# HAPTEN-SPECIFIC DELAYED HYPERSENSITIVITY TO ε-2,4-DINITROPHENYL-L-LYSINE-FICOLL IN GUINEA PIGS IMMUNIZED WITH 2,4-DINITROPHENYL-KEYHOLE LIMPET HEMOCYANIN

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Delayed skin hypersensitivity in guinea pigs was first described by Koch (1) who noted that an extract of tubercle bacilli could induce a prolonged skin reaction when appropriately inoculated into animals which had been made allergic to mycobacteria. Subsequently, Chase (2) showed that the tuberculin-delayed type of allergy could be transferred by giving leukocytes derived from immunized animals to otherwise untreated recipients. More recently, Benacerraf and Gell (3–5) and others (6–11) examined the conditions required to induce and elicit delayed sensitivity to hapten protein complexes. They found that immunization with relatively small amounts of these complexes, such as 1–10 μg, produced a delayed type of allergic response that could be elicited with about 10 μg of the immunizing hapten protein complex, but not with the same hapten on another protein carrier (3).

However, when they immunized with large amounts of the hapten-protein complex (1 mg), then they could elicit a delayed type of response with that hapten on another protein carrier provided they challenged the animals with a sufficiently large quantity, 100 μg per skin test site (4).

It was of interest to extend these studies further to determine if animals immunized with a hapten-protein conjugate could respond with a delayed type of skin reaction when tested with the same hapten coupled to a carbohydrate carrier. Such a response to a hapten carbohydrate conjugate could then be employed to study further the importance of the carrier and the epitope density in delayed responses.

We were also interested in the tolerogenic ability of the hapten carbohydrate complex to inhibit the formation of antibody and skin hypersensitivity in animals immunized with a hapten-protein conjugate. For these purposes the experiments described below were undertaken.

## Materials and Methods

*Animals.* Guinea pigs of the Hartley strain, strain 13, strain 2, NIH strain and NIH C4-deficient subline guinea pigs were supplied by the Rabbit and Rodent Section of the NIH. They were fed Feed A pellets, along with kale and water, all ad lib.

*Antigens.* A sucrose polymer made with epichlorohydrin, Ficoll, Pharmacia Fine Chemicals, Inc., Piscataway, N. J.), lot 2300 (average mol wt 400,000), was coupled to ε-2,4-dinitrophenyl (DNP)[1]-lysine by cyanuric choloride to make dinitrophenyl-L-lysine (DNPL)-Ficoll conjugates as described elsewhere (12).[2] The DNP content was measured by optical absorption at 365 nm and the carbohydrate content by the phenol sulfuric acid test (13).

Dinitrophenyl conjugates with keyhole limpet hemocyanin (DNP-KLH) were prepared by adding drop by drop while stirring 1 ml of a 10% dinitrofluorobenzene (DNFB) in absolute ethanol to 1 g KLH in 40 ml 3% $KHCO_3$, pH 9–10. 2 mmol of glycylglycine was then added to the reaction mixture and stirred for 2 h before it was dialyzed to remove excess free DNFB. The epitope density was determined by measuring the optical density of 280 and 365 nm assuming a mol wt of 50,800 for KLH (14, 15). In addition, DNFB was coupled to mycobacteria as described by Benacerraf and Gell (3).

Oxazalone-KLH ($ox_n$-KLH) was prepared according to Askenase (16). The nitrogen concentration of the product was assayed.[3] In addition the oxazalone content was measured spectrophotometrically at 352 nm. The final product was calculated to be $ox_{7.5}$-KLH, by assuming a molar extinction coefficient of 18,400.

*Active Immunization.* For immunization with DNP-KLH or DNP-Ficoll, 15 ml of the antigen solution, usually at a concentration of 1 mg of antigen/ml was emulsified with 5 ml of Freund's complete adjuvant composed of Bayol F (Humble Oil; Refining Co., Houston, Tex.), Arlacel A (Atlas Chemical Industries, Inc., Wilmington, Del.), 80/20 (vol/vol) containing *Mycobacterium tuberculosis* $H_{37}$ R v at a concentration of 4 mg/ml of the oil Arlacel phase. 1 ml of a freshly prepared emulsion was then inoculated intradermally (i.d.) in small portions in each foot and in the nuchal and posterior scapular area.

For immunization with DNP coupled to mycobacteria, 5 mg of the DNP-mycobacteria was suspended per ml of the oil phase in incomplete adjuvant and an emulsion of that made with 3 vol of physiologic saline. This was inoculated as above.

The procedure of Askenase (16) was followed for immunization with oxazalone.

*Passive Immunization.* For passive transfer tests with antibody, pooled antisera that had been stored at −20°C was warmed to room temperature and 5 ml given intravenously (i.v.) or intraperitoneally (i.p.).

*Skin Tests.* At suitable intervals after immunization, the guinea pigs were skin tested by inoculating 0.1 ml of the DNPL-Ficoll, Ficoll, cyanuric-Ficoll, DNP-KLH or $ox_n$-KLH, test material intradermally on the flank. To test with oxazalone, the material was applied to the flank skin as described by Askenase (16). The tests were observed at intervals, usually at 2, 4, and 24 h and sometimes in addition at 1/2, 1, 3, 12, and 18 h. Unless the experiment was terminated at 24 h, they were usually measured at 48 and 72 h also. The diameter of the soft swelling, hemorrhage, erythema and induration, and the thickness of a double-skin fold were recorded.

Sections of skin were prepared for histological examination by fixing a radial strip of a full thickness piece of skin either in 10% formalin or 0.2 M HgCl and 0.2 M sodium acetate, embedding it in paraffin and cutting sections 6 μm thick, and then staining either with hematoxylin and eosin or Giemsa. Other sections were fixed in glutaraldehyde for the preparation of 1-μm Epon-embedded sections for microscope examination especially for basophils as described elsewhere (17).

*Antibody Titers.* To obtain serum for antibody determinations, the animals were anesthetized with pentobarbital and bled by cardiac puncture. Once the serum had been separated from the retracted clot, it was stored at −20°C. Anti-DNP titers were measured by the Farr (18) test with radioactive ε-DNPL using the calculated 30% end point as the final titer (19).

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[1] *Abbreviations used in this paper:* DNFB, 2,4-dinitrofluorobenzene; DNP, 2,4-dinitrophenyl; DNPL, 2,4-dinitrophenyl-L-lysine; KLH, keyhole limpet hemocyanin; $ox_n$-KLH, oxazalone coupled to KLH: PPD, purified protein derivative of *Mycobacterium tuberculosis.*

[2] McMaster, P. R. B., J. D. Owens, and W. E. Vannier. 1976. The preparation and characterization of a thymic independent antigen: ε-Dinitrophenyl-L-lysine-Ficoll. Manuscript in preparation.

[3] Kindly performed by Paula Parisius, Section on Microanalytical Services and Instrumentation, Laboratory of Chemistry, National Institutes of Allergy and Infectious Diseases, Bethesda, Md.

## Results

When skin tested with DNPL-Ficoll, groups of Hartley guinea pigs that were immunized with DNP-KLH in complete Freund's adjuvant, quickly developed an intense degree of soft subcutaneous swelling. This area of edema expanded in size for 1-3 h and then faded away. Sometimes 1 or 2 h after the start of the test, this was accompanied by a centrally located dark red area of hemorrhage that did not blanch upon pressure. In addition, in these experimental animals, as in the controls, an area of mild erythema often appeared 1-2 h after the DNP-Ficoll was injected. However, by contrast with the controls, this erythematous component in the experimental animals did not fade away. Instead it began to increase in intensity and size 3-12 h after the start of the test. Furthermore, from about 12 h onward, the skin about the injection site became superficially indurated which caused it to feel firm and stiff. By 12 h, the erythema and the induration were coincident in size, and remained so thereafter. Usually, at 24 h, the erythema and induration had reached their maximum size and intensity. In many animals the lesion had almost disappeared by the 2nd day, but in certain others, it remained florid for 48 h, and occasionally, was still prominent at 72 h. Table I shows the diameter of the erythema at selected hours in such animals skin tested with two concentrations of $DNPL_{71}$-Ficoll, and for comparison, gives the course of the skin reaction in other animals similarly immunized, but tested with purified protein derivative of *Mycobacterium tuberculosis* (PPD). In addition, the responses to identical inoculations in unimmunized animals are included to provide a base-line measure of irritation resulting from the injection.

To ascertain if other strains of guinea pigs could also produce prolonged skin reactions, five strain 13 and three strain 2 guinea pigs were immunized with DNP-KLH and tested with DNPL-Ficoll. The average diameter of early erythema and subsequent induration and erythema at 24 and 48 h which developed in those animals appears in Table II.

*Histological Appearance of Lesions in Actively Immunized Animals.* Histological sections of the lesions appeared characteristic of an immediate reaction mixed with a more prolonged type of skin reaction (Fig. 1). Often numerous polymorphonuclear leukocytes were present within and about the blood vessels. Many of these were neutrophils, but eosinophils were also present and in selected animals may have constituted a majority. In addition, there were many mononuclear cells about the small vessels and scattered through the dermis. Although plasma cells were sometimes present most of the mononuclear cells appeared to be lymphocytes and macrophages. Focal erythrocyte extravasation and moderate to extensive interstitial fibrin deposition in the reticular dermis were regular findings. To evaluate the quantity of basophils, 1-$\mu$m thick Epon-embedded sections of skin fixed in glutaraldehyde were studied. This prolonged type of lesion, elicited with DNPL-Ficoll, contained only occasional basophils, no more than could be expected in a standard tuberculin reaction to PPD.

*Induction of Skin Hypersensitivity with Other DNP-Conjugates.* To determine if a DNP-conjugate other than DNP-KLH could induce prolonged skin responsiveness to DNPL-Ficoll, animals were immunized with DNP coupled to killed mycobacteria. Those tested 5 days later with DNPL-Ficoll did not re-

TABLE I

*Skin Response to $DNP_{71}$-Ficoll and PPD in Hartley Strain Guinea Pigs Immunized with DNP-KLH and in Control Non-Immunized Guinea Pigs*

| Immunized with | Skin test with | Skin response (average diameter, *mm*) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Erythema | | | | | Induration and erythema 24 h |
| | | 2 h | 3-4 h | 6 h | 8 h | 12 h | |
| DNP-KLH in complete Freund's adjuvant | 2.9 mg $DNP_{71}$-Ficoll | 0 | 24 | 25 | 25 | | 55 |
| | | 0 | 15 | 18 | 18 | | 59 |
| | | 0 | 23 | 23 | 25 | | 60 |
| | | 20 | 22 | 22 | 25 | | 50 |
| DNP-KLH in complete Freund's adjuvant | 0.29 mg $DNP_{71}$-Ficoll | 0 | 14 | 0 | 0 | | 32 |
| | | 0 | 3 | 15 | 16 | | 32 |
| | | 14 | 18 | 20 | 25 | | 32 |
| | | 0 | 0 | 20 | 0 | | 23 |
| Not immunized | 2.9 mg $DNP_{71}$-Ficoll | 12 | 11 | 11 | 12 | | 8 |
| | | 0 | 0 | 0 | 0 | | 0 |
| | | 0 | 0 | 0 | 8 | | 3 |
| | | 0 | 0 | 0 | 0 | | 0 |
| | | 0 | 0 | 0 | 5 | | 6 |
| DNP-KLH in complete Freund's adjuvant | PPD | | 0 | | 22 | 30 | 32 |
| | | | 0 | | 18 | 15 | 32 |
| | | | 0 | | 22 | 29 | 31 |
| | | | 0 | | 20 | 26 | 32 |
| | | | 0 | | 13 | 26 | 39 |
| Not immunized | PPD | | 0 | | 0 | 0 | 0 |
| | | | 0 | | 0 | 8 | 7 |
| | | | 0 | | 7 | 10 | 7 |

For immunization the animals were inoculated with 1 mg of DNP-KLH in complete Freund's adjuvant. For skin tests, twice second strength PPD was used.

spond. Nevertheless, three of four tested 3 wk later gave responses 33-36 mm in diameter at 24 h. Similarly 1 mg of $DNPL_{30}$-Ficoll in adjuvant produced a similar degree of prolonged skin hypersensitivity.

*Effect of Interval between Immunization and Skin Test.* In other animals, the required time between immunization and skin testing needed to induce prolonged skin reactivity was studied. After immunization with 1 mg $DNPL_{7.9}$-KLH in complete adjuvant, no response appeared when the animals were tested 5 days later, but a response of modest degree appeared in three of five tested on the 9th day. As before, large reactions appeared in similarly immunized guinea pigs by 4-6 wk. Immunization with 10 $\mu$g of $DNPL_{7.9}$-KLH did not lead to responsiveness to DNPL-Ficoll, indicating that intense immunization was needed to obtain such a response.

*Control Skin Tests.* When skin tested with DNPL-Ficoll, unimmunized control animals developed a small area of soft edema within an hour. This sometimes expanded slightly in size during the next hour. Then it gradually faded away so that it was at most a few millimeters in diameter the next day. In

TABLE II

*Prolonged Skin Hypersensitivity to DNPL-Ficoll in Strain 13 and Strain 2 Guinea Pigs Immunized with DNP-KLH and Complete Freund's Adjuvant*

| Immunized with | Strain of guinea pigs | Skin response (average diameter, *mm*) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Erythema | | | Induration and erythema | | |
| | | 3 h | 6 h | 12 h | 24 h | 48 h | 72 h |
| DNP-KLH in adjuvant | 13 | 20 | 23 | 29 | 34 | 0 | |
| | | 23 | 26 | 18 | 22 | 0 | |
| | | 0 | 18 | 32 | 33 | 0 | |
| | | 22 | 21 | 27 | 32 | 0 | |
| | | 8 | 33 | 30 | 32 | 25 | |
| Not immunized | 13 | 8 | 13 | 14 | 12 | 8 | |
| | | 4 | 10 | 10 | 7 | 0 | |
| | | 0 | 0 | 6 | 8 | 0 | |
| | | 10 | 0 | 6 | 4 | 0 | |
| | | 12 | 12 | 14 | 14 | 6 | |
| DNP-KLH in adjuvant | 2 | 0 | 0 | | 41 | 15 | 8 |
| | | 0 | 0 | | 43 | 22 | 12 |
| | | 22 | 22 | | 34 | 0 | 12 |
| Not immunized | 2 | 0 | 0 | | 11 | 0 | 0 |
| | | 0 | 0 | | 13 | 0 | 0 |
| | | 0 | 0 | | 0 | 0 | 0 |

Strain 13 skin tested with $DNPL_{13}$-Ficoll 1.7 mg; Strain 2 skin tested with $DNPL_{71}$-Ficoll 2.8 mg.

addition, a mild erythema of lesser extent sometimes appeared 1-2 h after the DNPL-Ficoll was injected. This then gradually faded away so that the lesions were less than 10 mm in diameter at 24 h. For immunized controls, one group was immunized with 1 mg of KLH without DNP in adjuvant and tested with DNP-KLH and DNPL-Ficoll 6 wk later. The responses to DNP-KLH at 24 h ranged from 33 to 45 mm but those to 2.4 mg of $DNPL_{25}$-Ficoll were less than 3.5 mm in diameter.

In addition, the possibility that Ficoll without DNP could elicit reactions in DNP-KLH-immunized animals was tested by giving selected amounts up to 50 mg intradermally. These tests were all negative, as were tests with cyanurate-Ficoll without DNP. Finally, the possibility that DNPL-Ficoll could prolong an immediate response to another antigenic determinant was examined. For this purpose, animals that were hyperimmunized to type III pneumococcal polysaccharide were injected on the left flank with that polysaccharide and on the right with it mixed with $DNPL_{13}$-Ficoll at a concentration of 17 mg/ml. There was no evident difference in the immediate component of the reactions and no evident lesion at 24 h even in those given the type III polysaccharide and DNPL-Ficoll together.

*Effect of Quantity of DNPL-Ficoll and Epitope Density upon the Skin Reactions.* The molecular requirements needed to evoke the prolonged component of the skin test to DNPL-Ficoll were evaluated in DNP-KLH-immunized ani-

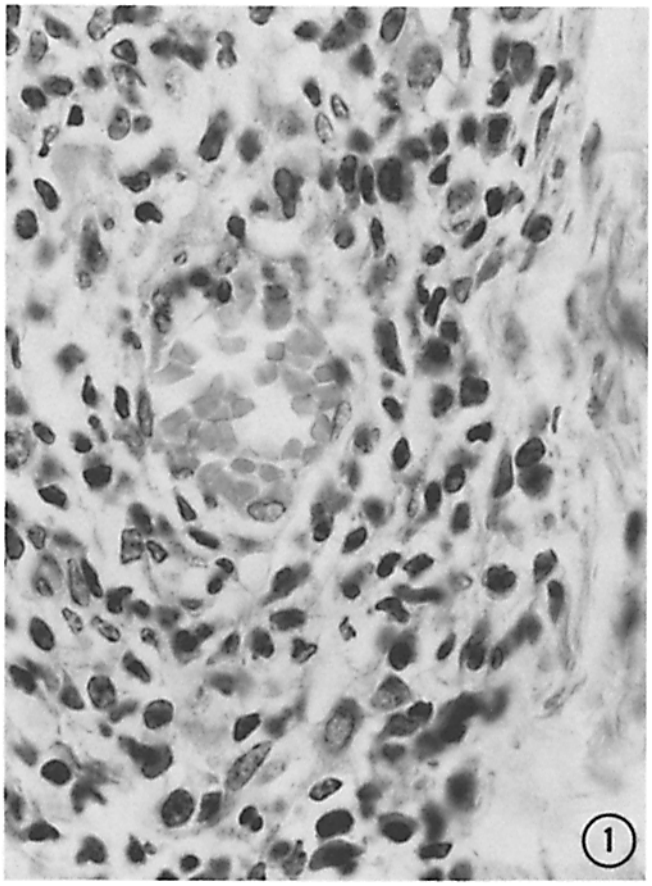

FIG. 1. 72-h skin reaction to DNPL-Ficoll in a guinea pig immunized with DNP-KLH. Hematoxylin and eosin × 500.

mals. Initially, animals were tested with different quantities of $DNPL_{30}$-Ficoll. Table III shows the diameter of induration and erythema at 24 h in these studies. The largest quantity of $DNPL_{30}$-Ficoll (3.2 mg per site) elicited the largest response (35-52 mm). In another group, tests with 100 $\mu$g were also positive (23-35) but were not as large or as florid. By contrast, 10 $\mu$g failed to elicit any response at all.

The relative importance of the epitope density vs. the number of carbohydrate molecules with DNP was also studied. When animals were tested with DNPL-Ficoll with low substitution ratios, their response to 100 $\mu$g of $DNPL_{1.5}$-Ficoll or 200 $\mu$g of $DNPL_{0.8-1.5}$-Ficoll was negative, whereas the comparative tests with similar quantities of $DNPL_{30}$-Ficoll in the same animals were positive. To determine if the molecules with a low epitope density could elicit a reaction if sufficient quantity were injected, one group was tested with 7.9 mg of $DNPL_{0.8}$-Ficoll. Three of five gave positive responses. In addition, another group was tested on one side with 1.7 mg of $DNPL_{13}$-Ficoll and on the other with $DNPL_{1.5}$-Ficoll adjusted so as to have the same concentration of DNP. The skin reactions with both DNPL-Ficoll preparations were positive, and of similar size.

TABLE III

*Prolonged Skin Hypersensitivity Reactions to DNPL-Ficoll Preparations with Selected Epitope Densities in Hartley Guinea Pigs Immunized with DNP-KLH*

| Skin test inoculum | | | Guinea pig group | Skin response (average diameter, *mm*) Induration and erythema at 24 h in individual animals | | | | |
|---|---|---|---|---|---|---|---|---|
| mg DNPL-Ficoll | μmol DNP | Epitope density | | | | | | |
| 3.17 | 0.237 | 30 | A | 52 | 48 | 50 | 35 | |
| 0.2 | 0.015 | 30 | B | 25 | 30 | 25 | 0 | 6 |
| 0.1 | 0.0075 | 30 | C | 33 | 23 | 23 | 35 | |
| 0.01 | 0.00075 | 30 | A | 0 | 3 | 0 | 0 | |
| 7.9 | 0.016 | 0.8 | D | 22 | 28 | 27 | 0 | 5 |
| 0.2 | 0.0004 | 0.8 | B | 6 | 9 | 3 | 0 | 0 |
| 0.1 | 0.00038 | 1.5 | C | 0 | 0 | 0 | | |
| 1.7 | 0.055 | 13 | E | 43 | 34 | 38 | 42 | |
| 14.7 | 0.055 | 1.5 | E | 42 | 33 | 33 | 34 | |

Animals of groups A, B, C, D, E skin tested simultaneously on both sides. Guinea pigs immunized with $DNP_{10.7}$-KLH 1 mg/ml in Freund's complete adjuvant.

*Induction of Tolerance by DNPL-Ficoll.* The tolerogenicity of DNPL-Ficoll in $DNP_{12}$-KLH-immunized animals was also evaluated by giving 10-15 mg of $DNPL_{30}$-Ficoll, $DNPL_{57}$-Ficoll, or $DNPL_{17}$-Ficoll, either at the time of immunization or 5 days later. The response in these tolerized animals was then compared 6 wk later with the response in others immunized but not tolerized (Table IV). The skin reaction and the antibody titer were greatly reduced in all but one of the animals injected with a tolerogenic dose of DNPL-Ficoll. By contrast, all those which were simply immunized gave strong skin reactions and produced high antibody titers.

*Skin Reactions to DNPL-Ficoll after Passive Immunization with Antibody.* In addition to studying reactions to DNPL-Ficoll in actively immunized animals, we also examined the skin tests in others given serum from DNP-KLH-immunized animals. Several attempts to transfer a prolonged reaction to DNPL-Ficoll were made by giving the antisera i.p. or i.v.; they all failed except one. The one success came using a pool of sera from Hartley and strain 13 guinea pigs that had been immunized 9 wk earlier. 25 ml of this antiserum pool was given i.p. to five recipients which were skin tested shortly thereafter with $DNPL_{71}$-Ficoll, 2.8 mg per site. No erythema was evident 3 h later in four out of five, but at 24 h, some very mild erythema was present in all and was accompanied by a little soft swelling of equal diameter. These lesions ranged in diameter from 20 to 40 mm. None of them felt indurated (Table V). At 22 h, one of these recipient animals was tested on the other side with DNPL-Ficoll. It gave a similar positive response with a small central hemorrhage in 2 h. Histological sections of these tests differed from those in actively immunized animals, for these skin tests (in animals give antibody) contained a few polymorphonuclear leukocytes but no lymphocytes or macrophages (Fig. 2). In the controls, three had no erythema, whereas two had some erythema which was 5 and 10 mm in diameter, respectively. Most of the sections of the control skins were devoid of inflammatory cells except for an occasional polymorphonuclear leukocyte.

TABLE IV
*Induction of Tolerance to DNP by DNPL-Ficoll in Guinea Pigs Immunized with DNP-KLH in Complete Freund's Adjuvant*

| Tolerized with | Given days after immunization | Skin response (average diameter, *mm*) | | 30% $Log_2$ antibody titer |
|---|---|---|---|---|
| | | Erythema 2–3 h | Induration and erythema 24 h | |
| 0 | | – | 40 | 12.8 |
| | | – | 43 | 11.3 |
| | | – | 38 | 11.2 |
| | | – | 42 | – |
| 15 mg $DNPL_{30}$-Ficoll | 0 | – | 4 | <0 |
| | | – | 8 | <0 |
| | | – | 30 | 8.4 |
| | | – | 6 | <0 |
| | | | | <0 |
| 0 | | 20 | 43 | 13.6 |
| | | 20 | 44 | 13.1 |
| | | 22 | 43 | 12.6 |
| | | 25 | 44 | 14.1 |
| 15 mg $DNPL_{57}$-Ficoll | 0 | 12 | 10 | <0 |
| | | 12 | 5 | <0 |
| | | 13 | 8 | <0 |
| | | 10 | 6 | – |
| 0 | | 12 | 5 | 1.5 |
| | | 20 | 34 | 11.6 |
| | | 23 | 22 | – |
| | | 0 | 33 | – |
| | | 22 | 32 | – |
| | | 8 | 33 | 11.6 |
| 10 mg $DNPL_{17}$-Ficoll | 5 | 7 | 0 | 2.0 |
| | | 9 | 0 | – |
| | | 7 | 0 | 1.7 |
| | | 8 | 0 | 5.7 |
| | | 0 | 0 | 1.7 |

–, Not measured.

*Comparison of DNPL-Ficoll Skin Reactions to Delayed, in Time, Oxazalone Reactions.* It was of interest to compare the DNPL-Ficoll reaction in actively and passively immunized guinea pigs with the delayed in time reaction to oxazalone which some have attributed to serum antibody (16). For this purpose guinea pigs were immunized by painting oxazalone on their ventral surface. Later they were tested by painting oxazalone on one side and by injecting the other side with oxazalone coupled to KLH. The side painted with oxazalone had no reaction at 2 or 4 h, but gave reactions ranging from 0 to 33 mm in diameter at 24 h. These were slightly pink, and had some soft swelling, but no real

TABLE V

*Skin Reactions to 2.8 mg of $DNPL_{71}$-Ficoll in Individual Guinea Pigs Given I.P. 25 ml of Pooled Anti-DNP-KLH Antisera*

| Amount of antiserum given | Skin lesion (average diameter, *mm*)* | | | | | | |
|---|---|---|---|---|---|---|---|
| | Soft swelling, 3 h | Pale erythema, 6 h | Soft swelling, 6 h | Pale erythema, 6 h | Soft swelling, 24 h | Pale erythema, 24 h | H‡ |
| 25 ml | 25 | 18 | 25 | 0 | 32 | 28 | 4 |
| " | 15 | 0 | 8 | 0 | 30 | 30 | 3 |
| " | 22 | 0 | 18 | 0 | 40 | 40 | 8 |
| " | 22 | 0 | 12 | 0 | 38 | 38 | 6 |
| " | 20 | 0 | 21 | 0 | 22 | 20 | 8 |
| None | 20 | 0 | 21 | 0 | 2 | 0 | 0 |
| " | 22 | 0 | 22 | 0 | 3 | 0 | 0 |
| " | 20 | 0 | 22 | 0 | 2 | 0 | 0 |
| " | 22 | 0 | 27 | 0 | 5 | 5 | 0 |
| " | 20 | 0 | 15 | 0 | 10 | 10 | 0 |
| " | 9 | 0 | −§ | | 2 | 2 | 0 |

Antisera collected 9 wk after immunization with $DNP_{10.4}$-KLH, 1 mg/ml in Freund's complete adjuvant. The 30% $\log_2$ titer to DNP was 11.2.

* No duration was evident during the observation interval.

‡ H, Hemorrhage.

§ Not measured.

induration. The reactions to oxazalone-KLH on the other side appeared by 2 h and ranged in size from 25 to 35 mm at 4 h. They were still about this size at 24 h. They, too, were slightly erythematous and had some soft swelling beneath the erythema but no palpable induration. As such, the lesions differed from those prolonged reactions in actively (DNP-KLH) immunized animals tested with DNPL-Ficoll, for the prolonged reactions in actively immunized animals were much more florid and were firmly indurated.

## Discussion

In earlier studies Benacerraf and Gell showed that the specificity of delayed reactions with hapten-protein conjugates included part of the carrier molecule as well as the hapten determinant after immunization with modest quantities of antigen in Freund's complete adjuvant (3). They also found that a delayed type of hypersensitivity could be elicited in animals intensely immunized to a picryl protein complex if they tested with the same hapten joined to another protein, provided that a large quantity was used both to immunize and elicit the response (4).

Recently other experiments[4] suggested that DNPL-Ficoll could not only act as an immunogen and tolerogen in guinea pigs, but could when injected intradermally in suitable quantity evoke immediate and prolonged skin reactions in guinea pigs immunized with the same material. In that work, 100 $\mu$g $DNPL_{30}$-

[4] McMaster, P. R. B., J. D. Owens, R. Weichbrod, and R. Asofsky. 1976. Immunogenic, tolerogenic, and allergenic effects of dinitrophenyl-L-lysine coupled to the sucrose polymer - Ficoll - in guinea pigs. Manuscript in preparation.

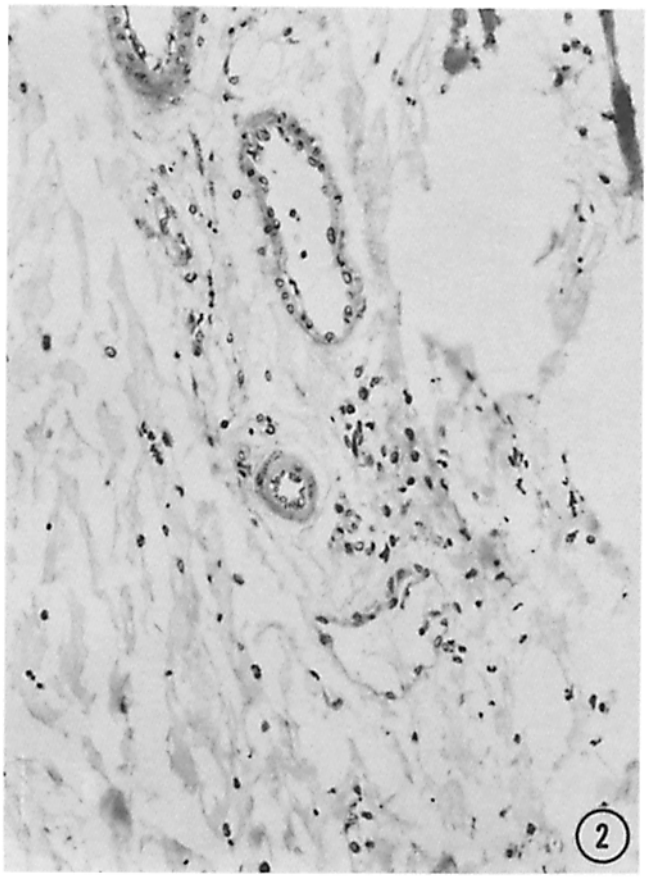


FIG. 2. 24-h skin reaction to DNPL-Ficoll in a guinea pig passively immunized to DNP-KLH with 25 ml of antisera. Hematoxylin and eosin × 250.

Ficoll sufficed to elicit strong reactions whereas 10 $\mu$g did not do so. When tests were performed with preparations with less DNP per Ficoll molecule, the total amount of the DNPL-Ficoll complex needed to produce the skin reaction was roughly inversely proportional to the amount of DNP per DNPL-Ficoll molecule, indicating that the amount of DNP in the complex injected was the determinant factor. Control tests with Ficoll alone and with Ficoll coupled to cyanuric chloride without DNP did not give such reactions in the immunized animals. These findings suggested that the reactions observed were specific for the hapten. Accordingly the present experiments were undertaken to determine if DNPL-Ficoll molecules could induce hapten-specific reactions in animals immunized with DNP attached to a totally different carrier such as a protein like KLH. When adequate amounts of this DNPL-Ficoll were injected intradermally into immunized animals, immediate and prolonged skin reactions appeared. As in the previous experiments the amount required to do so was about the same in animals immunized with DNP-KLH as in others immunized with DNPL-Ficoll. Just as the hapten substitution ratio determined, in part, the amount needed for a skin test in animals immunized with DNPL-Ficoll, that ratio was also influen-

tial in skin tests in animals immunized with DNP-KLH. As in those immunized with DNPL-Ficoll, those immunized with DNP-KLH could be tolerized by an injection of DNPL-Ficoll at the time of immunization, for such animals failed to make nearly as much antibody or skin reactivity to DNPL-Ficoll as those immunized with DNP-KLH, but not tolerized. These findings suggested that the reactions were indeed hapten specific.

Of these reactions the prolonged component of the skin reaction to DNPL-Ficoll was of particular interest, for it was similar to a delayed hypersensitivity reaction to PPD in the guinea pig.

The appearance of the prolonged reaction to DNP-Ficoll, like the PPD reaction, was red or pink to red at 24 h. Like the PPD reaction, the DNPL-Ficoll reaction often lasted 48–72 h. Moreover, in the actively immunized animal the DNPL-Ficoll reaction developed a firm superficial induration of the epidermis by 24 h. By contrast, another type of prolonged response, that to oxazalone in oxazalone sensitized animals, was paler in color and soft rather than indurated.

In the past, the histological appearance of skin reactions has been employed to clarify their pathogenesis. Accordingly, 24- and 72-h skin reactions induced with DNPL-Ficoll in DNP-KLH-immunized animals were examined histologically by light microscopy. The appearance of the sections confirmed the gross clinical impression of an immediate reaction for it contained numerous polymorphonuclear cells even in sections taken at 24 h. Interestingly, many appeared to be eosinophils rather than neutrophils. The sections also showed that lymphocytes and macrophages had collected about the blood vessels and in the epidermis, in the pattern so characteristic of delayed reactions and of cutaneous basophilic hypersensitivity (16, 17, 20-23). Because prolonged chronic basophilic skin reactions may be confused clinically and histologically with delayed reactions unless a search for basophils is made, sections of 24-h lesions were prepared and examined in 1-$\mu$m Epon-embedded sections. They did not reveal an unusual number of basophils. Other sections of the skin reactions fixed in mercuric acetate (24) and stained with Giemsa also revealed only an occasional cell with basophilic granules. Similar numbers of these cells appeared in control sections of PPD reactions. Moreover there was extensive dermal fibrin deposition of the type occurring in certain immediate hypersensitivity reactions and in classic tuberculin hypersensitivity but lacking in chronic basophilic hypersensitivity (25). Consequently, the nature of the prolonged skin response to DNPL-Ficoll in intensely immunized animals bore several similarities to a classical delayed PPD type reaction. However, the distinct possibility still existed that the prolonged reaction was some other type of allergic reaction. Since Ficoll derivatives might persist for a long time in the skin, they might enable serum antibody to produce a more continuous long-lasting reaction than would be elicited with a material that vanished quickly. Therefore, attempts to transfer the reaction with serum were made. Most pools of serum failed to produce any reaction in recipients. However, one pool did confer upon recipients the ability to respond to a DNPL-Ficoll skin test with a very pale erythema and some soft swelling of the skin that was still present 24 h after the skin was inoculated. The skin lesions in the passively immunized animals differed from those in actively immunized animals grossly in two respects. In the passively immunized animals, they were much paler and softer than in actively immunized animals in

which the lesions were florid and firmly indurated. In addition, histological sections of the lesions in animals passively immunized with antibody differed from those in actively immunized animals. The lesions in animals given antibody contained only a few polymorphonuclear cells, whereas those in actively immunized animals had in addition collections of lymphocytes or macrophages about the blood vessels. As such, the prolonged lesion induced by antibody appeared histologically compatible with a serum antibody type of reaction, despite its duration, confirming the clinical observation that it differed from the indurated lesion in the actively immunized animals.

The possibility that the skin reactions to DNPL-Ficoll occurred partly because the carrier molecules, KLH, mycobacteria, and Ficoll had one or more structurally similar regions seems unlikely, particularly since the Ficoll was devoid of nitrogen. That they might have developed because of a common contaminant in the preparations used to immunize and to skin test also seems most unlikely for other reasons. The time needed to dissolve the reagents before coupling them was too short for any bacterial multiplication, and the coupling conditions were most unconducive to bacterial growth. To prevent the dinitrobenzene coupling agent from combining with any contaminant in the dialysis sac, or with the animals tissue, we mixed glycyl-glycine with several of the DNP-KLH preparations 2 or more hours before putting it in the boiled washed dialysis sac to eliminate as much remaining free DNFB coupling agent as possible. Finally, were there, nevertheless, free DNFB available to couple with the animal's tissues and thus immunize the animal so as to yield positive skin tests with DNPL-Ficoll, then painting dinitrochlorobenzene repeatedly on the skin should have done the same. However, that process did not sensitize the animals so as to give positive skin tests with DNPL-Ficoll, although it did make them very reactive to challenge with 1% dinitrochlorobenzene in oil.

All these factors suggest that the prolonged component of the skin reaction to DNPL-Ficoll in DNP-KLH-immunized animals is a form of delayed hypersensitivity. Such a conclusion might imply that one of the in vitro correlates of delayed hypersensitivity should yield a positive test when the cells of DNP-KLH-immunized animals are challenged with DNPL-Ficoll. Recent evidence suggests that this is the case for the migration of leukocytes of such animals was inhibited by DNPL-Ficoll.[5]

The fact that an in vitro correlate test is positive with this apparently hapten-specific system may make it popssible to analyze the mechanism further to determine the role of B and T cells (26). It is entirely possible that these roles may differ from that in systems which require not only the exposure of the sensitized cells to the hapten but to the adjoining area of the carrier as well. If it becomes possible to demonstrate such a difference, the results would add to the evidence that more than one type or class of delayed hypersensitivity exists.

## Summary

After active immunization with 2,4-dinitrophenyl-keyhole limpet hemocyanin (DNP-KLH), 2,4-dinitrophenyl-L-lysine (DNPL)-Ficoll may elicit indurated, er-

[5] McMaster, P. R. B., J. D. Owens, and R. Asofsky. 1976. Hapten-specific leukocyte migration inhibition. I. Inhibition of cells from animals immunized with DNP-KLH by ε-DNP-L-lysine Ficoll. Manuscript in preparation.

ythematous skin reactions lasting 24-72 h. Histological sections of these reactions, examined by microscope techniques, showed they contained polymorphonuclear leukocytes and perivascularly situated lymphocytes and macrophages, but had very few basophils. Consequently, the reaction was interpreted as having an immediate component and a component typical of delayed hypersensitivity; this indicated that the delayed reaction could be specific for the DNP hapten. Although this delayed type of skin reaction was not transferred to recipients with anti-DNP-KLH serum, one pool of that serum did sensitize guinea pigs so that they could respond with a different skin reaction after challenge with DNPL-Ficoll. This reaction was soft, pale pink, and lasted for 24 h. Histologically, it contained only a few polymorphonuclear leukocytes. It differed from the delayed reaction in actively immunized animals in that it lacked induration, and was devoid of lymphocytes and macrophages.

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