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INVESTIGATIVE REPORT

An Immunohistochemical Analysis of Cytokine Expression in Allergic and Irritant Contact Dermatitis

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The purpose of this study was to investigate whether the specific and non-specific inflammatory responses to allergens and irritants give rise to immuno-histochemical detectable differences in the cytokine profile in the skin. Skin biopsies taken at 0, 6, 24 and 72 h from contact allergic reactions to nickel and from irritant reactions to sodium lauryl sulphate were analysed. The main finding was that the dermal cells expressed similar patterns of cytokines (IL-1α, IL-1β, IL-2, IL-4, IL-6 and IL-10) in both types of contact reaction at 72 h. However, two differences were observed. Staining for the IL-1 receptor antagonist was more prominent in the dermis at the late stages of the allergic reaction compared with the late stage of the irritant reaction. The other difference was an increased interferon-γ staining of infiltrating mononuclear inflammatory cells in the dermis in the sodium lauryl sulphate group compared with the nickel group. A more rapid general onset of cytokine production was found in the sodium lauryl sulphate group than in the nickel group. The main conclusion of this study was that after 6 h the cytokine patterns did not differ between the specific and the non-specific inflammatory responses in the skin.

Key words: cytokine; contact dermatitis; rheumatoid arthritis.

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Exposure of the skin to different chemical substances can result in allergic or irritant contact dermatitis. These two types of contact dermatitis cannot be macroscopically or histologically distinguished, despite their induction by different mechanisms (1). The allergic reaction is a specific immune reaction and follows sensitization with the allergen in question. The irritant reaction, on the other hand, does not need a previous sensitization. In previous immunohistochemical studies, these two conditions have been compared for the presence of specific cell surface markers without any major differences being reported (2–4).

Considering the important function of cytokines in immunoregulation it is of interest to know whether an antigen-induced contact allergic reaction differs from an irritant lesion in regard to local cytokine production. There is only limited information available to answer this question. In one study using *in situ* hybridization and immunohistochemistry for the detection of IL-1α and IFN-γ in nickel-provoked skin from nickel-allergic and normal persons (5), an upregulation of IFN-γ was recorded within 4 h after application of the nickel in the skin biopsies from sensitized persons but not from controls. IL-1α was detected in both groups.

We have previously used an immunohistochemical technique (6) to detect cytokine production in biopsies of synovial membrane from rheumatoid arthritis patients. This is a technique in which a combination of saponin treatment and application of monoclonal anti-cytokine antibodies enables the antibodies to penetrate the cell membrane to detect intracellularly located cytokines in the Golgi apparatus.

In using this immunohistochemical method, we aimed to investigate the dynamics of cytokine expression in allergic and irritant contact reactions after induction of these reactions. The molecules that we analysed in the skin were IL-2, IL-4, IFN-γ, IL-6, IL-10, IL-1α, IL-1β, TNF-α and IL-1 receptor antagonist.

PATIENTS AND METHODS

Patients and volunteers

Eight healthy volunteers (4 females and 4 males, 23–58 years) with no known allergy or skin disease and 8 females (20–53 years) with previously diagnosed contact allergy to nickel were included. Skin reactions were induced on the gluteal area in both groups. Irritant contact reactions were induced with 4% sodium lauryl sulphate (SLS) in distilled water (w/v) (SLS, Heneckel-Niopco AB, Gothenburg, Sweden). SLS (15 μl) was applied under occlusion with 8 mm Finn Chambers® for 6, 24 and 48 h. After intradermal injection of 0.1 ml Lidocain with adrenaline, skin punch biopsies (diameter 4 mm) were taken from the exposed skin sites. Specimens were obtained immediately after 6 and 24 h of occlusion and at 24 h after removal of the 48 h of occlusion, i.e. at 72 h after test application. One biopsy was also taken from non-exposed control skin. Contact allergic reactions were induced with 5% nickel sulphate in petrolatum using 8 mm Finn Chambers®. Times for exposure and biopsies were the same as for the SLS group.

As a vehicle control four persons were exposed to 15 μl of water or to petrolatum under occlusion with 8 mm Finn Chambers®. These same persons were also exposed to empty Finn Chambers®. Non-exposed skin was used as normal control. Biopsies were taken at 24 h.

The experiments were approved by the Ethics Committee at the University Hospital in Uppsala.

Handling of biopsies

Biopsies were immediately snap frozen in Isopentane on dry ice. All tissues were stored at −70°C until sectioned.

Preparation of tissue sections

For each monoclonal antibody, two cryostat sections, 6–8 μm thick, were mounted on gelatine-coated glass slides (Cel-Line, Newfield, USA). The sections were cut at different levels of the biopsy at

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intervals of least 60 μm. Fixation was performed with 2% cold formaldehyde for 20 min or with 50% cold acetone for 30 sec followed by 100% cold acetone, depending on the staining procedure used. The formaldehyde-fixed slides were used for the cytokine staining (6, 9, 11, 12) and the unfixed slides for the cell characterization staining.

Immunohistochemistry for cytokine detection

A summary of the procedure is as follows: The frozen, fixed sections were thawed and then washed in Ca^{2+} and Mg^{2+} containing Earls Balanced salt solution (EBSS) (Gibco Ltd, Paisley, Scotland). Endogenous peroxidase activity was blocked by incubation of the specimens in the dark for 60 min at +20°C in 1% (v/v) H_2O_2 and 2% (w/v) NaN_3 dissolved in EBSS supplemented with 0.01 M Hepes buffer and 0.1% (v/v) saponin (Riedel de Haen AG, Seelze, Germany) as a detergent (from now on referred to as EBSS saponin). Saponin reversibly permeabilizes cell membranes and must be present throughout the whole staining procedure in order to allow intracellular access of antibodies. Following three additional washes in EBSS saponin the slides were incubated overnight at room temperature in a humid chamber with 100 μl of a panel of carefully selected cytokine-specific mAb at a concentration of 2–5 μg/ml in EBSS saponin (Table I). Control staining was performed in parallel with species-matched and isotype-matched myeloma proteins.

The slides were then washed three times in EBSS saponin and incubated with 1% (v/v) normal goat serum in EBSS saponin for 15 min at room temperature in order to reduce background signals potentially caused by IgG Fc-interactions from the biotinylated second-step goat antibodies. Biotin-labelled secondary antibodies, absorbed against human Ig (biotinylated goat anti-mouse IgG1, Caltag Laboratory, South San Francisco, CA; biotinylated goat anti-rat IgG, Vector Laboratory, Burlingame, CA) were diluted in EBSS saponin containing 1% normal goat serum and incubated with sections for 30 min at room temperature. Following subsequent washes in EBSS saponin the slides were incubated with avidin-biotin-horseradish peroxidase (Vectastain Elite, ABC-HP-kit, Vector Laboratory) for 45 min at room temperature. After one wash with EBSS the colour reaction was developed with 0.5 mg/ml diaminobenzidine (DAB, Vector Laboratory) for 7 min. The slides were finally counterstained with Mayer's hematoxylin and mounted in PBS glycerin (diluted 1:9) (Merck, Darmstadt, Germany).

Table I. *Cytokine-specific antibodies used for tissue staining*

Cytokine	Designation and source of antibodies	Isotype
IL-1α	1277-89-7,1277-82-29, 1279-143-4 Immunokontact, Switzerland	mouse IgG1
IL-1β	2-D-8, 1437-15 Immunokontact, Switzerland	mouse IgG1
IL-1 rec. antagonist	1384.92.17.8, 1390.29.53 Biomedicals AG, Switzerland	mouse IgG1
IL-2	17 H12 PharMingen, San Diego, CA, USA	rat IgG2a
IL-4	MP4-25D2 PharMingen, San Diego, CA, USA	rat IgG1
IL-6	6A3 PharMingen, San Diego, CA, USA	rat IgG2a
IL-10	JES3-19F1, JES-12G8 PharMingen, San Diego, CA, USA	rat IgG2a
TNF-α	mab 1, mab11 PharMingen, San Diego, CA, USA	mouse IgG1
IFN-γ	DIK1, 7B6 Mabtech, Stockholm, Sweden	mouse IgG1

Characterization of cell surface markers in the skin biopsies

The slides were fixed in acetone immediately after removal from storage at −70°C. The initial fixation was performed for 30 sec at +4°C in 50% (v/v) acetone, followed by an incubation with 100% acetone for 3 min at +4°C. Endogenous peroxidase activity was then blocked with 0.3% H_2O_2 in PBS. Cell surface staining was performed during an overnight incubation with CD-specific mAb specific monoclonal anti-CD1a, CD3, HLA-DR (Becton-Dickinson, Mountain View, CA), CD106 (VCAM) (PharMingen, San Diego, CA) or CD54 (ICAM-1) (Serotec, Oxford, UK) antibodies dissolved in PBS containing 1% bovine serum albumin and 0.02% NaN_3. Control staining was performed in parallel with species-matched and isotype-matched myeloma protein mouse IgG1 (X0931, Dakopatts, Copenhagen, Denmark). After washes in PBS the sections were incubated for 15 min with 1% normal horse serum in PBS in order to prevent Fc interactions with the secondary antibodies, followed by an exposure to the second step biotinylated antibody (biotinylated horse anti-mouse IgG, Vector Laboratory, CA) for 30 min in the presence of 1% horse serum in PBS. Following subsequent washes in PBS the slides were incubated for 45 min with Avidin-biotin-horseradish peroxidase (Vectastain ABC-HP-kit standard, Vector Laboratory or Extra Avidin Peroxidase EAP, Sigma) and the reaction was developed for 15 min with AEC (3 amino-9-ethylcarbazol) generating a strong red colour reaction. The slides were finally lightly counterstained with Mayer's hematoxylin and mounted in glycerin-gelatin.

Specificity test

In order to test the specificity of the staining of cytokine-producing cells, blocking experiments were also performed for all antibodies used in the experiments. Purified natural or recombinant cytokines were incubated with primary antibody at 10 times excess of the antibody concentration (20 μg/ml) at 4°C, overnight. Subsequent staining was performed as described. In all cases the staining reaction disappeared completely or was reduced after blocking with the relevant recombinant cytokine.

Microscopic quantification of cells

Skin sections were analysed with a Reichert-Jung Polyvar microscope at 200× magnification. Positively stained cells were studied in high power fields (400× to 1000×). The whole section was analysed. All microscopic evaluations were performed by one observer (AU) and repeated on three different occasions. Another observer (ML) also confirmed the criteria for evaluation. Infiltrating positively stained cells were evaluated semi-quantitatively.

Statistical analysis

In order to evaluate the dynamics of cytokine production in general, we also compared the total number of positively stained sections (with at least one clearly positive cell) for the various anti-cytokine antibodies in the SLS and Ni groups at various times after challenge. A non-parametric analysis (Mann-Whitney U test) was used for this comparison.

RESULTS

Clinical evaluation of the contact reactions

At 6 h, clinical signs of dermatitis were observed in the SLS but not in the Ni group. After 24–72 h the clinical appearance was similar in both groups. In the control group no reactions were evident. After 72 h, SLS-exposed skin presented a more dry, scaly surface than the nickel-exposed skin. No reactions were apparent in the control group.

Cell surface markers in irritant and allergic reactions

In normal skin, no T-cells were evident in the epidermis. A few $CD3^+$ cells were observed in the epidermis after 24 h and 72 h of exposure to SLS or nickel. In normal skin a few $CD3^+$ cells were recorded in the dermis. The number of $CD3^+$ cells was increased in SLS-exposed and also in nickel-exposed skin at 72 h in the dermis. In the 24-h-exposed skin only a few $CD3^+$ cells were recorded after both types of provocation.

ICAM-1 staining was evaluated in three different respects; for expression by keratinocytes in the epidermis, on endothelial cells and on mononuclear cells. In normal skin ICAM-1 was only expressed on a few endothelial cells. After SLS exposure there was an increased ICAM-1 expression at 6 h on endothelial cells and also after 24 h on inflammatory cells in areas where $CD3^+$ cells were noted. After 72 h there was in addition a staining for ICAM-1 on certain keratinocytes. ICAM-1 expression for the allergic reactions was very similar to that for SLS-induced lesion.

Cytokine detection in irritant and allergic reactions

When evaluation was conducted the entire area of the section was evaluated unless stated otherwise. A group of four vehicle controls was provoked with water, petrolatum, empty chamber and without provocation. The sections from this vehicle control group gave only IL-4 staining. The observed cytokine staining was mainly in mononuclear inflammatory cells in the dermis and no staining was recorded in the keratinocytes in the epidermis.

Expression of IL-2 was detected in nickel-exposed skin at 24 h and 72 h. IL-2-positive cells were also present in SLS-exposed skin but at lower numbers than in nickel-exposed skin. IFN-γ positive cells were apparent in the SLS-exposed skin, but not in the nickel-exposed skin. At 6–72 h IL-4-positive cells were increased in both SLS-exposed and nickel-exposed skin. Staining for TNF-α was recorded in a small number of cells in the skin after exposure to both SLS and nickel. IL-6 was expressed in a small number of biopsies at 6–72 h in both the nickel-exposed and SLS-exposed skin.

IL-1α-positive cells were observed in both types of skin exposure, and the numbers of stained cells were higher in the SLS than in nickel-exposed skin from 6 until 72 h. IL-1β was only expressed by a low number of cells for both SLS and nickel biopsies throughout the exposure. IL-1 receptor antagonist expression was increased in nickel-exposed skin compared to SLS-exposed skin. This difference was particularly clear after 72 h of exposure. Expressing IL-10 was recorded in both nickel-exposed and SLS-exposed skin after 6 h and persisted until 72 h in both kinds of lesion.

Finally, we compared the irritant and antigen skin reactions for the combined presence of all measured cytokines. When comparing all the cytokines of the SLS and Ni groups at 0, 6, 24 and 72 h with the Mann-Whitney U test, a tendency of more cytokine expression in the SLS group was recorded at 6 h ($p = 0.0068$).

DISCUSSION

The design of this study was to provoke two types of contact reaction so that ensuring comparable inflammatory lesions, as evaluated clinically and by the extent of infiltrating lymphocytes, could be analysed for local cytokine production. The concentration chosen for SLS exposure was 4%, which has previously been reported to give a clinical response comparable to that obtained by the current nickel exposure regimen (7, 8). In macroscopic terms the reactions obtained with the irritant and the allergen were similar at 72 h, whereas at 6 h the SLS-exposed skin exhibited stronger reactions. There was also a similar amount of CD1a and CD3 cells in the irritant and allergic reaction at 72 h in the microscopic evaluation. This implicates a time difference in the development of the reaction types in this study.

With the method used intracellularly located cytokines can be detected. This method gives an opportunity to identify cytokine-producing cells rather than cytokine-binding cells and is in contrast to the situation with acetone fixation in which the golgi apparatus is not exposed in a similar fashion (6, 9–12).

In earlier studies of allergic and non-allergic contact reactions it has not been possible to detect any major differences between these two reactions concerning cell surface/activation markers (1, 4). The present study confirms previous findings of an early upregulation of ICAM-1 expression on keratinocytes and other cell types (7). ICAM-1 is an adhesion molecule that is implicated in T-cell aggregation, activation and migration. Another feature of the allergic reaction which we confirmed was that of infiltrating, T-cells expressing IL-2 receptors (13). This activation may be due in part to the production of IL-1 from keratinocytes (14, 15). Using the present technique we did not detect any cytokine production in the epidermal keratinocytes.

The T-cell cytokine most readily detected in our study was IL-2, which was present in both SLS-exposed and nickel-exposed skin. However, we did not detect any IFN-γ in the biopsies from nickel-exposed skin, whereas a minor IFN-γ production was observed in the irritative lesions. This is surprising, as other authors have reported IL-2 and IFN-γ mRNA as well as intact IFN-γ and IL-2 protein in nickel-exposed skin (5). One possible explanation of this discrepancy may be methodological: the present method detects only intracellularly located and thus locally produced cytokine, whereas most other immunohistochemical methods make no distinction between internally produced cytokines and cytokines that are produced elsewhere. It is also possible that PCR-based methods used for detecting mRNA in the skin are more sensitive to small amounts of molecules than our current immunomorphological method.

The saponin method has not previously been tested *in vitro* or *in vivo* for evaluation of the keratinocyte production of cytokine. In this study we could not detect cytokine production in the keratinocytes, possibly because saponin could not penetrate the subcellular compartments of the epidermis.

IL-6, which is a major mediator of the acute phase response, was present in early stages of both SLS-induced and nickel-induced inflammation. This is in agreement with earlier studies in which an increase in keratinocyte IL-6 expression in allergic patch test reactions (16) and induced irritant reactions (17) has been reported.

On comparing the two types of skin reactions, one could draw the conclusion that the allergic and irritant skin reactions differ surprisingly little with regard to the presence

of various cytokines, at least during the 6–72 h after provocation. The currently used method for cytokine detection can be used to analyse cytokine patterns in skin inflammatory lesions. It does not give information to compare different types of skin lesions. This demonstrates the need for still further molecular investigations if we are to understand at what levels the two types of agents, the irritant and the allergen, give rise to different local skin responses and at which level final common pathways are in action.

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