

IN VITRO LYMPHOCYTE RESPONSES IN CONTACT HYPERSENSITIVITY III*

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ABSTRACT

Guinea pigs were sensitized to one of two contact allergens: dinitrofluorobenzene (DNFB) or paraphenylenediamine (PDA). Sensitization was accomplished by footpad injections of the hapten conjugated to an extract of soluble guinea pig epidermal proteins in Freund's complete adjuvant. Lymphocytes from animals with contact hypersensitivity to DNFB or PDA were cultured *in vitro* with guinea pig epidermal proteins conjugated to DNFB or PDA. Responses to these antigens, as measured by incorporation of tritiated thymidine into DNA, indicated that lymphocyte transformation *in vitro* can distinguish between sensitivity to either of these allergens.

Previous reports originating in this laboratory indicate that lymphocyte transformation *in vitro*, as quantitated by the uptake of tritiated thymidine, can serve as an indicator of contact hypersensitivity (1, 2). First it was reported that sensitization of guinea pigs with a hapten conjugated to an extract of epidermal protein could serve as both an immunizing agent and an *in vitro* eliciter of sensitivity in sensitized donors (1).

A later report demonstrated that this technique was capable of distinguishing between sensitivity to different haptens such as dinitrofluorobenzene (DNFB) or paraphenylenediamine (PDA). Sensitization was accomplished by the injection of unconjugated hapten in Freund's complete adjuvant, but tested with conjugates of the hapten and epidermal protein extracts. It was felt that, in this instance, conjugation of the hapten with the host's proteins occurred in the injection site (2).

This was in accordance with current theory that contact hypersensitivity is a form of cellular immunity in which the antigen is composed of a conjugate between a hapten and epidermal proteins (3).

In an effort to determine whether immunization with such conjugates would also result in donor lymphocytes which could demonstrate sensitivity and specificity by means of lymphocyte transformation, guinea pigs were sensitized appropriately and their lymphocytes tested *in vitro*.

MATERIAL AND METHODS

Guinea pigs. Inbred Hartley strain guinea pigs weighing 300-500 grams were used. They were individually caged and maintained on Purina Chow with daily lettuce supplementation.

Antigen preparations. Skin extracts were prepared as

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described previously (1). The standard diluent for all materials was Tris buffer pH 8.4. Next 0.07 ml of 3% by volume DNFB in dioxane was added to 2 ml skin extract at a concentration of 2000 mcg/ml by a slow drop method with gentle stirring with a magnetic mixer over a 15 minute period. This solution was then allowed to conjugate at room temperature for 15 minutes, then dialyzed against 500 ml Tris buffer overnight in a refrigerator.

0.25 ml of 1% oxidizing aqueous PDA was added to 2 ml skin extract at a concentration of 1000 mcg/ml (2). This solution was then allowed to conjugate in a 37° C waterbath for 2½ hours and dialyzed similarly to DNFB. A lower concentration of skin extract was found to be necessary for good sensitization. When conjugating PDA at this lower concentration of skin extract a precipitate may form; however, it does not seem to interfere with sensitization.

After dialysis the conjugates were then diluted with Tris buffer to concentrations required for immunization or testing. All test doses for the conjugates are based on the concentration of skin protein.

Sensitization. Guinea pigs were sensitized in the heel of a hind footpad with a 0.4 ml emulsion of equal volumes of complete Freund's Adjuvant (2 mg Tbc/ml) and a hapten (DNFB or PDA) skin protein conjugate at a concentration approximately 150 mcg skin protein.

Skin test. DNFB: On the sixth day following sensitization both experimental and control guinea pigs were tested by dropping 0.01 ml of 0.1% by volume DNFB in oil (Wesson) onto an area of the guinea pig's flank which had been clipped free of hair.

PDA: On the seventh day following sensitization 0.01 ml of 1.0% (by weight) PDA in oil was applied as indicated for DNFB above.

Reading of skin test site. Reactions were read 24 hours after skin testing. Guinea pigs showing erythema and swelling at test site were considered sensitized. Criteria for evaluation of skin reactions were as reported previously (1). Those guinea pigs showing the most marked erythema and swelling were used for tissue culture.

Cell collection. After a lethal intraperitoneal injection of pentobarbital, the animal's popliteal and inguinal nodes draining the injected footpad were dissected free and minced in Waymouth's medium containing penicillin 50 units/ml and streptomycin 50 µg/ml. The lymphocyte-rich suspension was gassed with 10% CO₂ in air to a pH of approximately 7.0 to 7.3. The cell suspension was then diluted with Waymouth's medium containing 20% fetal calf serum until the cell concentration was 2.10 × 10⁶ cells/ml.

Cell plating. Antigens, protein preparations, and con-

controls were all added to test vials in 0.1 ml volumes of Tris buffer. All test samples were done in quadruplicate. A 2 ml cell suspension was placed in each of these vials (4.5 ml glass vials with rubber-lined screw caps) and incubated in 10% CO₂.

Test doses. DNFB conjugate was tested at a concentration of 50 µg/vial and PDA conjugate was tested at 5 mcg/vial. Plain skin protein controls were tested at 50 µg/vial and 5 mcg/vial. PWM (Pokeweed Mitogen) control vials contained 0.03 ml PWM + 0.1 ml Tris buffer to evaluate the transformation potential of each animal's lymphocytes. Another control set of six vials contained 0.1 ml Tris buffer only, two of which served as Label Controls.

Cell harvest. Cells were harvested after 72 hours of incubation according to our previously reported procedure (2).

RESULTS

Forty-four guinea pigs were immunized for this study, 21 with DNFB and 23 with PDA. None of the 13 non-immunized control animals reacted to DNFB or PDA. By our criteria, 18/21 DNFB sensitized guinea pigs and 15/23 PDA sensitized guinea pigs were positive. Of the 33 positive animals, 10 DNFB and 13 PDA guinea pigs were used for tissue culture. Results from a representative 9 of each are shown in Tables I and II. Sensitivity is measured by ratios of hapten-skin protein conjugates to its appropriate skin protein control.

The responses of sensitized animals to the appropriate conjugate ranged from 279 to 15,069 cpm. Each animal seemed to vary considerably in its response, not only in stimulated cultures but in control cultures as well. One animal's response to antigen stimulus was occasionally less than a control response of another animal. Another consideration for this variation is that since skin proteins themselves are complex mixtures of antigens (4), they could nonspecifically stimulate lymphocytes *in vitro*; hence response to conju-

gates of haptens and skin proteins would be unduly elevated. For this reason, it was felt that a comparison of each animal's response to the skin conjugate with its response to unconjugated skin would be more meaningful. The ratio in sensitized animals ranged from 0.6 to 34.8. Ratios of response to the alternate conjugate ranged from 0.2 to 2.0. If the ratio of 2.5 is arbitrarily selected as a measure of contact hypersensitivity, 17 of the 18 guinea pigs (94.5%) in Tables I and II demonstrated both sensitivity and specificity. (Inclusion of the 5 animals not tabulated would increase the above figures to 22 of 23 guinea pigs or 95.7%.)

DISCUSSION

The results obtained indicate that guinea pigs sensitized to DNFB or PDA produce lymphocytes which respond to DNFB-skin protein conjugates (DNP-SPC) or PDA-skin protein conjugates (PDA-SPC) specifically.

The initial publication originating in this laboratory (1) showed that guinea pigs sensitized by DNP-SPC in complete Freund's Adjuvant produced lymphocytes that responded to DNP-SPC *in vitro*. At that time it was not possible to prove specificity in the reaction, since the alternate conjugate, PDA-SPC, had not yet been prepared. Also, it was not possible to distinguish between sensitivity to a conjugate or a non-specific reaction to an immunizing agent.

For that reason, our second publication dealt with the responses *in vitro* to animals sensitized with unconjugated DNFB or PDA in Freund's complete adjuvant (2). Presumably, in that case, the haptens combined with skin and other footpad proteins as well as the tuberculo-protein in the adjuvant.

In that publication we demonstrated that, indeed, lymphocyte transformation in the presence of the appropriate conjugate was significantly

TABLE I

Lymphocyte transformation of lymph node cells from guinea pigs sensitized to dinitrofluorobenzene conjugated to guinea pig skin protein

	#331	#332	#334	#351	#352	#607	#608	#609	#610
PWM	3,070	16,683	11,444	26,100	23,994	70,768	22,520	39,847	8,120
SP 50	260	353	255	959	490	1,410	265	350	345
SP 5	213	449	552	479	541	1,499	165	301	326
DNP-SPC 50	1,895	3,369	3,880	10,297	15,069	8,879	5,006	12,175	1,439
PDA-SPC 5	174	146	336	298	132	1,113	474	275	305
Control	368	367	559	232	258	873	289	282	359
Label	49	70	115	146	100	147	92	681	400
Ratios									
DNP-SPC/SP 50	7.3	9.5	15.2	10.7	32.0	6.3	18.9	34.8	4.2
PDA-SPC/SP 5	0.8	0.3	0.6	0.6	0.2	0.7	2.9	0.9	0.9

The figures represent counts per minute of tritiated thymidine in nucleic acid residues obtained by averaging 4 culture vials from the same animal. Columns represent different animals. The legend at the left margin refers to stimulating substances added to the cultures: PWM, pokeweed mitogen; SP 50 or 5, 50 or 5 µg skin protein; DNP-SPC 50, 50 µg of DNP-skin protein; PDA-SPC 5, 5 µg of PDA-skin protein; Control, no stimulus; Label, tritiated thymidine immediately prior to harvesting; DNP-SPC 50/SP 50, the ratio of the response to DNP-SPC compared with the response to SP; PDA-SPC 5/SP 5, the ratio of the response to PDA-SPC 5 compared with the response to SP 5.

TABLE II

Lymphocyte transformation of lymph node cells from guinea pigs sensitized to paraphenylenediamine conjugated to guinea pig skin protein

	#375	#376	#377	#396	#401	#764	#765	#774	#775
PWM	33,857	9,890	36,820	62,260	39,551	22,621	24,544	3,448	13,357
SP 5	415	425	738	557	747	586	479	137	348
SP 50	915	147	801	389	636	532	571	110	204
PDA-SPC 5	4,426	279	1,848	9,558	9,745	2,642	6,999	749	3,017
DNP-SPC 50	819	171	532	360	562	856	452	70	290
Control	913	326	315	264	583	1,218	617	122	224
Label	79	68	90	66	66	128	117	51	63
<i>Ratios</i>									
PDA-SPC/SP 5	9.3	0.6	2.5	17.2	12.9	5.0	14.6	5.5	7.5
DNP-SPC/SP 50	0.9	1.1	0.7	0.9	0.9	1.6	0.8	0.6	1.4

The figures represent counts per minute of tritiated thymidine in nucleic acid residues obtained by averaging 4 culture vials from the same animal. Columns represent different animals. The legend at the left margin refers to stimulating substances added to the cultures: PWM, pokeweed mitogen; SP 50 or 5, 50 or 5 μg skin protein; DNP-SPC 50, 50 μg of DNP-skin protein; PDA-SPC 5, 5 μg of PDA-skin protein; Control, no stimulus; Label, tritiated thymidine immediately prior to harvesting; DNP-SPC 50/SP 50, the ratio of the response to DNP-SPC compared with the response to SP; PDA-SPC 5/SP 5, the ratio of the response to PDA-SPC 5 compared with the response to SP 5.

greater than that of the alternate conjugate, thus demonstrating the specificity of the test.

The exact delineation of which of the numerous proteins, as outlined by Aoki (4), combine with haptens to produce the antigen in contact hypersensitivity, remains unsettled. Salvin and Smith (5), Gell and Banacerraf (6), and Parker (7), have all reported conflicting results. These findings may be explained by the hypothesis of Godfrey and Baer (8) who postulate two methods of sensitization: the "peripheral sensitization" of Macher and Chase (9), and direct drainage of the allergen into draining nodes. These papers do not concern themselves with conjugation nor do they rule it out. Peripheral sensitization has been invoked as the mechanism operative in another technique of lymphocyte transformation in guinea pigs sensitized to dinitrochlorobenzene. In this method, the lymphocytes are themselves conjugated with the DNP radical (10).

Recent work by Parker, Aoki, and Turk (11) implies that DNFB contact sensitization in guinea pigs can be accomplished by intramuscular injection of DNFB conjugates of a variety of fractions of epidermal proteins (including serum proteins) in complete Freund's adjuvant.

Preliminary work in this laboratory agrees with the above findings of Parker, Aoki, and Turk in that contact sensitization to DNFB can be induced with conjugates of serum proteins.

The conjugates employed in this study were made up of haptens combined with either 5 μg or 50 μg of skin protein. It was found that DNFB skin conjugates produced higher transformation ratios when the dosage of protein was 50 μg . Conversely, PDA skin conjugates in the 5 μg dosage were most effective.

We employed a relatively crude extract of epi-

dermal protein which was conjugated with either DNFB or PDA. Whether these different haptens attach to the same proteins is unknown. Much is known about the attachment of DNFB to proteins (12); little or nothing is known about PDA. Nevertheless, our work would indicate that hapten-skin protein conjugate immunization can produce sensitized animals that respond to topical application of the hapten, and produce lymphocytes which demonstrate conjugate-specific *in vitro* responsiveness.

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