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INVESTIGATIVE REPORT

Decreased *In Vitro* Cellular Response to Tetanus Toxoid and Tuberculin in Patients using Topical Corticosteroids

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Topical corticosteroids are the mainstay of treatment in inflammatory skin diseases. Corticosteroids penetrate human skin, especially when the penetration barrier is damaged. Whether long-term application of topical corticosteroids can lead to alteration of immune responses is not clear. We sought to examine the impact of topical corticosteroids on immune responses in patients using long-term topical corticosteroids. Peripheral blood mononuclear cell proliferation in response to tetanus toxoid and tuberculin stimulation was studied, and tetanus toxoid-specific antibodies were examined with ELISA. The results showed that, compared with the control group, the stimulation indices of patients' peripheral blood mononuclear cell to tetanus toxoid and tuberculin stimulations were lowered, which was especially significant in the tetanus toxoid group. No significant decrease was found in serum levels of tetanus toxoid-specific antibody. The results suggest that topical corticosteroids can suppress cell-mediated immune response in patients using long-term topical corticosteroids. ***Key words: skin; side effects; immune responses.***

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Topical corticosteroids are the mainstay of treatment in skin diseases such as atopic dermatitis, contact dermatitis and psoriasis (1). The use of more potent corticosteroids is associated with several untoward effects, the most important being loss of efficacy due to tolerance and atrophy due to inhibition of collagen synthesis (2). Furthermore, topical corticosteroids to some extent penetrate human skin, especially when the penetration barrier is damaged by inflammation (3, 4). Thus, in an earlier study the bone mineral density of patients using topical corticosteroids for atopic dermatitis was lowered when compared with that of healthy controls (5). In order to see whether long-term application of topical corticosteroid can also lead to alteration of systemic immune responses, we examined the cellular and humoral immune responses to tetanus toxoid and purified protein derivative (PPD) of tuberculin in patients using topical corticosteroids.

MATERIALS AND METHODS

Patients

Sixteen patients (13 women and 3 men; mean age 46.8 years, age range 32–64 years), who suffered from different skin diseases and who had, according to patient hospital records, intermittent usage of topical corticosteroids for at least 1 year before study, participated in the study. The data on health and medical treatments, including corticosteroids and vaccinations, were obtained from patient records and interviews. Fifteen normal healthy volunteers (10 women and 5 men; mean age 42.8 years, age range 27–60 years) without history of corticosteroid usage were included in the study as controls. All patients and controls had been vaccinated according to the national vaccination protocol of Finland, which includes tetanus and tuberculosis. Tuberculosis (Mantoux) vaccinations were done at birth and at 10–13 years of age. Further tuberculin vaccinations or testings are not done in Finland. Tetanus vaccinations are carried out three times in childhood and at least once every 10 years after that. Patients and controls were reported to have an interval of less than 10 years from their previous tetanus vaccination. The patients' clinical data are shown in Table I. The study was accepted by the Ethical Committee of the Skin and Allergy Hospital, Helsinki University Central Hospital.

Isolation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized venous blood of patients and controls by density gradient centrifugation over Ficoll Hypaque (Lymphoprep, Nycomed Phrama, Oslo, Norway). Cells from the interface were washed twice with phosphate-buffered saline (PBS) and resuspended in RPMI-1640 with glutamax-1, supplemented with 1 mM sodium pyruvate, 1% non-essential amino acid (GibcoBrl, Life Technologies, Scotland), 5% heat-inactivated human AB serum (Red Cross, Finland), 200 μg/ml streptomycin (Sigma, Germany), 200 IU/ml penicillin (Geepenil, Espoo, Finland) and 0.9 mM 2-mercaptoethanol (Sigma); thereafter referred to as complete medium.

PBMC proliferation in response to tetanus toxoid and PPD stimulation

One hundred microlitres of PBMC suspension containing 1×10^5 cells were cultured together with either 100 μl of tetanus toxoid (20 μg/ml, Statens Serum Institute, Denmark) or 100 μl of PPD (10 μg/ml, Statens Serum Institute) in triplicate in 96-well round-bottomed microtitre plates. Phytohaemagglutinin (PHA) was included as a positive control. After 5 days of incubation at 37°C and 5% CO_2, the cells were pulsed with ^{3}H-thymidine (1 μCi/well, Amersham Pharmacia Biotech UK) and harvested after 18 h onto a glass-fibre filter paper (Wallac, Turku, Finland). ^{3}H-thymidine uptake was determined by measuring the

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Table I. *Clinical data of 16 patients using topical corticosteroids.*

Age/sex	Diagnosis	Steroid use	Duration (years)
51/F	Chronic eczema	B, H, HB	>3
64/F	Chronic eczema	H	>10
52/F	Chronic eczema	HB	>1
46/F	Allergy	H	>2
54/F	Nickel eczema	B, H	>6
56/F	Chronic eczema	H, D	>4
32/M	Balanitis	H, D	>3
37/F	Chronic eczema	H	>3
38/M	Balanitis	H, D	>5
55/F	Chronic eczema	B, H	>4
33/M	Chronic eczema	B, H	>3
39/F	Chronic eczema	B, H	>20
55/F	Seborrhoeic eczema	B, H	>3
58/F	Chronic eczema	C, B, M	>20
32/F	Chronic eczema	H	>6
47/F	Atopic eczema	B, D, H	>40

B, betamethasone valerate; C, clobetasol propionate; D, desonide; H, hydrocortisone; HB, hydrocortisone butyrate; M, mometasone furoate.

radioactivity in a beta-liquid scintillation counter (Wallac). The stimulation index was defined by the ratio of mean cpm of stimulated to unstimulated cultures.

ELISA

Tetanus toxoid-specific antibodies were analysed by ELISA as described previously (6). Briefly, 96-well microtitre plates (NUNC-Immuno Plate, Denmark) were coated with 100 μl of tetanus toxoid (2 μg/ml) at 4℃ overnight. The plates were washed with washing buffer containing PBS and 0.05% Tween (Sigma) and blocked with 1% human serum albumin in 0.05 M carbonate buffer for 1 h. After washing with washing buffer, 100 μl of sample serum were added to each well and incubated for 2 h. Different serum dilutions were prepared for studying different antibody isotypes: 1:5 for IgE, 1:150 for IgG1, 1:80 for IgG2, 1:10 for IgG3 and 1:5 for IgG4. After incubation and subsequent washing, 100 μl of biotinylated goat anti-human IgE (diluted 1:1000, Vector, Burlingame, CA, USA) or biotinylated mouse anti-human IgG1, IgG2, IgG3 and IgG4 (all diluted 1:1000, Zymed, San Francisco, CA, USA) were added, respectively. This was followed by 100 μl of streptavidin-conjugated alkaline phosphatase (diluted 1:1000, Zymed) and colour substrate (1 mg/ml, Bio-Rad, Hercules, CA, USA). The colour formed was read as optical density (OD) at 405 nm with an automated ELISA reader (Titertek Mutisca; Eflab, Turku, Finland).

Statistics

The differences between groups were assessed with the unpaired Student's *t* test.

RESULTS

PBMC proliferation in response to tetanus toxoid and PPD

To study the effects of topical corticosteroids on the human immune system, *in vitro* stimulation of cultured PBMC with tetanus toxoid and PPD was employed. The results showed that the mean stimulation index of patients' PBMC to tetanus toxoid was significantly lower than that of the control group ($p=0.009$) (Fig. 1A). Similarly the stimulation index for PPD was also lower in the patient group, but the difference was not statistically significant compared with the control group ($p=0.139$) (Fig. 1B). No difference was found in PHA stimulation index between the patient and the control groups ($p=0.567$ (data not shown)).

Tetanus toxoid-specific antibodies

Tetanus-specific antibodies including IgE, IgG1, IgG2, IgG3 and IgG4 were checked with ELISA. There was no tetanus toxoid-specific IgE in either the patient or the control group. The levels of tetanus toxoid-specific IgG subtypes were not statistically different between the patient and control groups (data not shown).

DISCUSSION

This study comprised a heterogeneous patient population where intermittent topical corticosteroid usage of at least 1 year prior to study was the entry criterion. The patients did not report use of other medications or treatments affecting cell proliferation or immune responses prior to study. However, the type of corticosteroid used, the skin area where the drug was used, and the frequency and duration of treatment differed widely among the patients. It is also possible that the menstrual cycle may affect proliferation responses to bacteria in premenstrual women. Further, although all patients and controls had received their last tetanus vaccinations within the last 10 years, it was not possible to track their exact timing. The results show

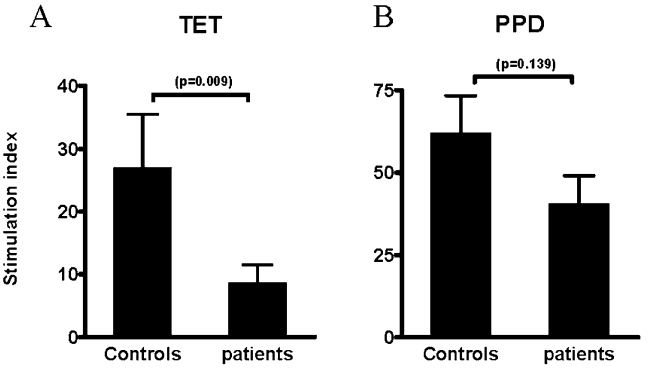

Fig. 1. The proliferation responses of cultured peripheral blood mononuclear cells to (A) tetanus toxoid (TET) and (B) purified protein derivative (PPD) stimulation in patients using long-term topical corticosteroids and a normal control group without history of corticosteroid use. The TET stimulation index of the patient group was significantly lower than that of the control group ($p=0.009$). Bars represent mean ± SE.

that the overall proliferation responses of PBMC to bacterial antigens were lower in the patient group using topical corticosteroids than in the control group with healthy skin, especially with regard to the tetanus response ($p < 0.01$). In a recent study, splenocyte proliferation responses to tetanus were significantly suppressed in mice that had their corticosterone levels increased (7). PBMC responses to PHA were unchanged in patients when compared to controls. As PHA responses reflect the overall function of PBMC, not antigen-specific immune responses, it seems that the lowered responses to bacterial antigen were related to acquired cellular immune responses only. In support of this, the specific antibody levels in the sera of patients using topical corticosteroids were comparable to the levels of healthy controls. Interestingly, a recent murine study showed that the antibody response to sheep blood erythrocytes, while decreased by high corticosteroid dosage, was actually increased by a low corticosteroid dosage (8). Also, the amounts of topical corticosteroids absorbed systematically in our patients may not have been sufficient to affect antibody production at all.

It has been known for a long time that the use of topical corticosteroids may result in local immunosuppression in the skin (9, 10). However, the findings in this study suggest that systemic (cell-mediated) immune responses could also be suppressed. Attention should therefore be given to long-term usage of topical corticosteroids, especially in children, as the use of topical corticosteroids in young children could cause even further systemic immunosuppression, because percutaneous penetration in children is two to three times higher than in adults (11). A new prospective study is warranted, where details of the patients' topical corticosteroid use are carefully controlled for and where the timing of tetanus vaccinations is identical in patients and controls. The new topical immunosuppressants tacrolimus and pimecrolimus should also be studied in this regard. Further research would ascertain whether current practices of very wide topical corticosteroid usage should be altered.

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