

In vitro Testing of Contact Sensitivity

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A new in vitro technique for the testing for contact sensitivity is described. The method is based upon the fact that in response to immunological stimuli, keratinocytes can start to synthesize and express class II histocompatibility antigens. Nickel sulphate (NiSO₄) stimulated lymphocytes from nickel-sensitive persons, and the supernatant of these cells thus induced expression of HLA-DR on keratinocytes when co-cultured with autologous normal skin biopsies. This was in contrast to what was the case in healthy controls. *Key words:* Contact sensitivity; In vitro test; Class II histocompatibility antigens. (Received March 14, 1987.)

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Epicutaneous tests that are used to verify the occurrence of delayed type of hypersensitivity reactions to different compounds, may give both false-negative and false-positive results (1) and may also induce sensitization in some individuals (2). More specific immunological tests are therefore desirable.

The work on the development of in vitro tests in patients with allergic contact dermatitis has mainly been focused on assays of lymphocytes, where the cells are stimulated to proliferate by different metal compounds. The results have been contradictory, however, and both specific and non-specific lymphocyte stimulation has been reported (review ref. 1).

We present here a different approach to in vitro tests for the delayed type of hypersensitivity, based upon the knowledge that in response to immunological stimuli, keratinocytes can start to synthesize and express class II transplantation antigens (3-9), whereas in normal epidermis these antigens are confined to Langerhans' cells (10, 11).

MATERIALS AND METHODS

Patients and controls

Two women and one man, with a history of eczema but without active skin disease were chosen for the study (Table I). They had been patch tested 2 years (patient 1) and 6 years (patients 2 and 3) before the present study. Finn chamber technique was used with 5% nickel sulphate and 1% cobalt chloride in petrolatum and all showed a strong (++) or more reaction to nickel sulphate and patient 3 also had a positive reaction to cobalt chloride. Three healthy, sex- and age-matched persons served as controls (Table I). These persons had no history of eczema.

Medium

RPMI 1640 (Flow Lab., Irvine, UK) supplemented with 1 mM L-glutamine, streptomycin (100 µg/ml), penicillin (100 U/ml), fungizone (1.25 µg/ml) and heat inactivated (56°C for 30 min) normal human ABO serum (exp. no. 1) or autologous plasma (exp. nos. 2, 3).

Separation of peripheral blood mononuclear cells (PBM)

30 ml heparinized blood samples were collected from each person. Mononuclear cells were isolated by centrifugation on Lymphoprep (Nyco, Oslo, Norway) as described by Bøyum (12).

Lymphocyte proliferation assay

Triplicate cultures were set up in 96 well flat bottomed microtitre plates (Flow Lab.) with 2×10^5 PBM cells in 200 μ l medium per well. Nickel sulphate (NiSO_4) (Merck, Germany) was prepared as 1% stock solution in distilled water, and thereafter diluted in the medium to the final concentrations in the cultures of 6.25, 12.5 and 25 $\mu\text{g/ml}$. Phytohemagglutinin (PHA) 5 $\mu\text{g/ml}$ (Wellcome Lab., UK) or the medium alone were used as controls. The plates were incubated for seven days at 37°C in a humidified atmosphere of 95% air and 5% CO_2 . During the final 18 hours of culture, each well was pulsed with 1 μCi of (^3H)-thymidine (Amersham, Buckinghamshire, UK). The cells were finally harvested on glass-fibre papers and processed for liquid scintillation counting. Results are expressed as stimulation indices (SI), where the mean counts per minute (cpm) of stimulated cultures were divided by the mean cpm of unstimulated cultures (13).

Preparation of lymphocytes and supernatants for skin cultures

10^6 fresh PBM cells in 1 ml medium were cultured in 24 well flat bottomed plates (Flow Lab.) with or without 12.5 $\mu\text{g/ml}$ NiSO_4 for 7 days in a humidified atmosphere of 95% air and 5% CO_2 . Supernatants were carefully pipetted and centrifuged to avoid any cell contamination. The cells were collected, washed three times and then counted. Combinations of cells and their supernatants or supernatants alone were then used for cultures of skin specimens as indicated below.

Biopsies

Two punch biopses (3 mm) from normal skin were taken from each individual 7 days after blood samples.

Culture of skin specimens

The fresh punch biopsies were washed in phosphate buffered saline (PBS) and subcutaneous tissue was trimmed off, whereafter they were divided into halves. The 4 specimens from each person were cultured with dermal side down in 96 well flat bottomed microtitre plates (Flow Lab.) together with (a) autologous NiSO_4 stimulated 0.8×10^6 PBM + their supernatant (exp. nos. 1, 2, 3), (b) this (a) supernatant alone (exp. nos. 1, 3), (c) autologous non-stimulated (0.8×10^6) PBM + their supernatant (exp. nos. 1, 2, 3), (d) this (c) supernatant alone (exp. no. 1), (e) the supernatant from allogeneic NiSO_4 -stimulated lymphocytes (exp. no. 2), or (f) the medium alone (exp. nos. 2, 3). The end-volume per well was 300 μ l and fresh antibiotics, L-glutamin as indicated above + 5% ABO serum or autologous plasma was added.

Table I. Response of peripheral blood mononuclear (PBM) cells stimulated with PHA and various concentrations of NiSO_4 in three patients with positive patch test to NiSO_4 and three healthy control persons

Induction of HLA-DR antigens in vitro on keratinocytes in normal skin specimens cultured for three days with autologous (12.5 $\mu\text{g/ml}$) NiSO_4 -stimulated PBM and their supernatant is also indicated. The PNM responses are given as stimulation indices (SI), where $\text{SI} = \text{mean cpm of stimulated cultures divided by the mean cpm of unstimulated cultures}$

	Age/ sex	Background (cpm) (PBM+medium)	PHA ($\mu\text{g/ml}$) (SI) 5	Conc. of NiSO_4 ($\mu\text{g/ml}$) (SI)			In vitro HLA-DR induction on autologous keratinocytes
				25	12.5	6.5	
Exp. no. 1							
Patient 1	31/M	4000	28.5	3.1	13.3	18.8	+
Control 1	30/M	4650	11.5	0.5	2.0	1.5	-
Exp. no. 2							
Patient 2	26/F	600	158.3	1.3	15.5	9.3	+
Control 2	24/F	960	182.3	0.2	1.0	0.6	-
Exp. no. 3							
Patient 3	40/F	1050	38.8	3.8	5.6	4.9	-
Control 3	42/F	1000	146.0	1.7	1.3	1.0	-

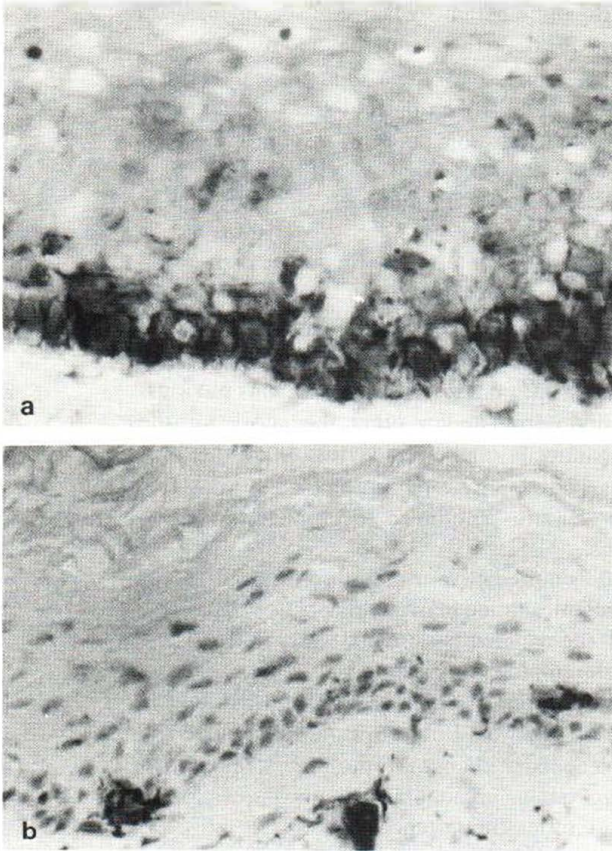


Fig. 1. Immunoperoxidase staining with the monoclonal antibody anti-HLA-DR (1.5 µg/ml) applied to frozen sections of (a) normal skin specimen from a nickel-sensitized person co-cultured for three days with autologous NiSO₄-stimulated peripheral blood mononuclear (PBM) cells + their supernatant. (b) Normal skin specimen from a healthy person co-cultured for three days with autologous NiSO₄-stimulated PBM + their supernatant.

The plates were incubated for 3 days at 37°C in a humidified atmosphere of 95% air and 5% CO₂ whereafter the specimens were dried briefly on filter papers, snap frozen in chilled isopentane and stored at -70°C until sectioned for immunohistochemical staining.

Immunohistochemical staining of skin sections

Acetone fixed 6 µm thick cryostat sections were investigated by means of a sensitive avidin/biotinylated peroxidase complex ('ABC') technique (14) using mouse monoclonal antibodies from Becton Dickinson Corp. (Sunnyvale, Ca., USA) denoted: anti-Leu 6 (diluted 1/64, specific for thymocytes, Langerhans' cells), anti-HLA-DR (diluted 1/128) and anti-Leu 10 (diluted 1/128, specific for HLA-DQ antigens).

RESULTS AND DISCUSSION

The following criteria for a positive *in vitro* response to NiSO₄ on day 6 of PBM culture have been presented by Al-Tawil et al. (13): (a) The experimental SI should be at least twice that of the controls tested simultaneously, when a NiSO₄ concentration of 6.25 µg NiSO₄/ml is used, (b) the SI of the patients should be more than 3 with use of this concentration and (c) the SI of the patients should be more than 6 with a concentration of 12.5 µg/ml. As seen in Table I two of our three patients (day 7 of culture) fulfilled all three criteria. Patient 3 fulfilled the first two criteria but not the third, thus making the nickel sensitivity of this person doubtful. Unfortunately, she was not available for re-testing or for a dilution test.

Immunohistochemical staining revealed an induction of HLA-DR (Fig. 1a) but not of

HLA-DQ antigens on keratinocytes in two of the three patients when skin specimens were co-cultured with NiSO₄-stimulated autologous PBM + their supernatant, but not with any of the other combinations. The control persons showed no induction of class II antigens in any of the tests. The patient (no. 3, Table I) who did not show induction of class II antigens on keratinocytes was the one with a questionable PBM response to NiSO₄ in vitro, mentioned above. In all these cases with HLA-DR-negative keratinocytes, class II antigens were confined to Langerhans' cells, as also were T6 antigens. The Langerhans' cells often showed a basal position (Fig. 1*b*).

When added to epidermal cell cultures, recombinant γ -interferon can induce expression of class II antigens on keratinocytes (15), as also can γ -interferon given locally in vivo (16). Another mediator, distinguishable from γ -interferon, produced by human allogeneic stimulated lymphocytes, can also induce class II antigen expression in a variety of different cell types (17). The supernatant of NiSO₄-stimulated PBM in nickel-sensitive persons obviously did not contain sufficient amounts of γ -interferon and/or other mediators for the induction of detectable amounts of class II antigens on keratinocytes, since this was observed only when the stimulated cells themselves also were present in the cultures. It is thus possible that the lymphocytes in the co-cultures continued to produce mediators necessary for the induction of class II antigens.

A similar experimental model has been used as a predictive test in vitro for graft versus host disease in patients with genotypic HLA-identical bone marrow transplants (18). Here pre-sensitized lymphocytes were cultured with the recipient's skin, and the majority of the co-cultured skin explants from patients later developing graft versus host disease showed histological changes compatible with this disease. In our experience, however, histological evaluation alone, as used in their investigation (18), would be too hazardous, since the culture method is hampered with the risk of secondary changes in the specimens, which might complicate the interpretation. For the particular immunohistochemical question in this study, on the other hand, such changes are of less importance.

The present investigation thus shows that in nickel-sensitized patients, in contrast to healthy controls, expression of HLA-DR antigens on keratinocytes can be induced in vitro by co-culture of normal skin biopsies with NiSO₄-stimulated autologous PBM.

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