

ATTEMPTS TO IDENTIFY THE CAUSATIVE ALLERGEN IN CASES OF ALLERGIC CONTACT DERMATITIS USING AN *IN VITRO* LYMPHOCYTE TRANSFORMATION TEST

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Abstract—The use of an *in vitro* lymphocyte transformation test for the identification of the causative allergen in cases of human allergic contact dermatitis is described. Peripheral blood mononuclear cells (PBMC) were prepared from the venous blood of seven nickel-sensitized (patch test-positive) patients and ten non-sensitized control donors, and induced proliferation was measured by [³H]thymidine incorporation following culture for 7 days in the presence of various concentrations of nickel sulphate. Nickel sulphate is non-specifically mitogenic for human lymphocytes and although PBMC from all donors exhibited a significant increase in proliferation with nickel sulphate concentrations of 20 µg/ml, those isolated from Ni-sensitive patients responded to lower concentrations of the allergen. Employing a stimulation index of 3 following culture with 5 µg NiSO₄/ml as an arbitrary indicator of specific proliferative responses to nickel, it was possible, using the lymphocyte transformation test, to separate Ni-sensitive from non-sensitized donors. In a limited series of experiments it was observed that lymphocytes prepared from each of two patients sensitized to neomycin sulphate mounted significant proliferative responses to this allergen *in vitro*; neomycin sulphate failed to induce similar changes in PBMC from non-sensitized donors. Finally, PBMC from one of two 2,4-dinitrochlorobenzene-sensitized donors were found to respond to autologous cells bearing the relevant dinitrophenol hapten.

Introduction

Allergic contact dermatitis (ACD) is a delayed-type immune hypersensitivity reaction induced in susceptible individuals following skin contact with allergic chemicals. The clinical diagnosis of occupational and environmental ACD is conventionally performed using a patch test, frequently with a battery of allergens. Not only are patch tests sometimes difficult to interpret, but they also carry the risk of inadvertent sensitization of a previously non-allergic individual (Agrup, 1968).

During the last twenty years many investigators have demonstrated that peripheral T lymphocytes from contact-sensitized individuals can mount a proliferative response when cultured with the relevant hapten. The majority of studies have examined the capacity of nickel salts to provoke T lymphocyte proliferation (Al-Tawil *et al.*, 1981; Everness, 1988; Everness *et al.*, 1989; Hutchinson *et al.*, 1972; Macleod *et al.*, 1970; Nordlind, 1984; von Blomberg-van der Flier *et al.*, 1987) and only a limited number of other allergens such as chromium (Yamada *et al.*, 1972), 2,4-dinitrochlorobenzene (DNCB) (Levis *et al.*, 1976; Miller and Levis, 1973; Soeberg and Andersen, 1976) and urushiol (Byers *et al.*, 1979) have been examined.

It is the purpose of our studies to investigate further the potential utility of lymphocyte transform-

ation tests as a means of identifying the causative allergen in cases of ACD.

Materials and Methods

Subjects. Patients with allergic contact dermatitis attending the patch test clinic at Manchester Skin Hospital, and control subjects with no history of skin allergy were examined. In the case of patients, blood was drawn immediately prior to patch testing.

Preparation of peripheral blood mononuclear cells. Venous blood was drawn into preservative-free heparin and peripheral blood mononuclear cells (PBMC) separated by density gradient centrifugation on Lymphocyte Separation Medium (Flow Laboratories Ltd, Scotland) using a modification of the method described by Boyum (1968). PBMC were washed in phosphate-buffered saline (PBS; pH 7.2) and resuspended in RPMI-1640 culture medium containing 400 µg streptomycin/ml and 400 µg ampicillin/ml. Cell viability was measured by exclusion of 0.5% trypan blue and the cell concentration adjusted to working values.

Lymphocyte transformation test—(a) *soluble antigens.* PBMC were seeded into 96-well microtiter plates at a concentration of 1.2×10^6 cells per ml in the presence of 10% autologous plasma. Various concentrations of allergen in PBS (nickel sulphate or neomycin sulphate (Sigma Chemical Co., St Louis, MO, USA)) or an equal volume of PBS alone were added to each well. Plates were cultured at 37°C in a humidified atmosphere of 5% CO₂ in air for 7 days, the final 24 hr in the presence of 2 µCi [³H]methyl thymidine (sp. act. 2.5 Ci/mmol; Amersham International, Amersham, UK). Culture was terminated by

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Abbreviations: ACD = allergic contact dermatitis; DNCB = 2,4-dinitrochlorobenzene; DNFB = 2,4-dinitrofluorobenzene; DNP = dinitrophenol; PBMC = peripheral blood mononuclear cells; PBS = phosphate buffered saline.

automatic cell harvesting and [^3H]TdR incorporation determined by β -scintillation counting.

(b) *Cell-associated antigen*. PBMC (5×10^6 per ml in RPMI supplemented with 10% autologous plasma) were incubated for 30 min at 20°C with 15 mM-2,4-dinitrofluorobenzene (DNFB; Aldrich Chemical Co. Ltd, Gillingham, UK) or 15 mM-4-ethoxymethylene-2-phenyloxazol-5-one (oxazolone; Sigma). Following haptenation cells were washed three times with an excess of PBS, irradiated (2000 R) and adjusted to working concentrations in RPMI-1640. Haptenated cells were added to wells containing untreated, autologous responder PBMC (1.2×10^6 cells per ml in RPMI containing 10% autologous plasma) to provide a stimulator:responder ratio of 1:10. Control wells received unhaptenated stimulator cells at the same ratio. Cultures were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air for 5 days and [^3H]TdR incorporation measured as described previously.

Skin tests. Patients were tested with a standard panel of allergens in petrolatum. Patches were removed after 48 hr and the test sites examined. Reactions were re-evaluated 2 days later.

Results

In initial studies the relative capacity of PBMC populations isolated from nickel-sensitive and non-sensitive individuals to mount proliferative responses following co-culture with a range of concentrations of nickel sulphate was examined. The data derived from studies with eight representative donors (five patients who gave a positive response to a nickel patch test and three who proved negative) are recorded in Table 1. In all cases co-culture of PBMC with one or more concentrations of NiSO_4 resulted in a significant enhancement of proliferative activity. Nevertheless, clear quantitative differences between patch test-positive and -negative patients were apparent. Thus, PBMC from Ni-sensitive patients exhibited significant increases in thymidine incorporation following co-culture with lower concentrations of NiSO_4 than did PBMC from their non-sensitized counterparts. In the Ni-sensitive group shown in Table 1, a significant increase in proliferation was, in all instances, observed with $1 \mu\text{g NiSO}_4/\text{ml}$, in four of five cases with $0.5 \mu\text{g NiSO}_4/\text{ml}$ and in two cases (donors 4 and 8) with $0.25 \mu\text{g NiSO}_4/\text{ml}$. In contrast, the concentration of NiSO_4 required to stimulate significant proliferative responses in PBMC from patch test-negative donors was $2 \mu\text{g}/\text{ml}$ (donor 12), $5 \mu\text{g}/\text{ml}$ (donor 6) and $20 \mu\text{g}/\text{ml}$ (donor 10). In some previous studies on the selectivity and sensitivity of lymphocyte transformation tests in nickel allergy, investigators have employed a stimulation index (usually a stimulation index of 2) to discriminate between nickel-sensitive and unsensitized individuals (Hutchinson *et al.*, 1972; Macleod *et al.*, 1970; von Blomberg-van der Flier, 1987). We adopted a similar policy and, on the basis of results available to date and data from a previous study by Everness (1988), determined that a stimulation index of 3 or greater following co-culture with $5 \mu\text{g NiSO}_4/\text{ml}$ was of value in defining positive responses. From the selected results recorded in Table 1, and the data from all nickel studies to date

Table 1. The lymphocyte transformation test. NiSO_4 -induced proliferative responses: a comparison between peripheral blood mononuclear cells isolated from nickel-sensitized (patch test-positive) and nickel-non-sensitized (patch test-negative) donors

NiSO_4 $\mu\text{g}/\text{ml}$	[^3H]TdR incorporation†											
	Donor 4		Donor 5		Donor 6		Donor 8		Donor 9		Donor 10	
	Ni patch test +VE	Ni patch test -VE	Ni patch test +VE	Ni patch test -VE	Ni patch test +VE	Ni patch test -VE	Ni patch test +VE	Ni patch test -VE	Ni patch test +VE	Ni patch test -VE	Ni patch test +VE	Ni patch test -VE
0	35.4 $\pm$ 7.5 (1.0)	32.3 $\pm$ 26.0 (1.0)	52.8 $\pm$ 26.3 (1.6)	28.8 $\pm$ 11.8 (1.0)	48.4 $\pm$ 21.2 (1.0)	47.9 $\pm$ 6.6 (1.0)	51.5 $\pm$ 9.8 (1.1)	35.2 $\pm$ 21.4 (1.0)	48.9 $\pm$ 16.2 (1.0)	62.3 $\pm$ 21.6 (1.0)	69.1 $\pm$ 7.7* (1.4)	92.1 $\pm$ 24.6 (1.5)
0.25	88.6 $\pm$ 15.6** (2.5)	56.9 $\pm$ 21.0 (1.8)	82.5 $\pm$ 27.6* (2.6)	37.7 $\pm$ 11.3 (1.3)	186.0 $\pm$ 69.1** (3.8)	144.0 $\pm$ 33.1** (3.0)	194.2 $\pm$ 34.9** (4.1)	40.0 $\pm$ 22.1 (1.1)	150.4 $\pm$ 40.8** (3.1)	78.7 $\pm$ 21.4 (1.3)	158.5 $\pm$ 22.4** (3.2)	84.3 $\pm$ 31.4 (1.4)
0.5	114.3 $\pm$ 27.5** (3.2)	140.2 $\pm$ 26.3** (4.3)	169.4 $\pm$ 34.6** (4.8)	29.1 $\pm$ 10.6 (1.0)	352.9 $\pm$ 90.6** (7.3)	228.3 $\pm$ 36.6** (4.8)	332.2 $\pm$ 40.1** (6.9)	38.5 $\pm$ 13.2 (1.1)	135.9 $\pm$ 28.4** (2.8)	106.7 $\pm$ 24.5* (1.7)	142.8 $\pm$ 23.4** (2.3)	169.1 $\pm$ 31.9** (2.7)
1.0	200.6 $\pm$ 20.1** (5.7)	169.1 $\pm$ 25.3** (5.2)	178.5 $\pm$ 27.8** (5.5)	39.5 $\pm$ 6.4 (1.4)	415.0 $\pm$ 176.3** (8.6)	478.2 $\pm$ 217.0** (9.9)	372.2 $\pm$ 25.9** (7.8)	51.4 $\pm$ 20.0 (1.5)	119.2 $\pm$ 11.2** (2.4)	142.8 $\pm$ 23.4** (2.3)	193.1 $\pm$ 32.3** (3.9)	205.2 $\pm$ 24.6** (3.3)
2.0	211.2 $\pm$ 19.7** (6.0)	199.5 $\pm$ 27.6** (6.2)	240.7 $\pm$ 32.1** (7.5)	48.0 $\pm$ 6.7* (1.7)	518.5 $\pm$ 130.3** (10.7)	566.4 $\pm$ 63.0** (11.7)	387.8 $\pm$ 42.3** (8.1)	65.3 $\pm$ 26.4 (1.9)	195.1 $\pm$ 26.0** (4.0)	205.2 $\pm$ 24.6** (3.3)	195.1 $\pm$ 26.0** (4.0)	225.7 $\pm$ 30.9** (3.6)
5.0	309.7 $\pm$ 46.5** (8.7)			65.7 $\pm$ 23.8* (2.3)	548.9 $\pm$ 104.6** (11.3)	383.5 $\pm$ 36.1** (8.0)		59.9 $\pm$ 35.7 (1.7)	130.4 $\pm$ 65.9* (2.7)			
10.0	354.2 $\pm$ 62.2** (10.0)			66.8 $\pm$ 8.9** (2.3)				78.1 $\pm$ 20.7* (2.2)				
20.0												

*Significantly elevated compared with control cultures (two-sided Student's *t*-test) ($P < 0.05$).

**Significantly elevated compared with control cultures (two-sided Student's *t*-test) ($P < 0.01$).

†cpm $\pm$ SD $\times 10^{-2}$. Stimulation index in parentheses.

Table 2. A summary of stimulation indices recorded for PBMC isolated from nickel-sensitized and nickel-non-sensitized donors cultured with 5 µg NiSO₄/ml

Donor	Nickel-sensitized (Ni patch test-positive)	Donor	Nickel-non-sensitized (Ni patch test-negative* or control† donors)
	Lymphocyte transformation test Stimulation index 5 µg NiSO ₄ /ml		Lymphocyte transformation test Stimulation index 5 µg NiSO ₄ /ml
1	3.0	2†	2.0
4	6.0	3†	1.4
5	5.5	6*	1.7
8	10.7	10*	1.9
9	7.8	12*	2.7
11	3.9	13*	1.2
17	4.7	15*	1.7
		18*	1.3
		19*	1.6
		20*	1.8
Mean ± SE	5.9 ± 1.0		1.7 ± 0.1

*Patients attending patch test clinic.

†Donors with no history of dermatitis.

summarized in Table 2, it is clear that this arbitrary definition of positivity successfully separated nickel-sensitized and non-sensitized individuals. It must be emphasized, however, that with the limited data available, the criterion selected for a positive response is in the nature of a testable hypothesis rather than an established rule. Indeed, further experience may require that this variable is modified to facilitate maximal discrimination.

We also examined if PBMC from patients sensitized to neomycin sulphate will mount a proliferative response to this allergen in culture, and found some evidence of its occurrence (Table 3). Lymphocytes from two patients with positive patch tests for neomycin sulphate exhibited significant proliferative responses following co-culture with concentrations of the allergen of 0.125 mg/ml or greater (donor 15) and 0.25 mg/ml or greater (donor 1). PBMC isolated from three patients who were patch test-negative failed to respond to any concentration of neomycin sulphate employed. In the case of neomycin sulphate-sensitive patients the highest stimulation indices recorded were 2.4 (donor 1) and 3.1 (donor 15). As the highest stimulation index recorded for patch test-negative donors in this small series of experiments was 1.6 (donor 10), and the mean stimulation index for non-sensitized patients at all concentrations of neomycin sulphate was 1.05, an index of 2 or greater may prove to be a reliable indicator of neomycin sensitivity.

The data in Tables 1, 2 and 3 reveal two additional points of interest. First, lymphocytes prepared from donor 1, who when examined with a patch test panel was found to be sensitized to both nickel and neomycin sulphate, responded to both allergens *in vitro* (stimulation indices of 3.0 with 5 µg NiSO₄/ml, Table 2; and of 2.4 with 2 mg neomycin sulphate/ml, Table 3). Secondly, with donor 15, the lymphocyte transformation test accurately reflected patch test results (nickel—negative; neomycin sulphate—positive) inasmuch as PBMC isolated from this patient only mounted a proliferative response against neomycin sulphate (stimulation indices of 1.7 with 5 µg NiSO₄/ml, Table 2; and of 3.1 with 1 mg neomycin sulphate/ml, Table 3).

We (Kimber *et al.*, 1986), and others (Miller and Butler, 1983; Phanuphak *et al.*, 1974; Thomas *et al.*, 1982), have previously demonstrated that lymphocytes prepared from mice sensitized to chemicals such as DNCB and 2,4,6-trinitrochlorobenzene (picryl chloride) will mount proliferative responses when co-cultured with autologous cells bearing the relevant hapten. In a single series of experiments a similar protocol was employed to examine two patients sensitized to DNCB. Peripheral blood mononuclear cells from such patients, and from a laboratory donor with no history of dermatitis, were cultured for five days with autologous irradiated PBMC that had been pre-incubated with either DNFB or oxazolone or were untreated. The results (Table 4) demonstrate

Table 3. The lymphocyte transformation test. Neomycin sulphate-induced proliferative responses: a comparison between peripheral blood mononuclear cells isolated from neomycin sulphate-sensitized (patch test-positive) and neomycin sulphate-non-sensitized (patch test-negative) donors

Neomycin sulphate mg/ml	[³ H]TdR incorporation†				
	Donor 1 NS patch test +VE	Donor 10 NS patch test -VE	Donor 12 NS patch test -VE	Donor 13 NS patch test -VE	Donor 15 NS patch test +VE
0	95.1 ± 17.5 (1.0)	35.2 ± 21.4 (1.0)	62.3 ± 21.6 (1.0)	91.5 ± 49.1 (1.0)	41.6 ± 4.6 (1.0)
0.125	151.5 ± 53.4 (1.6)	56.8 ± 19.9 (1.6)	71.1 ± 25.7 (1.1)	105.0 ± 33.1 (1.1)	60.4 ± 8.8** (1.5)
0.25	124.8 ± 10.4* (1.3)	44.8 ± 23.1 (1.3)	70.6 ± 29.3 (1.1)	87.4 ± 32.0 (1.0)	80.0 ± 12.2** (1.9)
0.5	126.5 ± 22.6* (1.3)	39.9 ± 29.2 (1.1)	69.8 ± 22.7 (1.1)	90.8 ± 40.6 (1.0)	81.5 ± 23.8** (2.0)
1.0	210.1 ± 25.0** (2.2)	22.4 ± 9.3 (0.6)	62.0 ± 28.6 (1.0)	97.5 ± 45.1 (1.1)	130.7 ± 20.1** (3.1)
2.0	227.7 ± 38.5** (2.4)	19.2 ± 12.5 (0.5)	82.8 ± 46.1 (1.3)	77.5 ± 32.9 (0.8)	117.0 ± 20.2** (2.8)

NS = neomycin sulphate

*Significantly elevated compared with control cultures (two-sided Student's *t*-test) (*P* < 0.05).**Significantly elevated compared with control cultures (two-sided Student's *t*-test) (*P* < 0.01).†cpm ± SD × 10⁻². Stimulation index in parentheses.

Table 4. The capacity of hapten-bearing, irradiated autologous PBMC to stimulate proliferation of DNCB-sensitized and control donor lymphocytes

Experiment	Responder	Irradiated Stimulator Cells	[³ H]TdR incorporation†
1	DNCB-sensitized patient A	PBMC‡	42.1 ± 4.2 (1.0)
		PBMC-Ox§	38.7 ± 6.4 (0.9)
		PBMC-DNP	162.2 ± 24.1* (3.9)
	Control	PBMC‡	39.3 ± 7.1 (1.0)
		PBMC-Ox§	48.4 ± 6.3 (1.2)
		PBMC-DNP	42.5 ± 11.1 (1.1)
2	DNCB-sensitized patient B	PBMC‡	55.1 ± 3.8 (1.0)
		PBMC-Ox§	49.8 ± 5.1 (0.9)
		PBMC-DNP	64.2 ± 7.0 (1.2)
	Control	PBMC‡	37.6 ± 6.5 (1.0)
		PBMC-Ox§	34.4 ± 2.1 (0.9)
		PBMC-DNP	40.3 ± 7.3 (1.1)

†cpm ± SD × 10⁻². Stimulation index in parentheses.

‡Irradiated PBMC.

§Irradiated PBMC treated with oxazolone.

||Irradiated PBMC treated with DNFB.

*Significantly elevated compared with control culture (two-sided Student's *t*-test) (*P* < 0.01).

that PBMC prepared from one of the two DNCB-sensitized donors, patient A, mounted a significant proliferative response to cells treated with DNFB and bearing the dinitrophenol (DNP) hapten. In contrast, irradiated PBMC coated with the antigenically-non-cross-reactive sensitizer, oxazolone, failed to stimulate proliferative activity compared with control cultures containing untreated stimulator cells. Lymphocytes from the second DNCB-sensitized donor, patient B, failed to respond to either DNFB- or oxazolone-bearing cells. In neither experiment did control PBMC respond to hapten-modified cells (Table 4).

Discussion

Experience to date with the lymphocyte transformation test suggests that this assay may be of value in the diagnosis of nickel sensitivity. It is apparent, however, that the distinction between Ni-sensitized and non-sensitized individuals is quantitative rather than qualitative, and that in practice an arbitrary stimulation index is necessary to define a 'biologically-relevant' level of proliferation. It is likely that the proliferation observed following co-culture of control PBMC with higher concentrations of nickel sulphate is attributable to the mitogenic properties of nickel salts for human lymphocytes (Al-Tawil *et al.*, 1981; Nordlind, 1984; Nordlind and Henze, 1984; Pappas *et al.*, 1970) rather than reflecting a latent or sub-clinical sensitivity to the metal. Thus, even human thymocytes and cord blood lymphocytes have been shown to exhibit a weak, but nevertheless significant, proliferative response in following exposure to relatively high concentrations of nickel sulphate *in vitro* (Al-Tawil *et al.*, 1981; Nordlind and Henze, 1984).

The limited data available for neomycin sulphate suggest that only lymphocytes isolated from sensitized donors will mount a proliferative response to this allergen. Cellular toxicity rather than non-specific mitogenicity may prove important here since, in two of three instances, 2 mg neomycin sulphate/ml caused a small (donor 13) or marked (donor 10) depression of thymidine incorporation by PBMC from patch test-negative donors.

Experience with the presentation of non-water soluble allergens to lymphocyte cultures is currently limited to two patients treated with DNCB for alopecia aerata; no patch tests were performed. The strategy adopted, based on previous studies in the mouse (Kimber *et al.*, 1986), was to conjugate allergen to autologous stimulator cells. In these experiments 2,4-dinitrofluorobenzene was chosen for cell haptentation since, although it is antigenically fully cross-reactive with DNCB, it possesses a greater protein reactivity. Similar protocols have been used by other investigators to successfully measure proliferative responses by human lymphocytes to the same hapten (Levis *et al.*, 1976; Miller and Levis, 1973; Soeberg and Andersen, 1976), urushiol (Byers *et al.*, 1979) and *p*-*N*-dimethylnitrosaniline (Soeberg and Andersen, 1976).

Lymphocytes from only one of the two patients examined mounted a significant proliferative response and this was specific for the DNP hapten; oxazolone-treated stimulator cells were without effect. For reasons that are unclear, PBMC from the second patient were unresponsive. Although the relevant information is unavailable, one may speculate that as *in vitro* responsiveness to both DNCB (Miller and Levis, 1973) and urushiol (Byers *et al.*, 1979) apparently declines with increasing time from sensitization, the period between exposure to DNCB and assay in the case of the second patient was too long.

Collectively the data indicate that, provided an appropriate method for delivering the hapten is available, the assessment of induced lymphocyte proliferative responses may be successfully extended to the examination of lipophilic allergens.

Although firm conclusions regarding the practical utility of the lymphocyte transformation test await the results of more extensive studies with a variety of skin allergens, the data contained within this report suggest that the method may provide an adjunct, and in some instances an alternative, to patch testing in the clinical assessment of allergic contact dermatitis.

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