Anamnestic Antibody Response in Rabbits to Topically Applied 2,4-Dinitrofluorobenzene[1] (34731)

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Since the demonstration of passive cellular transfer of delayed type hypersensitivity to simple chemical compounds (1), cellular phenomena in this allergic state have been investigated extensively. Only a few studies have been concerned with humoral antibody production following sensitization by topical application of chemicals. However, precipitating antibodies have been demonstrated in rabbits and guinea pigs in association with chemically-induced delayed type hypersensitivity (2–5). Also, various investigators have detected Prausnitz-Küstner type, passive cutaneous anaphylaxis producing and hemagglutinating antibodies in serum of guinea pigs following treatment with chemicals or chemical–protein conjugates (6–9).

The status of the induction of delayed-type cutaneous reactivity to simple chemicals is unclear. Eisen (10) noted in 1959 that contact skin sensitivity had not been produced in rabbits up to that time. Nevertheless, the mechanism of sensitization and antibody response has been considered by many workers (4, 5, 11, 12).

This report demonstrates that classical humoral antibodies and a characteristic anamnestic antibody response appear following appropriate topical application of 2,-4-dinitrofluorobenzene to rabbits. Further, the skin reactivity of these animals to a subsequent application of the sensitizing compound was noted.

Materials and Methods. Rabbits. New Zealand white male rabbits weighing 2.5 to 3.5 kg were employed. They were housed in individual cages and maintained on a diet of Purina rabbit chow and supplemental cabbage.

Sensitization. Six drops of a 2% alcoholic solution of 2,4-dinitrofluorobenzene (approx 3.2 mg) were applied to a shaved area of the nape of the neck, and were spread evenly over this area with a fire-polished glass rod. This treatment was repeated daily for either 6 or 12 times. No depilatories were employed.

Sampling. Animals were test bled from the central ear artery either daily (5 ml) or on alternate days (10 ml). Serum was collected and stored at −20°.

Skin tests. 2,4-dinitrofluorobenzene was diluted to 1, 0.5, and 0.1% concentrations in olive oil for testing. An area of the flank was clipped with an electric clipper. No depilatories were employed. One drop of each chemical dilution and an olive oil control was placed on each of four separate well-spaced sites, and was spread over an area 15 mm in diameter with a fire-polished glass rod (one rod for each dilution). The reactions were graded visually and recorded 24 and 48 hr after testing. The grading criteria were as follows:

− = no detectable reaction;
1+ = patchy erythema;
2+ = homogeneous erythema;
3+ = homogeneous erythema, induration; and
4+ = homogeneous erythema, induration, raised reaction site.

Conjugates. Protein samples were conjugated with 1-fluoro-2,4-dinitrobenzene (Eastman Organic Chemicals, 3× recrystal-

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lized from ether) by the method of Porter and Sanger (13). Protein was determined by the biuret method (14). The conjugate was sterilized by Millipore filtration (0.45 μ) and stored in sterile serum vials at —20°. Conjugates of autologous and homologous serum, bovine serum albumin, and lysine were prepared.

Precipitin tests. Ring or interfacial precipitin tests were used to detect precipitating antibodies. Twofold dilutions of autologous serum–dinitrophenyl conjugates were prepared in 0.15 *M* NaCl, pH 7.0. These dilutions were carefully layered over undilute serum samples in 4 mm (od) glass tubes. The presence of any precipitate at the interface within 60 min was recorded as positive.

Precipitin inhibition. Lysine–dinitrophenyl conjugate was mixed with an equal volume of serum known to contain precipitins. After incubation at 37° for 30 min, this mixture was overlaid with 2-fold dilutions of autologous conjugate, and the interfacial titer was determined.

Gel diffusion. The Ouchterlony gel diffusion technique (16) was employed with some of the sera. After addition of serum and conjugate dilutions, the plates were incubated at 37° for 48 hr and observed for precipitin bands.

Oakley-Fulthorpe (17) double diffusion tubes were employed for further study of precipitins in some of the sera. One percent agar was used to solidify the medium, and tubes were incubated at 37° until distinct bands had formed.

Passive hemagglutination. The tanned erythrocyte hemagglutination method of Boyden (18) was employed. Tannic acid (Mallinckrodt, A.R.) at a dilution of 1:10,000 was used to treat fresh sheep erythrocytes which were then coated with autologous conjugate (0.33 mg/ml). This antigen (2.5% suspension) was added in equal parts to a 2-fold dilution of serum in the Microtiter apparatus (Cooke Engineering Co.). Plates were incubated for 30 min at room temperature, shaken vigorously, and the incubation was continued for 2 hr. A homogeneous layer of agglutinated cells on the bottom of a well was considered positive.

Passive cutaneous anaphylaxis. A modification of the technique of Salvin and Gregg (19) was employed. One-tenth ml of serum was injected intracutaneously into the shaved abdominal surface of a 250–400 g guinea pig. Six sites were prepared on each animal with different dilutions of serum. After 30 min, the animal was injected intracardially with 1 mg of autologous conjugate mixed with 1 ml of Evans' blue dye solution (3 mg/ml). Reactions were read and recorded 30 min later.

Results. Figure 1 illustrates the typical serological response of the animals to topically applied 2,4-dinitrofluorobenzene (DNFB). This rabbit (No. 2) received applications of 2% DNFB for 7 consecutive days. Both precipitin and passive hemagglutination titers began to rise on day 8 and reached peak titers of 16 and 32, respectively, on day 14 for precipitins and passive HA antibodies. By day 20, the precipitin titer had dropped to 2, and the HA titer remained at 16.

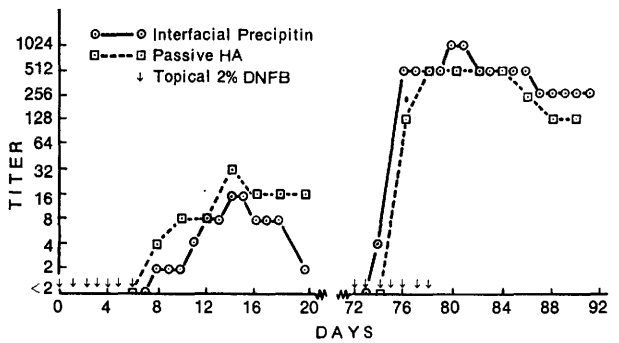

FIG. 1. Serological response of rabbit No. 2 to topically applied 2,4-dinitrofluorobenzene.

Figure 1 also shows typical results of a second series of paintings applied several weeks after primary sensitization. There was a dramatic rise in titer and the response was characteristic of an anamnestic response. The precipitin titer peak was 64 times greater than that elicited by the first painting series, and was "echoed" by the passive HA titer. The rise was apparent as early as 48 hr after secondary contact.

The effect of skin test amounts of chemical on a sensitized animal is shown in Fig. 2. This rabbit (No. 5) received two skin tests following initial treatment. These tests employed approximately 1/40th the amount of chemical used in the painting series, or approximately 0.5 mg. The peak titers obtained following the first skin testing were of the same magnitude as those induced by the first painting series. However, peaks were reached 5 to 6 days following the skin test, compared with 9 to 12 days after initial painting was started. The precipitin and passive HA titers were again comparable. The response to the second skin test was 4-fold lower than that to the first one, but the times necessary to reach peak titers were the same.

The effect of extending the initial painting period from 7 to 12 days was examined. Four animals given this extended treatment did not show significantly higher titers than those painted a shorter time. Rabbit No. 8 showed a typical response. This treatment retarded the time of initial peak titer by approximately 2 days. Rabbit No. 8 was skin tested at days 16 and 67, and the response to a second skin test was typical of an anamnestic response. Any response to the first skin test was presumably masked by the initial immune response. These earlier skin tests did not seem to affect the anamnestic response to a second painting series initiated by day 110. In no case was a reaction noted when autologous serum carrier was employed as an antigen control in precipitin tests.

Comparison of the peak titers following initial versus second painting reveals a significant difference. Titer peaks after initial painting ranged from less than 2 to 64 with the median at 16. In contrast, peak titers after a second painting ranged from 64 to 1024 with the median titer at 256. The peak titer distributions following first and second painting series were clearly separated, overlapping only at a titer of 64, and the two populations means differ significantly at the 1% level by Student's t test. The peak titers due to skin testing range over the other two distributions. The precipitin and passive HA titers did not differ significantly.

Comparison of the times following treatment at which peak titers were reached revealed another significant difference. A peak precipitin titer was reached 10.9 days (mean) following the start of an initial painting series. In contrast, a peak titer was reached only 5.3 days following a skin test, and 5.9 days following a second painting series initiation. The peak titer times following skin tests and second paintings differ from the primary painting peak times at the 1% level of significance.

Precipitin inhibition tests showed the antibodies to be directed solely against the DNP group. One precipitation line was obtained in gel diffusion studies, suggesting the presence of one antibody. However, a specific IgM, if

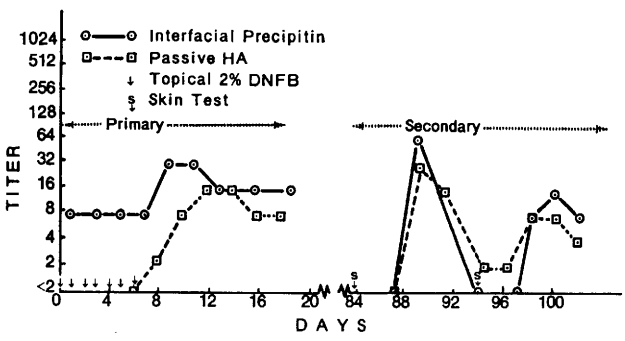

FIG. 2. Serological response of rabbit No. 5 to topically applied 2,4-dinitrofluorobenzene.

present, might have been expected to react well under these conditions. Positive PCA reactions were obtained only with the peak titer sera from a second painting series.

The typical skin test response in the rabbit to topically applied DNFB is somewhat different from that seen in the guinea pig. The reaction is barely apparent at 6 hr, although some animals exhibit a mixed reaction characterized by whiteness and edema at this time. At 24 hr, the reaction area is characterized by erythema and induration, and in many animals the site is quite raised. By 48 hr, the redness has usually darkened and the elevation has subsided. The reaction site usually shows tissue damage which remains as a dark area for a week or more.

No correlation was found between the intensity of the skin test and the titer of precipitins. These developments were apparently concomitant, but related only in their specificity.

Discussion. The present results support the hypothesis that reactive dinitrophenyl compounds couple to body proteins and thereby acquire the "carrier molecule" necessary for antigenicity.

The anamnestic character of this antibody response has been established. The drastic increase in antibody due to repainting and the shortened time necessary for a repainted animal to produce a peak titer are anamnestic characteristics. The titer reached upon re-exposure to DNFB appears to be dose dependent, since significantly higher titers were obtained following a second painting series versus a skin test. The length of the rest period also seems to be an important factor. A second painting series appears to induce maximal anamnestic titers if it is initiated on or after the 28th day following initial topical exposure of the animals to the chemical.

A state of cutaneous reactivity was detected in all the painted rabbits tested. Whether this reactivity was directed against the hapten alone or against the autologous protein carriers is difficult to ascertain.

Summary. Most rabbits treated topically with 2,4-dinitrofluorobenzene produced circulating precipitins and passive hemagglutinins. Animals sensitized by a primary painting series and re-exposed topically to DNFB all demonstrated anamnestic antibody response. The time required for a peak titer to be reached after a second contact with the chemical was approximately one-half of that following the first application series. The relative titers following a skin test versus a second painting series suggested that the magnitude of the anamnestic response may have been dose dependent. The antibodies produced by this treatment were apparently directed only against the dinitrophenyl group. No precipitating antibodies against the autologous carrier were detected. No correlation was seen between the amount of precipitating antibody present and the intensity of the skin test. The development of contact hypersensitivity to a simple chemical compound in the rabbit was shown.

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