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IN VITRO LYMPHOCYTE RESPONSES IN CONTACT HYPERSENSITIVITY IV*

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ABSTRACT

Sixteen patients were sensitized to dinitrofluorobenzene (DNFB) by topical application. Of these, 6 were determined to be 4+ sensitized, in that the cutaneous application of 2,000 μg of DNFB resulted in vesiculation, erythema, and edema beyond the border of the test site. Six unsensitized patients were used as controls. The 22 subjects had their lymphocytes cultured in the presence of conjugates of DNFB and human skin (DNP–SP). Responses were quantitated by measuring the incorporation of tritiated thymidine into nucleic acids of the cells in culture. Using a ratio of 3.0 (numbers indicate the ratio of thymidine incorporation in cultures containing DNP–SP to those containing only skin protein [SP]), 5 of 6 4+ patients were classified as sensitive, and none of the 6 control patients responded to this extent. Of the remainder of the sensitized patients, only 2 of 10 were positive. This indicates that our method, at present, is quite effective in detecting contact sensitivity in strongly positive patients, but insensitive in weakly sensitized patients.

The diagnosis of contact dermatitis is usually accomplished by taking a history and performing a physical examination. A positive patch test serves to confirm the clinical diagnosis. Patch testing, first developed by Jadassohn in 1895 [1], has had only minor modifications in the ensuing three-quarters of a century, although this method of testing can exacerbate an existing dermatitis, sensitize an individual not already sensitized [2], cause discomfort, or produce postinflammatory pigmentary changes at the site of testing.

Since the primary reactive cell in contact hypersensitivity is the lymphocyte, testing for contact hypersensitivity should be more suitably done with the lymphocyte than the skin. Consistent with classical theory, the antigen selected should be a hapten–skin protein conjugate [3]. The responses of sensitized lymphocytes can be quantitated by measuring the incorporation of tritiated thymidine into the nucleic acids of the lymphocytes undergoing transformation [4]. This method has been found to be both specific and sensitive in the guinea pig [5].

MATERIALS AND METHODS

Immunization

Human volunteers, ages 23–65, were topically immunized (day 0) with 0.1 ml of 2% (by volume) 1-fluoro-2,4-dinitrobenzene (DNFB) (Eastman Organic Chemicals) in acetone (2000 nl of DNFB) on their medial upper arm within an area approximately 20 mm in diameter. After evaporation of the acetone, the area was occluded with gauze and plastic tape for 48 hr.

Skin Test

Seven days postimmunization, each volunteer was topically skin tested with 0.025 ml of 2% (by volume) DNFB in acetone (50 nl) to an area approximately 12 mm in diameter on the medial forearm opposite the arm used for immunization. The acetone was left to evaporate, and the area was occluded with gauze and plastic tape for 24–72 hr. Reactions were read 24, 48, and 72 hr after the application of DNFB.

Skin test reactions were graded as follows:

±	Moderate erythema or several small vesicles with erythema around hair follicles
++	Erythema and/or small vesicles, edema
+++	Marked erythema, vesiculation, and edema
++++	Total vesiculation, erythema, and edema beyond borders of test site

Antigen Preparations

All antigens and proteins were prepared and diluted with Tris–HCl buffer (pH 8.4).

Skin extracts were obtained from fresh surgical amputations. The skin was scraped, by scalpel, to what appeared to be dermal connective tissue and collected in Tris–HCl buffer (pH 8.4). After being frozen and thawed twice, the resulting suspension was filtered twice through Whatman #3 filters, twice through 0.45-μ Millipore filters, and once through 0.22-μ Millipore filters. Soluble skin protein (SP) concentrations were determined by the Lowry technique. (Concentrations of SP usually ranged from 1800 μg/ml to 4000 μg/ml.) SP concentration was adjusted to near 2000 μg/ml, the material was frozen in the vapor phase of a liquid nitrogen bank and was stored in liquid nitrogen until needed.

DNP-conjugates. SP was quick thawed in 37°C water-bath and adjusted to concentration of 1000 μg/ml with Tris–HCl buffer (pH 8.4). 3% (by volume) DNFB in dioxane was added by a slow-drop technique with gentle mixing to a final concentration of 31,000 nl DNFB/ml SP. The mixture was stirred for 15 min and the conjugation was allowed to proceed for an additional 15 min at room temperature. The mixture was dialyzed against 500 ml Tris–HCl buffer (pH 8.4) overnight in the refrigerator.

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Controls. Pokeweed mitogen (PWM) (Grand Island Biological) 0.03 ml/2 ml vial was used as a nonspecific stimulant. Tris-HCl buffer (pH 8.4), 0.1 ml, was added to the nonstimulated controls and was also added to the PWM and label controls.

Cell Preparations

40-ml samples of heparinized venous blood were placed in 50-ml round-bottomed centrifuge tubes. Lymphocytes were partially purified by differential centrifugation around 35 × *g*, according to the method of Mangi and Mardiney [6]. The supernate of cells and plasma was collected and counted. Cells were plated at a concentration of 1×10^6 cells/ml in Waymouth's medium with penicillin (50 U) and streptomycin (50 μg/ml). Plasma concentration was adjusted to as near 15% as possible (range: 12.5–15%). Average cell counts were:

Lymphocytes	89%
Monocytes	6%
Polymorphonuclear leukocytes	5%

2.0-ml samples of the above suspension were cultured in 4.5-ml glass vials with rubber-lined screw caps and incubated at 37°C in 10% CO_2.

Test Doses

All test doses were 0.1 ml per culture vial and were based on the concentration of skin protein. All test samples were done in quadruplicate. DNP-SP conjugate was tested at 100 μg and 50 μg per culture vial. Unconjugated skin protein controls were tested to match the above dosages of conjugates.

Cell Harvest

Cells were harvested after 120 hr of culture with tritiated thymidine added for the final 6 hr, according to our previously reported procedures [5].

RESULTS

Sixteen patients were sensitized to DNFB, as described in *Materials and Methods*. The patients were first tested by patch testing at 7 days post-sensitization. Reactions were recorded as the maximal at 24, 48, or 72 hr. Five to 7 days after testing, the patients' lymphocytes were tested as outlined above. If, for technical reasons, a patient's cultures were unusable, he would be recalled for retesting. In some cases, several months had elapsed since initial sensitization. These patients were retested to insure sensitization, and these responses were then recorded (Tables I–III).

Of the 16 patients, 6 were 4+ by cutaneous testing, 2 were 3+, 5 were 2+, 2 were 1+, and 1 gave no reaction (0+). Six patients without known sensitization to DNFB were used as controls. They were not tested by cutaneous application, to avoid inadvertent sensitization.

The 22 subjects' lymphocytes were cultured as described above, and their responses were recorded as the ratio between the response to test doses of DNFB conjugated to 50 μg of skin protein (DNP-SP50) and the response to 50 μg of unconjugated skin protein (SP50). It will be noted that Table I shows the results of the patients who were 4+ by cutaneous testing. Their in vitro ratios ranged from 2.4 to 9.3. The controls ranged from 0.9 to 2.7.

Setting an arbitrary ratio of 3.0 as indicating a positive response, 5 of 6 4+ patients were positive, while 0 of 6 controls were positive. Of the 3+ patients, 0 of 2 were positive; of the 2+ patients, 3 of 5 were positive; and of the 0 and 1+ patients, none of the 3 was positive.

TABLE I

Lymphocyte transformation of peripheral leukocytes from humans sensitized to dinitrofluorobenzene with skin tests of 4+

Subject	B.M.	W.S.	R.S.	C.K.	E.R.	T.G.
PWM	33,463 (±4,678)	25,055 (±6,445)	89,650 (±13,715)	43,568 (±24,647)	33,524 (±262)	66,402 (±12,313)
SP50	348 (±126)	743 (±949)	830 (±239)	479 (±119)	451 (±100)	188 (±41)
DNP-SP50	1,945 (±545)	4,421 (±1,588)	5,315 (±2,210)	2,680 (±510)	1,100 (±593)	1,744 (±343)
Control	232 (±34)	321 (±39)	1,157 (±461)	335 (±182)	362 (±68)	138 (±20)
Label	79 (±1)	59 (±1)	106 (±9)	48 (±3)	72 (±12)	50 (±0)
S.T.	4+	4+	4+	4+	4+	4+
Ratios: DNP-SP50/SP50	5.6	6.0	6.4	5.6	2.4	9.3

In this and subsequent Tables, the figures represent mean counts per min ± one standard deviation of tritiated thymidine in nucleic acid residues obtained by averaging 4 culture vials from the same patient. Columns represent different patients. The legend at the left margin refers to stimulating substances added to the cultures: PWM, pokeweed mitogen; SP50, 50 μg of DNP-skin protein; Control, no stimulus; Label, tritiated thymidine immediately prior to harvesting; S.T., the cutaneous response to the application of DNFB; DNP-SP50/SP50, the ratio of the response to DNP-SP compared with the response to SP.

TABLE II

Lymphocyte transformation of peripheral leukocytes from humans sensitized to dinitrofluorobenzene with skin tests of 3+, or less

Subject	PWM	SP50	DNP-SP50	Control	Label	S.T.	Ratio DNP-SP50/SP50
R.C.	22,656 (±2,483)	250 (±80)	988 (±185)	241 (±41)	80 (±5)	2+	4
A.K.	61,359 (±15,718)	154 (±36)	458 (±279)	160 (±69)	71 (±1)	2+	3.0
W.S.	49,209 (±11,193)	547 (±129)	741 (±82)	377 (±46)	89 (±4)	2+	1.4
C.S.	23,227 (±3,526)	548 (±74)	938 (±156)	547 (±138)	125 (±3)	3+	1.7
G.F.	31,351 (±10,869)	707 (±124)	1,045 (±296)	803 (±161)	105 (±17)	2+	1.5
J.M.	97,071 (±31,396)	1,417 (±422)	3,756 (±739)	818 (±245)	86 (±15)	1+	2.7
P.B.	465,402 (±29,181)	274 (±53)	1,403 (±725)	309 (±48)	55 (±6)	2+	5.1
S.D.	16,684 (±5,043)	159 (±42)	462 (±92)	202 (±36)	43 (±6)	3+	2.9
D.S.	32,466 (±4,189)	280 (±69)	708 (±312)	285 (±108)	86 (±7)	1+	2.5
J.R.	106,832 (±6,507)	468 (±72)	1,075 (±274)	472 (±88)	69 (±6)	±	2.3

TABLE III

Lymphocyte transformation of peripheral leukocytes from humans unsensitized to dinitrofluorobenzene

Subject	R.K.	H.B.	J.P.	R.H.	F.R.	C.S.
PWM	23,778 (±4,943)	22,651 (±4,378)	11,500 (±1,537)	21,776 (±7,921)	13,908 (±2,596)	100,252 (±14,606)
SP50	239 (±37)	196 (±52)	145 (±16)	400 (±142)	143 (±16)	1,287 (±264)
DNP-SP50	638 (±221)	444 (±144)	198 (±44)	364 (±96)	192 (±38)	1,622 (±298)
Control	209 (±42)	89 (±13)	137 (±19)	494 (±174)	142 (±32)	353 (±34)
Label	44 (±3)	43 (±1)	40 (±10)	86 (±9)	38 (±1)	132 (±29)
S.T.	*N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Ratios: DNP-SP50/SP50	2.7	2.3	1.4	0.9	1.3	1.3

* Not done

DISCUSSION

The results indicate that our method of testing is successful in detecting contact hypersensitivity in strongly sensitive patients. Use of a ratio of 3 to indicate sensitivity is arbitrary, but it separates our sensitized subjects from controls in such a way as to indicate sensitivity in 5/6 (83%) 4+ patients, and none of 6 controls. Our method as yet does not detect the weakly sensitized patients, as evidenced by the negative responses from those patients 3+ or less.

The use of in vitro methods of diagnosis of contact dermatitis in humans has now been attempted by numerous investigators. Their methods can be grouped into two broad categories: [1] direct testing of lymphocyte response, and [2] indirect testing by means of the effect of lymphokines on other cells. This discussion will concern itself only with the first category.

In vitro testing for metal contact hypersensitivity has been a problem for over a decade. Aspergren and Rorsman [7] reported that nickel was a nonspecific stimulator of lymphocytes in culture. Their work was felt to be confirmed by Pappas et al [8] in 1970. The nonspecificity of

lymphocyte transformation in the presence of nickel was challenged by Schöpf et al [9] in 1969 and, more recently, by Millikan et al [10] in 1973.

The first report of a non-metal antigen was that of Kingery and Provost, who tested one patient to benzocaine and paraphenylenediamine. They measured both tritiated thymidine uptake and "blast" transformation. They felt that the method was restricted to the use of antigens "soluble in water to be utilized in the lymphocyte culture medium" [11]. They did not form conjugates of hapten and epidermal protein. Unfortunately, there have been no further reports of their technique being employed on a series of patients with paraphenylenediamine or benzocaine sensitivity. Levene [12] reported in 1972 that the lymphocytes of 1 of 3 dinitrochlorobenzene (DNCB)-sensitive patients would transform in the presence of DNCB added to the cultures in an unconjugated form.

Miller and Levis [13] conjugated DNCB to leukocytes, erythrocytes, or tissue culture cells, and extracted a lyophilized conjugate which, when added to cultures of sensitized patients, resulted in the detection of sensitivity as measured by the uptake of tritiated thymidine.

The necessity for conjugation of contact allergens is an unsettled point at this time. Although the classical conjugate is that of the hapten with epidermal proteins [3], other substances have been conjugated to haptens, with reports of success by a number of investigators [14, 15]. It is, of course, possible that conjugation occurred in vitro with proteins in the tissue culture, as pointed out by Levene [12].

Our work supports neither point of view since it concerns itself only with DNFB, a highly reactive compound. Our previous publication [5] examined both DNFB and PDA (paraphenylenediamine) conjugates in culture. In the guinea pig, conjugates, with the soluble products of skin scraping, were effective in indicating contact hypersensitivity in both a sensitive and specific manner. Whether DNFB or PDA conjugates can dissociate and produce haptens, which subsequently attach to cellular or soluble proteins in culture, is in dispute.

The "skin protein" used in these studies undoubtedly contains proteins from dermal sources, such as extravascular compartmental proteins, and plasma proteins as well; hence, the skin protein conjugate is a mixture of many carriers forming hapten–protein conjugates.

Further work must be directed toward two areas: simplifying the complex antigens used in testing for contact hypersensitivity in vitro, and increasing the sensitivity of the method to detect lesser degrees of sensitization.

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