

Keratinocyte-expression of Interleukin-6 but Not of Tumour Necrosis Factor-alpha is Increased in the Allergic and the Irritant Patch Test Reaction

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The cytokine expression on epidermal cells in the allergic patch test reaction (APR) and irritant patch test reaction (IPR) was studied using antibodies against rIL-6, rTNF α , rIL-1 α and rIL-1 β in a histochemical biotin-avidin technique. Nikkel sulphate was used for APR in 5 nickel allergic patients and sodium lauryl sulphate for IPR in 5 healthy individuals. The individuals served as their own control. Enhanced keratinocyte expression of IL-6 was observed in APR and IPR, whereas staining for TNF α remained unaltered compared with non-tested and petrolatum-tested skin. Staining for IL-1 α and IL-1 β proved negative in all specimens. Double-staining experiments demonstrated that epidermal and dermal OKT-6 (CD1) positive Langerhans cells (LC) remained negative for all cytokines. These results demonstrate that enhancement of keratinocyte-bound IL-6 does not induce TNF α , IL-1 α/β or IL-6 expression by LC during APR or IPR, and that enhanced keratinocyte expression of IL-6 fails to distinguish between these two reactions. **Key words:** Cytokines; Delayed hypersensitivity; Epidermal cells; Langerhans' cells.

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Contact dermatitis comprises either an allergic or an irritant mechanism, distinguished by the demonstration of a delayed-type allergy in the patient (1). Although often morphologically (microscopically and macroscopically) similar (2), the pathogenesis of the irritant contact dermatitis, experimentally illustrated by the irritant patch test reaction (IPR), has been considered non-immunological in contrast to the cutaneous delayed hypersensitivity demonstrated by the allergic patch test reaction (APR) (1).

Langerhans' cells (LC) and the keratinocytes both

participate in antigen presentation and the production by these cells of lymphocyte activating cytokines appears to be centrally involved in these processes (3–6). Keratinocyte-associated cytokines have been shown to influence maturation (7) and the stimulatory capacity of LC (8). Various cytokines are expressed and produced by keratinocytes (9), whereas interleukin-1 (IL-1) is the only LC-associated cytokine yet described (10).

ETAF (epidermal thymocyte activating factor) is considered to be a heterogeneous population of cytokines, active in the thymocyte co-stimulatory assay. ETAF includes IL-1 α , IL-1 β , tumour necrosis factor alpha (TNF α) and IL-6 (9). IL-1 and TNF α are potent inducers of IL-6 (11), and Helle et al. reported that IL-6 induces T-cells to become more responsive to IL-2 (12). In addition, Tosato & Pike presented evidence that IL-6 directly stimulates T-cell proliferation in an IL-2 receptor independent fashion (13). The demonstration of enhanced ETAF activity in the APR (14) and unaltered ETAF activity in IPR (15) indicates potentially differences with regard to cytokine expression in these two reactions.

IL-1 and TNF α induce intercellular adhesion molecule-1 (ICAM-1) on endothelial cells and fibroblasts in vitro (16, 17). ICAM-1 is involved in adhesion of mononuclear cells during immunoinflammatory reactions, and it is expressed by keratinocytes in the APR. This, in contrast to the IPR (18), again indicating differences in the molecular biology of the two reactions.

We have previously documented the in vivo expression of IL-6 and TNF α in keratinocytes, but not in LC, in normal human skin (19, 20). Furthermore keratinocyte expression of both these cytokines was increased after UVB irradiation (19), which interferes with antigen presentation of epidermal cells; this effect may be explained by alterations in cytokine production (21).

The present study compares the *in vivo* keratinocyte and LC expression of IL-6 and TNF α during the APR and IPR.

MATERIALS AND METHODS

Patients and design

Four unmedicated female and one male patient with a mean age of 44 years (range 26–64) were selected for allergic patch testing. All patients had had a positive nickel sulphate patch test within the last year (tested according to regulations of International Contact Dermatitis Research Group (ICDRG) (22). None of the patients had active eczema at the time of investigation.

Three healthy, non-mediated female and 2 male individuals with a mean age of 37 years (range 36–42) participated in irritant patch testing. All individuals gave their informed consent.

The patch testing was performed and read according to the guidelines of ICDRG. Finn chambers (10 mm) (Epitest, Ltd. Oy, Pajjala, Finland) were used for occlusion and fixed to the skin of the inner aspect of the upper arm by Scanpore Tape (Norges Plaster, Oslo, Norway) for 48 h. The vehicle was petrolatum, and the active ingredients were 5% nickel sulphate applied on nickel-allergic patients (APR) and 5% sodium lauryl sulphate (SLS) (23) applied on the healthy individuals (IPR). As controls, simultaneous patch tests with petrolatum were performed symmetrically on the other arm. Reading was performed after 48 h and scoring was performed to the guidelines of ICDRG (22).

Punch biopsies

Immediately after reading, simultaneous skin punch biopsies (4 mm) were obtained from the patch tests of both arms and from a non-tested area on the upper arm adjacent to the area tested with petrolatum, using infiltration anaesthesia with 2% lidocaine in a ring around the biopsy site.

Cytokines

Human recombinant IL-1 (rIL-1 α) (10^8 U/mg) and human rIL-1 β (10^7 U/mg) were kindly donated by Dr S. Gillis (Immunex Corp, Seattle, Wash. USA), human rIL-6 (10^9 U/mg) by Dr Hirano (Osaka, Japan), and human rTNF α (4×10^7 U/mg) by Dr G. R. Adolph (Boehringer, Vienna, Austria). In each case, the cytokines were tested for bioactivity using mouse thymocytes (rIL-1 α and rIL-1 β), B9 hybridoma cells (rIL-6) (24), and L-M fibroblasts (rTNF α). Bioactivities of the cytokines were compared with corresponding international interim reference preparation (Nat Inst Biol Standard Control, London, England).

Production of specific, polyclonal antisera to human monokines

The antisera to human rIL-1 α , rIL-1 β , rIL-6, and rTNF α were generated by repeated immunizations of high-responder rabbits (Dakopatts, Glostrup, Denmark) with 5–20 μ g of the purified cytokines (25) and tested for reactivity with their respective cytokines by Quchterlony double diffusion technique, ELISA and immunoblotting. Biochemical and

biological neutralization tests, using several different human cytokines confirmed the monospecificity of these antibodies.

Biotin-avidin technique for demonstration of cytokines

4–6 μ m sections of the skin were cut in a cryostat. The samples were air dried for 5 min, fixed in acetone for 1 min, and incubated for 5 min with 3% hydrogen peroxide in distilled water. The slides were placed in a humidity chamber for 20 min with 10% normal goat serum (Vector Laboratories, Burlingame, Calif. USA) diluted in phosphate-buffered saline (PBS) (pH 7.2). Excess serum was removed, and the slides treated for 30 min (20 $^\circ$) with rabbit anti-cytokine-antisera at two-fold dilutions from 1:40 to 1:1280. The specimens were washed in PBS (3 \times 5 min) and incubated for 30 min (20 $^\circ$ C) with biotinylated goat anti-rabbit immunoglobulin (Ig) (1:400 in PBS) (Vector). After washing in PBS (3 \times 5 min), incubation was performed with ABCComplex (Vector) for 30 min (20 $^\circ$ C) and washed as mentioned above. Specimens were incubated with 0.038% 3-amino-9-ethylcarbazol (Sigma, Chem. Co., St Louis, Mo, USA) in acetate buffer (pH 5) and 0.014% hydrogen peroxide for 5 min, washed in water for 10 min, counterstained with hematoxylin for 2 $\frac{1}{2}$ min and, finally, washed in water for 10 min. The samples were mounted with Crystal mount (Biomedica, Foster City, Calif.).

Double staining for CD1 (T6)-positive LC and epidermal membrane bound cytokines

Double labelling was carried out by indirect immunofluorescence (IF) staining for the CD1 antigen and biotin-avidin-peroxidase staining for the cytokines. All specimens were air dried for 5 min and fixed in acetone for 1 min. The specimens were incubated in a humidity chamber for 20 min with 10% normal goat serum (Vector) in PBS. Excess serum was removed, and the specimens incubated for 1 h with rabbit anti-cytokine antiserum (1:80 TNF α , 1:160 IL-6). Hereafter, the specimens were washed (3 \times 5 min) in PBS, incubated with mouse monoclonal against CD1 (OKT6) (1:100) (Ortho Diagnostic Systems, N.J., USA), washed again (3 \times 5 min) in PBS, incubated for 30 min at 20 $^\circ$ C with a horse FITC-labelled anti-mouse Ig (Vector) (1:20), washed 3 \times 5 min in PBS and, finally, incubated for 30 min at 20 $^\circ$ C with biotinylated goat-anti-rabbit Ig (Vector) diluted to 1:300 in PBS. After washing (3 \times 5 min), the specimens were processed with the ABCComplex as described above.

Fluorescence microscopy

The slides were read in Zeiss Microscope (Oberkochen, West Germany) equipped with a high-pressure mercury lamp (HBO 100 W/2), and an incident illumination system with exciter filter, beam splitter and barrier filter adapted for FITC.

Specificity controls

Two specimens from normal skin, two from IPR and two from APR were treated with antisera absorbed by preincubating the anti-TNF α antiserum with rTNF α (4×10^4 U per 0.5 μ l antiserum) and the IL-6 antiserum with rIL-6 (10^4 U

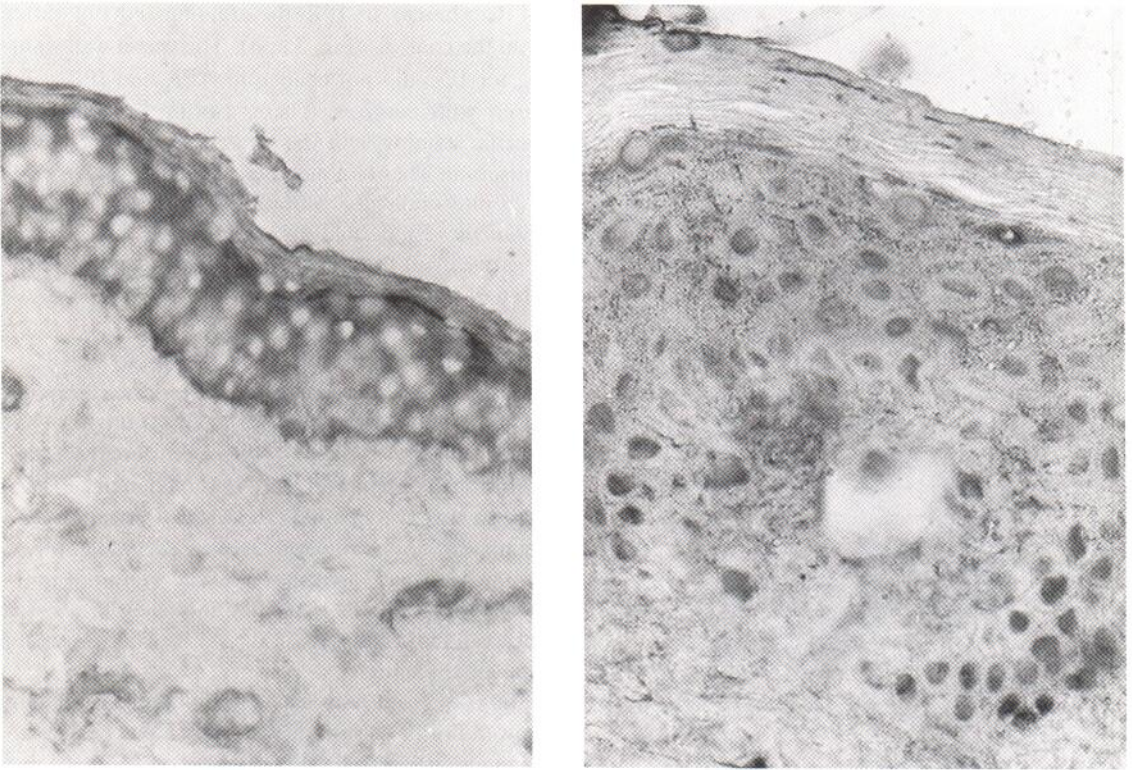


Fig. 1. (a) Interleukin 6 ($\times 200$) and (b) tumour necrosis factor-alpha ($\times 400$) demonstrated in normal skin by biotin-avidin-peroxidase technique.

per 0.5 μ l antiserum) at 20°C for 1 h followed by incubation overnight at 4°C. Staining was quenched in all cases.

The primary layer antiserum was replaced by preimmune serum in every specimen and all dilutions used, moreover, in one specimen from each biopsy the primary layer antiserum was replaced by PBS. The specific staining was lost if either the primary layer (the anti-rIL-6 and anti-rTNF α antiserum) or the secondary layer antibody was omitted (performed in two specimens), or if the primary antibody was replaced by PBS or preimmune serum.

Autofluorescence was negligible in specimens without added FITC-conjugated antibody performed in all specimens.

RESULTS

Patch test reactivity

APR: At the 48 h reading one patient reacted with redness only (1+), the other 4 with redness and infiltration (2+). *IPR*: Four individuals reacted with 2+ reaction and one with a 1+ reaction. Testing with petrolatum was negative in all cases.

Staining of skin sections for cytokines and CD1 (T6) positive LC

Biopsies from non-tested skin and petrolatum-tested skin showed similar staining patterns in all individuals. Staining for IL-6 and TNF α were located in stratum granulosum and spinosum in an intercellular irregularly distributed, granular pattern, in some areas associated with membranes of single cells in others with groups of cells. Using anti-rTNF α antiserum, the cytoplasmic (but not the membrane-associated) staining was difficult to distinguish from the unspecific staining obtained with preimmune serum. However, the antiserum to rIL-6 caused pronounced and apparently specific cytoplasmic staining of scattered groups of cells in the stratum spinosum. Specific staining of basal cells was present only occasionally (Fig. 1 a, b). Maximum dilutions giving rise to specific staining were for the anti-rTNF α antiserum in the range 1:40–1:80 and for the anti-rIL-6 antiserum, 1:60–1:640 with the 1:80 dilution giving reliable discrimination from the preimmune serum in all cases. In some biopsies a slight, scattered, streaky



Fig. 2. Interleukin-6 demonstrated in dermal vessel walls of normal skin. Biotin-avidin-peroxidase technique ($\times 800$).

stratum corneum staining for both cytokines was observed. A prominent specific staining of fibroblasts and vessel walls was detected with the anti-rIL-6 antiserum (Fig. 2), whereas the anti-rTNF α antiserum caused a diffuse (unspecific) staining of the connective tissue. There was no specific epidermal or dermal staining using the anti-rIL-1 α or anti-rIL-1 β antisera.

In biopsies from both APR and IPR staining for TNF α was unchanged compared with non-tested and petrolatum-tested skin. However, when using the anti-rIL-6 antiserum, the cytoplasmic staining was increased. All viable epidermal cell layers were markedly stained. The most obvious change was that the intracellular staining intensity was increased, resulting in a more extended and 'tightly packed' cytoplasmic staining. Moreover, staining could now be detected in dilutions up to 1:1280. The viable epidermal cell layer staining was similar for APR and IPR. However, the stratum corneum revealed pronounced, streaky and granular staining in all cases of

APR, whereas in IPR the staining did not differ from the controls (Fig. 3 a, b). The vessel wall staining for IL-6 did not change in APR and IPR compared with controls. There was no demonstrable specific staining with the anti-rIL-1 α or anti-rIL-1 β antiserum. The staining patterns did not show significant inter-individual variations. *Double staining experiments:* Epidermal and dermal LC were clearly negative in all specimens when stained with the anti-rIL-6 or anti-rTNF α antiserum.

Histology of hematoxylin/eosin stained specimens

Biopsies from both IPR and APR showed varying degrees of epidermal spongiosis. This, however, was more pronounced in the APR. Occasionally, a few infiltrating lymphocytes were observed in the epidermis. In two IPR, parakeratosis of the stratum corneum was observed. Perivascular accumulation of mainly small mononuclear cells and scattered polymorphonuclear cells extending into the dermis was observed most intensely in the APR. There were no signs of vasculitis.

Biopsies from non-tested petrolatum tested skin showed normal histology with only a few perivascular mononuclear cells (Fig. 4).

DISCUSSION

The present study shows that IL-6 and TNF α are expressed by keratinocytes in normal skin, but not by epidermal or dermal LC. During APR and IPR the expression of IL-6 per cell as well as number of cells expressing this cytokine, were significantly enhanced in a similar manner. The only difference between the two reactions was observed in the expression of IL-6 in the stratum corneum, which appeared more pronounced in the APR. Epidermal and dermal LC did not express the two cytokines. Unlike others (26), we failed to detect IL-1 α or IL-1 β expression; this has been discussed previously (20) and might be explained by differences in antibodies and/or the histological techniques. Freshly isolated LC have been shown to increase their stimulatory capacity for primary T cell-dependent immune responses when cultured with keratinocytes (8). It is not known to what degree this is facilitated by keratinocyte-bound/secreted cytokines (individually or in combination) (7-9). Recently, however, Belsito et al. showed that a variety of cytokines, including IL-6 and IL-2, cause a significant increase in the Ia expression on LC in vitro (27).

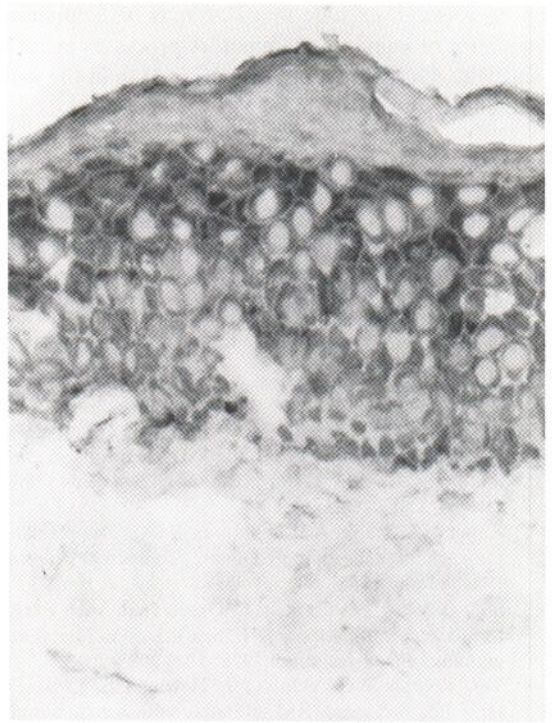
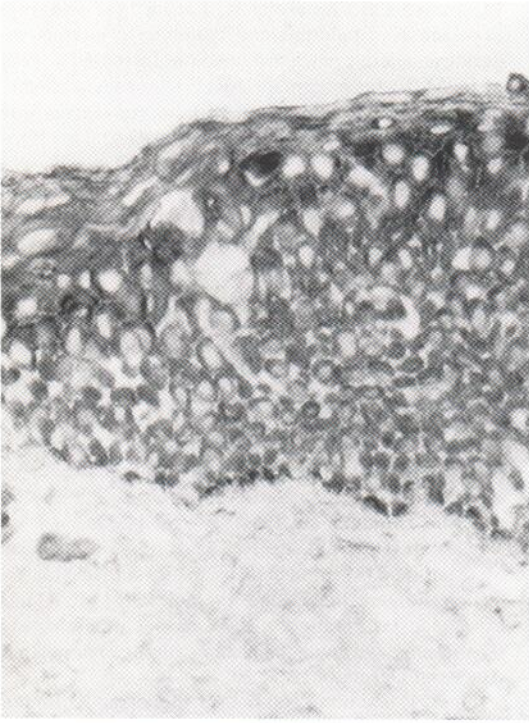


Fig. 3. Interleukin-6 demonstrated in (a) allergic patch test reaction, and (b) irritant patch test reaction by biotin-avidin-peroxidase technique ($\times 400$).



Fig. 4. Negative control. Normal skin. The primary layer antiserum was replaced by preimmune serum. Biotin-avidin-peroxidase technique ($\times 400$).

Measuring supernatants from homogenized epidermal sheets in the thymocyte proliferation assay Larsen et al. showed increased ETAF-activity/cm² epidermis overlying an APR compared with pre-testing values (14), using the same assay these investigators failed to demonstrate alterations in ETAF activity during the IPR (15). The ETAF demonstrated in these studies probably includes the function of several cytokines, including IL-1, IL-6 and TNF α , and this therefore does not allow direct comparison with our findings. However, in the increased stratum corneum expression of IL-6 in APR compared with IPR may nevertheless account for some of the increase in ETAF activity as measured in a thymocyte co-stimulatory assay.

The overall increase in IL-6 expression during the IPR was similar to that observed in APR even though this reaction has been regarded as 'non-immunologic'. Thus, both immunologic (LC-hapten bearing complex) and non-immunologic processes appear to induce IL-6 expression on keratinocytes either directly or via stimulation of other mediators, and enhanced keratinocyte IL-6 expression does not distinguish between these two reactions. This, how-

ever, does not exclude that IL-6 may be a common mediator of immunoinflammatory processes in APR and IPR. Only direct application of IL-6 in the skin will clarify this assumption.

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