THE SPECIFICITY OF ALLERGIC REACTIONS

III. Contact Hypersensitivity

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Contact hypersensitivity induced by simple chemicals is believed analogous to delayed hypersensitivity because of outstanding similarities between the two. (*a*) The inflammatory response to surface application of the hapten requires about 24 hours to reach its maximum. (*b*) The histological appearance of the two types is similar. (*c*) Passive transfer of contact and delayed hypersensitivities has not been carried out with serum, but can be readily accomplished by lymphoid cells. (*d*) Patients with agammaglobulinemia develop both contact and delayed types of hypersensitivity. Several characteristics of delayed hypersensitivity have not yet been achieved after sensitization with simple contact chemicals. (*a*) The cytotoxic effect of antigen on isolated cells from donors with specific delayed reactivity has not been reported with simple chemicals and cells from animals with contact hypersensitivity. (*b*) Delayed or tuberculin shock has not yet been duplicated in animals with contact hypersensitivity by injection of the homologous simple chemicals or protein conjugates. (*c*) Contact skin hypersensitivity to simple chemicals has not yet been induced in rabbits, although delayed hypersensitivity has (1).

Haptens or low molecular substances such as dinitrofluorobenzene or picryl chloride can induce both contact hypersensitivity and circulating antibody in guinea pigs. They are, however, believed to react first with protein *in vivo* to form a complete antigen (1–3). Intradermal injection of such a hapten in Freund's adjuvant (with or without mycobacteria) induces in guinea pigs a hypersensitivity wherein later surface application of the same chemical produces a local inflammatory response. This contact type responsiveness is believed to be related to, or identical with, delayed hypersensitivity (1).

The simple chemicals that induce such hypersensitivity are usually capable of combining *in vitro* with protein. A proportionality, moreover, exists between the rate of reaction with protein *in vitro* and the capability of inducing contact skin hypersensitivity (1). Hapten-protein conjugates prepared *in vitro*, however, do not generally induce contact hypersensitivity to the hapten, but do induce

delayed and Arthus types of sensitization to appropriate conjugates (4, 5). Exceptions are the findings that contact hypersensitivity may be induced in guinea pigs by injection (*a*) of homologous picrylated red blood cell stromata (6), (*b*) of heterologous picrylated proteins in massive doses (18), or (*c*) of homologous picryl albumin (19).

In addition, primary injection of homologous protein induces an anamnestic response to subsequent administration of conjugate, whereas primary injection of homologous hapten–heterologous protein does not (5). Since a change in specificity is associated with a change in the type of allergic response, the possibility exists that alterations in specificity may be the basis to some of the inconsistencies in the contact response such as the inability to induce contact reactions with conjugates. The present paper describes experiments that attempt to elucidate this problem.

Materials and Methods

Animals.—Guinea pigs of the Hartley strain weighing 350 to 450 gm were used for studies on sensitization and immunization. White or albino guinea pigs weighing from 300 to 400 gm were employed for passive cutaneous anaphylaxis.

Antigens.—

Hen egg albumin (HEA): Five times recrystallized hen egg albumin was obtained from the K & K Laboratories, Inc., Jamaica, N. Y.

Bovine gamma globulin (BGG): Armour purified bovine gamma globulin was used without further treatment.

Picryl chloride (PiCl), or 1-chloro-2,4-dinitrobenzene (DCB), or 1-fluoro-2,4-dinitrobenzene (DFB): These simple chemicals were obtained from Eastman Kodak Laboratories.

Stromata.—Red blood cell stromata were prepared according to the method outlined by Landsteiner and Chase (6): Red blood cells from citrated whole blood were washed by centrifugation in physiologic saline, resuspended in 3 volumes of saline, and heated at 56–58°C for 40 minutes. The cells were then dialyzed against several changes of distilled water. After isotonicity had been restored by addition of 10 per cent NaCl solution, the stromata were washed in saline until the supernatant liquid was colorless.

Skin.—The back and sides of 300 gm guinea pigs were shaved and cleansed with 70 per cent ethanol. Shortly thereafter, when the animal was sacrificed, the skin was excised, minced in Tyrode's solution,[1] homogenized in a Waring blendor, filtered through cheese-cloth to remove coarse particles, and centrifuged at 30,000 RPM (average centrifugal force 78,400 *g*) for 1 hour in the cold to remove particulate material. The supernate containing soluble protein was conjugated with hapten. Similar solutions of conjugated skin were also prepared from rabbits. The solutions were dialyzed against frequent changes of distilled water, in an attempt to eliminate uncombined hapten. A control solution was prepared by dissolving in physiologic saline the same quantity of hapten as was used for conjugation with protein and then dialyzing the solution in parallel with the conjugate.

Conjugates.—DFB or PiCl was conjugated with HEA, BGG, red blood cell stromata, or skin solutions (6, 7). After extensive dialysis against distilled water with stirring in the cold, the conjugates were centrifuged to remove insoluble material and analyzed (*a*) by evaporation

[1] NaCl, 8 gm, KCl, 0.20 gm, $CaCl_2$, 0.20 gm, $M_gCl_2 \cdot 6H_2O$, 0.10 gm, $NaH_2PO_4 \cdot H_2O$, 0.05 gm, $NaHCO_3$, 1 gm, glucose, 1 gm, distilled H_2O, 1000 ml, and pH, 7.3–7.35.

of measured aliquots to dryness and subsequent weight determination and (*b*) by micro-Kjeldahl technique.

Sensitization.—Protein antigens were dissolved in physiologic saline plus 1 per cent normal guinea pig serum. Both haptens and protein antigens were emulsified with an equal volume of Freund's adjuvant (Difco), without mycobacteria. Guinea pigs were sensitized with 5 μg protein, 15 μg conjugate or hapten, or in some instances as much as 50 μg hapten, in water-in-oil emulsion by injection of 0.5 ml into the digits of the feet.

Skin Tests.—Guinea pigs were tested on the sides (*a*) intradermally with 0.1 ml of antigen containing 50 μg/ml protein or protein conjugate, or (*b*) by contact with 0.05 ml of 0.5 per cent hapten dissolved in 4 parts acetone–1 part corn oil. Reactions were observed and diameters of areas of induration measured at 4 hours and at 18 to 24 hours. The strength of the contact reactions was recorded depending on the elevation and pinkness of the sites of hapten application (8). Normal guinea pigs were simultaneously tested on the sides with hapten, in order to serve as negative controls with which to compare experimental animals.

Antibody determination.—Guinea pigs were bled just prior to skin testing, and the sera assayed for antibody. The passive cutaneous anaphylaxis (PCA) reaction was used for this purpose (9). Herein, 0.1 ml test serum was injected intradermally in the flank of a normal guinea pig. About 3 hours later, 350 μg protein in 0.5 ml physiologic saline and 0.5 ml of a 1 per cent Evans blue solution in physiologic saline were introduced intravenously. 15 to 30 minutes later, the areas of pigmented skin were examined and the results recorded.

RESULTS

Reactions after Sensitization with Hapten.—15 μg PiCl or 50 μg DFB emulsified in Freund's adjuvant (without mycobacteria) were introduced into the digits of guinea pigs, and the animals tested periodically for hypersensitivity to the hapten and to the conjugate of hapten + guinea pig serum. Reactivity to the hapten was determined after application onto the surface of the animal skin, whereas reactivity to the conjugate was measured after intradermal injection. The guinea pigs were bled prior to skin testing so that serum might be obtained for antibody determination.

Animals sensitized with PiCl developed a contact hypersensitivity to the hapten on the 5th day after sensitization, which reactivity persisted for the duration of the experiments, *i.e.*, 15 days (Table I). Skin testing with conjugate, picrylated guinea pig serum (Pi·GPS), however, elicited equivocal delayed reactions, but did induce definite Arthus reactions as of the 9th day. The Arthus reactions were accompanied by the appearance of circulating antibodies, as determined by PCA tests with Pi·GPS as antigen. When picrylated hen egg albumin (Pi·HEA) was employed as antigen in PCA tests, circulating antibody was not readily detected. Similar results were obtained in guinea pigs sensitized with DFB (Table II), and skin-tested with DFB and DFB conjugated to guinea pig serum (DFB·GPS). Again, contact hypersensitivity to DFB appeared about 4 days before Arthus reactions and circulating antibody to DFB·GPS could be detected.

Reactions in Guinea Pigs Sensitized with the Conjugates DFB·GPS or Pi·GPS.—Twenty guinea pigs injected in the digits with 15 μg Pi·GPS in

adjuvant developed delayed reactions to the homologous antigen on about the 5th day, and Arthus reactions on the 10th day. Antibody was also present from the 10th day on, not only to the homologous Pi·GPS but also to the

TABLE I

Reactions in Guinea Pigs Sensitized (in the Digits) with 15 μg PiCl in Freund's Adjuvant

Day tested	No. of guinea pigs	No. of positive reactors after skin test with	
		PiCl (contact reactions)	Pi·GPS (Arthus reactions)
5	2	1	0
6	6	6	0
8	5	5	0
9	5	5	2
10	5	5	1
11	7	7	3
12	5	4	1
13	5	5	3
14	8	8	7
15	6	5	6

TABLE II

Reactions in Guinea Pigs Sensitized in the Digits with 50 μg DFB in Freund's Adjuvant

Day tested	No. of guinea pigs	No. of positive reactors after skin test with	
		DFB (contact reactions)	DFB·GPS (Arthus reactions)
5	2	2	0
6	2	2	0
7	2	2	0
8	5	5	1
9	7	7	4
10	6	6	6
11	4	4	3
12	2	2	2
15	5	5	5

homologous hapten–heterologous protein conjugates, picrylated hen egg albumin (Pi·HEA), or picrylated horse serum (Pi·HoS). At no time from the 4th to the 18th day postinoculation could contact type hypersensitivity be detected on surface application of PiCl. Similar results were obtained in 30 guinea pigs sensitized with DFB·GPS, in that delayed hypersensitivity ap-

peared on about the 5th day and Arthus reactions on the 10th, and contact hypersensitivity did not appear on surface application of DFB.

Hypersensitivity after Administration of Hapten–Red Blood Cell Stromata (DFB·GPRBC).—Conjugates made *in vitro* with red blood cell stromata induce both contact hypersensitivity and circulating antibody (6). Thus, contact hypersensitivity can be produced with a substance which could be considered a typical complete antigen and which ought to induce antibodies. In an attempt to add to the foregoing information, experiments were initiated wherein 50 μg of a conjugate prepared from DFB and guinea pig red blood cell stromata (DFB·GPRBC) were introduced in Freund's adjuvant into the digits of the four feet. Starting about the 5th day after sensitization, application of DFB onto the surface of the skin caused definite contact reactions to appear as indicated by thickening and pinkness of the epidermis. Intradermal injection of the conjugates DFB·GPS or DFB·GPskin from the 5th to the 8th day induced equivocal delayed responses, possibly because the protein of the conjugate was not identical with the *in vivo*–conjugated protein. DFB·GPRBC was not used as skin-testing antigen because particulate antigens are not satisfactory for this purpose. However, on about the 9th day, Arthus reactions could be induced by a variety of DFB conjugates and circulating antibody detected by PCA tests. When a conjugate of DFB and horse red blood cell stromata (DFB·HoRBC) in adjuvant was used as the sensitizing antigen, similar hypersensitive responses could be elicited. Circulating antibody, however, was not detected until 11 to 12 days postsensitization.

Reactions in Guinea Pigs Sensitized with a Conjugate of Soluble Skin Coupled with DFB (DFB·GPskin).—125 guinea pigs were injected in the digits with 15 to 50 μg conjugate of DFB and extract of guinea pig skin in adjuvant. On the 5th day after inoculation, delayed hypersensitivity appeared to the homologous conjugate, as well as to DFB·GPS. At the same time, contact hypersensitivity was detected in 6 out of 11 cases on the surface application of the hapten, DFB (Table III). On the 8th day after sensitization, when Arthus reactions and circulating antibody appeared to DFB·GPskin and to DFB·GPS, contact hypersensitivity to DFB persisted, but delayed hypersensitivity was masked by Arthus reactions. Control animals sensitized with saline to which DFB had been added and then dialyzed in parallel with the conjugate did not show any kind of hypersensitivity. Thus, sensitization with the conjugate, DFB·GPskin, is capable of eliciting delayed and later Arthus hypersensitivity to the homologous conjugate and contact hypersensitivity to surface application of the hapten alone. The contact reactions were typically distinct, pink, and elevated.

Anamnestic Response with DFB.—A primary injection of a protein followed by a second of the same protein plus hapten results in an anamnestic response to the conjugate (5, 10). Similar experiments were initiated to determine what

TABLE III

Reactions in Guinea Pigs Sensitized with a Conjugate of DFB and Extract of Guinea Pig Skin

Day tested after sensitization	Total No. of guinea pigs	Contact reaction to DFB	Delayed reaction to DFB·GPS or DFB·GPskin	Arthus reaction to DFB·GPS
4	4	2	0	0
5	11	6	8	0
6	17	14	9	0
7	10	5	8	0
8	15	11	4*	9
9	12	9	2*	10
10	13	9	1*	10
11	12	8	0	12
12	11	6	0	11
13	7	2	0	5
14	2	2	0	2
15	5	4	0	5
16	2	0	0	2
19	2	0	0	1
20	2	1	0	2

* These animals did not have detectable circulating antibody.

TABLE IV

Anamnestic Response to Intradermal Injection of a Hapten (DFB)

Primary injection	Secondary injection	Day after sensitization	Total No. of guinea pigs	Numbers with antibody to DFB·GPS
1.0 μg DFB·HEA	50 μg DFB in adjuvant	6	3	0
		7	5	1
		8	5	1
		9	5	1
		10	6	6
1.0 μg DFB in saline	50 μg DFB in adjuvant	7	6	0
		8	6	1
		9	6	5
		10	6	6
1.0 μg DFB·GPskin in saline	50 μg DFB in adjuvant	4	6	1
		5	6	2
		6	6	4
		7	6	5
		8	6	6
1.0 μg DFB·GPS in saline	50 μg DFB in adjuvant	5	6	0
		6	6	2
		7	6	4
		8	5	4
		9	5	5

conjugate, if any, would hasten the antibody response to DFB, on the assumption that the protein portion of the conjugate would be similar to the host protein with which the DFB combines to form an antigen.

Intradermal injection of 50 μg DFB in adjuvant induces contact hypersensitivity in about 5 days and antibody to DFB·GPS in 9 to 10 days. When a primary injection of 1.0 μg DFB·HEA in saline was followed 10 days later by a secondary injection of 50 μg DFB in adjuvant, antibody appeared at about the same time (Table IV), namely, about 10 days after the secondary injection. One μg DFB in saline as the primary dose was equally ineffective in shortening the inductive period when followed by a secondary injection of 50 μg DFB in adjuvant. When, however, 1.0 μg DFB·GPskin in saline was the primary inoculum, antibody response to an injection of 50 μg DFB in adjuvant occurred in 5 to 7 days. Primary injection of 1.0 μg DFB·GPS followed by a secondary of 50 μg DFB in adjuvant resulted in the appearance of detectable antibody by the 6th to 8th day. DFB·GPskin, therefore, could induce not only an anamnestic response to DFB, but also contact hypersensitivity to DFB.

DISCUSSION

A conjugate of DFB and soluble extract of guinea pig skin can induce contact hypersensitivity in the guinea pig. Such reactions to DFB do not occur when the animals are sensitized with other conjugates such as DFB·GPS or DFB·HEA. Since the conjugate of DFB·GPskin may be dissolved to produce a water-clear solution, since preparations were exhaustively dialyzed in parallel with control solutions, and since the conjugates were active in sensitizing doses of 15 μg, the activity seems not to be due to free DFB in the solution. In addition to contact hypersensitivity, the DFB·GPskin conjugate also produces delayed hypersensitivity to itself and to DFB·GPS, antibody to a variety of DFB conjugates, and an anamnestic response to a secondary injection of DFB.

The foregoing evidence adds considerable weight to the hypothesis that contact and delayed allergies are closely related, since the specificity of delayed hypersensitivity is also directed toward the protein portion of the conjugate (5). Therefore, on the basis of recognized features of delayed hypersensitivity, the following immunologic events might be anticipated: (*a*) Either percutaneous application of hapten or injection of the specific hapten conjugate should produce delayed hypersensitivity to the conjugate, contact hypersensitivity to the hapten, and antibody to the hapten group (4, 5, 11). (*b*) An anamnestic response in antibody formation to hapten should be produced after primary sensitization with minimal dose of either injected conjugate or percutaneous hapten (10). (*c*) Injection of a conjugate of hapten with unrelated protein should not produce contact hypersensitivity to the hapten, delayed hyper-

sensitivity to the specific hapten-skin conjugate, or an anamnestic response to either one. (*d*) Injection of skin conjugate or contact application of hapten should not produce delayed hypersensitivity to conjugates of the hapten with heterologous protein.

With the DFB system as antigens, general conformity has been found experimentally to the above, although exceptions do exist. For example, with regard to *a*, guinea pigs sensitized with DFB·GPskin show delayed reactions to DFB-skin and contact reactions to DFB, while animals sensitized with DFB develop contact hypersensitivity but only equivocal delayed responses. With regard to *b*, initial injection of DFB·GPskin prepares the animal for an anamnestic response to either DFB or DFB·GPskin, but initial sensitization with DFB does not seem to prime the animal for an anamnestic response to either one. With regard to *c*, contact hypersensitivity has been produced to DFB guinea pig RBC stromata, but the antigen is particulate, contains lipid, and may therefore have uncombined hapten. With regard to *d*, delayed hypersensitivity to DFB·GP serum after sensitization to DFB·GPskin may be due to the skin extract containing serum proteins as well as the specific protein responsible for contact sensitization.

Recent evidence (1, 12, 13, 19) supports the view that the combination of hapten with protein is an early essential step in contact hypersensitivity. With possibly three exceptions (6, 14, 18, 19), these conjugates, however, have been ineffective in producing contact hypersensitivity.

Three explanations have been offered as to why synthetic conjugates fail to induce contact allergy. (*a*) Haptens coupled with proteins *in vivo* have different antigenic determinants from those coupled *in vitro*. Such is probably not the case since studies have showed that dinitrophenyl linkages created in the skin are similar to those produced in the test tube (7, 15–17). (*b*) Haptens with high reactivity for proteins are concentrated in lymph node cells to a much greater extent than are protein-hapten conjugates, and, either because of a quantitative difference or because of conjugation with peculiar lymph node proteins, cause contact hypersensitivity (12). (*c*) Contact hypersensitivity is a result of conjugation of a hapten with a particular protein which has not been present in any of the preparations which were assayed. Production of contact hypersensitivity with a soluble *in vitro* conjugate would support this hypothesis. Both picrylated RBC stromata (6) or procollagen-adjuvant-hapten emulsions (14) have induced contact allergy, but they are particulate in nature and could possibly contain free hapten dissolved in the lipid of the disperse phase.

Sensitizing doses of 1 mg of picrylated bovine gamma globulin or picrylated guinea pig gamma globulin have induced contact hypersensitivity to picryl chloride (18), but here trace amounts of free picrate might have been present in the protein conjugate preparations. Most recently (19), contact hypersensitivity was described after sensitization with micro quantities of picryl protein conjugates, but not with soluble DFB conjugates.

One of the characteristics of delayed hypersensitivity is that the specificity is directed toward the carrier protein rather than toward the attached hapten (4, 5, 11). A definite carrier specificity exists in the case of delayed hypersensitivity to picryl conjugates (19). This specificity seems even sharper in sensitization with DFB conjugates and may explain previous failures to produce contact hypersensitivity with DFB conjugates. The reasons for this striking specificity of DFB and its conjugates are not clear, but may be related to a greater affinity of DFB for protein as compared with the picryl group. For example, after prolonged dialysis, picrylated proteins continue to produce free picryl groupings into the external buffer, whereas DFB conjugates do not.

That the DFB·GPskin conjugate should produce contact allergy but DFB conjugates with other proteins should not is consistent with the concepts (*a*) that contact and delayed hypersensitivities are related and (*b*) that the specificity of delayed hypersensitivity and probably contact hypersensitivity is directed toward the protein portion of the conjugate.

SUMMARY

Intradermal injection of a simple hapten (*e.g.*, 1-fluoro-2,4-dinitrobenzene) in water-in-oil emulsion results in contact hypersensitivity to surface application of the homologous hapten and, after appearance of circulating antibody, in Arthus type hypersensitivity to a conjugate of homologous hapten with guinea pig serum. Intradermal administration of this conjugate induces delayed and subsequently Arthus hypersensitivity to the conjugate, but no evidence of a contact reaction to the hapten alone. When a conjugate of hapten plus solubilized guinea pig skin is used as the sensitizing antigen, both contact hypersensitivity to the hapten and delayed and/or Arthus reactions to the conjugate develop. These observations are consistent with the hypothesis that the specificity of contact sensitivity is directed toward some particular protein of the skin which has been modified by combination with hapten.

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