 hours after intradermal injection of 100 μ g of PicHSA.

DISCUSSION

The ability to produce both delayed hypersensitivity and antibody formation by footpad injection of picryl chloride in guinea pigs suggested that this contactant might be used in man to study immune competence to a single well-defined antigenic determinant. However, skin application of picryl chloride in guinea pigs and man at tolerable concentrations failed to produce or maintain production of antibody. When irritative doses were used in guinea pigs, hemagglutinating and anaphylactic antibodies were produced. Such doses did not seem suitable in man for study of immune competence.

There had been a wide experience in human beings with contact allergens¹⁴ which are known to conjugate to tissue or serum proteins *in vivo*¹⁵ without report of subsequent autoimmune responses directed against the skin or other organs. Accordingly, conjugation of hapten to human serum albumin *in vitro* afforded the opportunity to immunize human beings with a defined antigen likely to produce both delayed and immediate hypersensitivity. Studies of the half-life of circulating albumin in sensitized subjects failed to show any immune response against the carrier protein. The 4 initial volunteers have been followed for 3 years without appearance of untoward "autoimmunity."

Response to the PicHSA conjugate was evident in delayed reactions within the first week after immunization. Subsequent appearance of the antibody in relatively low titer occurred almost invariably (24:26). The immune response was limited to delayed reactivity and 19S antibody production. While the 2 sera which contained some mercaptoethanol-resistant antibody suggested that manipulation of dose, route, and time might overcome the deficiency of 7S antibody production, we were concerned about the liabilities of hyperimmunization and did not manipulate these parameters.

In 4 patients hapten-specific wheal and flare antibody appeared. Three of these patients had histories of atopic disease, with pollenosis to ragweed in 2 and grasses in the third. Previous reports¹⁶ have indicated that immunization of normal or atopic individuals with protein antigens resulted in production of wheal and flare reactivity in a significant proportion of patients.

Delayed hypersensitivity in guinea pigs is specific for the immunizing conjugate and not for the hapten alone.¹⁷ In the present studies, patients immunized to PicHSA demonstrated delayed skin reactions only after injection of PicHSA

and not at sites injected with PicHGG, even in those with wheal and flare reactivity to a homologous-hapten-heterologous-carrier conjugate.

To test immune competence in man, the ideal antigen must be safe; it should be artificial so that chance prior exposure is impossible; and it must produce in all sensitized persons the whole spectrum of immune responses. The degree of response, while readily apparent, should not be so great that minor host deficiencies would be masked. PicHSA fills many of these criteria. It seems safe; it is artificial, although cross-reactivity with natural antigens cannot ever be excluded. The gamut of immune responses are produced; however, 7S antibody responses were meager, which may limit the use of PicHSA in studying this aspect of immune events.

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