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T lymphocytes and altered keratinocytes express interferon-γ and interleukin 6 in lichen planus

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Abstract **Lichen planus is asumed to represent a delayed hypersensitivity reaction, in the course of which cytokines control the proliferation and differentiation of cytotoxic T lymphocytes which attack the epidermis and cause apoptosis of undifferentiated keratinocytes. Since interferon-γ and interleukin 6 are known to be markedly generated in lichen planus, we investigated the cellular localization of these cytokines in affected skin/oral mucosa biopsy specimens using in situ hybridization for interferon-γ and in situ reverse transcription-polymerase chain reaction for interleukin 6 mRNA. In the upper subepithelial connective tissue interferon-γ mRNA was noted within proliferating $CD3^+$ T lymphocytes. In this tissue compartment interleukin 6 mRNA was detected in infiltrating $CD4^+$ and $CD8^+$ T lymphocytes. In the epithelium, expression of interferon-γ mRNA and interleukin 6 mRNA was observed in the basal and suprabasal keratinocytes of altered skin/oral mucosa. In contrast, normal skin did not reveal any interferon-γ or interleukin 6 expression, although a few $CD4^+$ and $CD8^+$ T lymphocytes were noted in the dermis as well as the epidermis. These findings indicate that in lichen planus the proinflammatory cytokines interferon-γ and interleukin 6 are produced not only by activated T lymphocytes but also by altered keratinocytes, and suggest that stimulated keratinocytes may amplify the course of lichen planus.**

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Introduction

Lichen planus (LP), the prototype lichenoid interface dermatitis, is a common disorder of the skin and the oral mucosa with a prevalence of approximately 1% in the general population (Taaffe 1979). LP is histologically characterized by a prominent band-like infiltrate of lymphocytes and histiocytes in the upper subepithelial connective tissue associated with apoptosis of the basal/suprabasal epithelium in the epidermis and/or mucous membrane. Several studies have indicated that LP represents a delayed-type hypersensitivity (Th1) reaction to an as-yet-undetermined neoantigen (for review see: Patterson 1991; Shai and Halevy 1992). Th1 immune responses require the direct participation of both $CD4^+$ T helper and $CD8^+$ cytotoxic T lymphocytes (CTL). Whereas activated T helper cells promote the recruitment, proliferation, and differentiation of inflammatory cells, the functional activity of CTL results in apoptosis of target cells (Ju et al. 1994; Liu et al. 1996).

In LP maturation of helper and CTL from undifferentiated precursors and the complex interplay between infiltrating lymphocytes and basal/suprabasal (undifferentiated) keratinocytes are controlled by cytokines that serve as messengers of cell-to-cell communication (Patterson 1991; Shai and Halevy 1992). Interferon-γ (IFNγ) and interleukin 6 (IL-6) have been shown to be two cytokines strongly generated in LP lesions, but not in those with nonspecific chronic inflammation (Yamamoto and Osaki 1995; Yamamoto et al. 1994). Based on this background, we analyzed the site of IFNγ mRNA and IL-6 mRNA production by in situ hybridization (ISH) and in situ reverse transcription-polymerase chain reaction (in situ RT-PCR), respectively, in biopsy specimens from patients with LP. It was shown that both cytokines are expressed not only in inflammatory T lymphocytes but also in basal/suprabasal keratinocytes.

Materials and methods

Tissue samples

Studies were performed on skin/oral mucosa biopsy specimens from lesional skin of LP patients (n = 15). Normal trunk skin biopsy specimens (n = 3) and surgically removed lymph nodes (n = 3) served as controls. The tissue samples were fixed in 4% formaldehyde and embedded in paraffin. Sections (5- to 10-μm thick) were mounted on silanized slides (Perkin Elmer, Langen, Germany). After deparaffinization, the sections were stained histochemically (H&E, Giemsa). In addition they were analyzed by ISH and in situ RT-PCR as well as by indirect immunofluorescence and immunohistochemistry.

Subcloning of IFNγ cDNA, preparation of IFNγ cRNA and ISH

For preparation of riboprobes, a cDNA fragment derived from the 3'-terminal region of the human IFNγ (position 381–600 of the coding cDNA) was subcloned into pBluescript II KS^+ phagemid (Stratagene, Heidelberg, Germany). The subcloned fragment served as a template for in vitro transcription of digoxigenin-11-dUTP-labeled antisense and sense probes, which were generated using T3 and T7 polymerase according to the manufacturer's instructions (Boehringer, Mannheim, Germany). ISH was performed according to the method described by Breitschopf et al. (1992). Signal was detected using a sheep polyclonal antibody $F(ab)_2$ fragment against digoxigenin conjugated with alkaline phosphatase (1:500; Boehringer). Alkaline phosphatase activity was detected using 5-bromo-4-chloro-3-indolyl phosphate as substrate and nitroblue tetrazolium as coupler (Boehringer). A light microscopic examination followed. Positive cells showed strong cytoplasmic staining around clearly demarcated nuclei.

After signal detection, specimens were either mounted in Aquamount or subjected to double-staining for indirect immunofluorescence for CD3 or for immunohistochemistry for Ki-67. For each tissue specimen, sense riboprobes were applied as controls and proved to be negative.

In situ RT-PCR for IL-6 mRNA

After deparaffinization sections were incubated three times for 5 min in a bath of 0.01 *M* citrate buffer (pH 6) in a microwave oven set on high power (600–700 W). In situ cycling and labeling of the PCR products were then performed as described by Peters et al. (1997). Signals were detected as described for ISH. Specimens were then mounted in Aquamount and viewed under the light microscope. Positive cells showed strong cytoplasmic staining around clearly demarcated nuclei.

Two slides were run as controls for each in situ RT-PCR experiment. Normal lymph node sections served as positive controls. They were run under cycling conditions and reaction mixes as described above. They were proven to exhibit positive signals in the compartments described by Peters et al. (1997). LP sections served as negative controls. They were run without primers, and were proven to be negative.

Indirect immunofluorescence and immunohistochemistry

Antibodies

The primary polyclonal antibody against CD3 and the primary monoclonal antibody against CD8 were obtained from Dako (Hamburg, Germany). The primary monoclonal antibody against CD4 was purchased from Novocastra (Dossenheim, Germany), and the primary monoclonal antibody against the cell proliferation-associated antigen Ki-67 from Dianova (Hamburg, Germany). The primary antibodies were applied at working dilutions of 1:50 (CD3) and 1:20 (CD4, CD8 and Ki-67). To visualize bound primary antibodies, the following detector components were applied as recommended by the manufacturer: a biotin-streptavidin-amplified (B-SA) system containing biotinylated secondary antibody (multilinker; 1:20), alkaline phosphatase-conjugated streptavidin (label; 1:20), fast red (chromogen) and naphthol phosphate (substrate) (Biogenex, Hamburg, Germany) for immunohistochemistry; and FITC-conjugated mouse antirabbit antibody (Dako; 1:20) for immunofluorescence.

Double-staining of the same sections subjected to ISH

Immediately after ISH specimens were double-stained for indirect immunofluorescence for CD3 or for immunohistochemistry for Ki-67. For indirect immunofluorescence sections were incubated for 2 h with the polyclonal primary antibody against CD3. Samples were then incubated with a FITC-conjugated secondary antibody for 1 h. Finally, they were mounted in Fluorescent Mounting Medium (Dako), and analyzed by fluorescence microscopy. For immunohistochemistry sections were incubated three times for 5 min in a bath of 0.01 *M* citrate buffer (pH 6) in a microwave oven set on high power (600–700 W) and incubated for 2 h with the primary antibody against Ki-67. The biotin-streptavidin system was applied to detect and visualize the bound anti-Ki-67 antibody. Samples were mounted in Aquamount and analyzed by light microscopy.

Immunohistochemistry on serial sections from materials subjected to in situ RT-PCR

Sections were pretreated in a microwave oven set on high power (600–700 W) and incubated for 2 h with the primary antibodies against CD4 and CD8 as described above. The biotin-streptavidin system was applied to detect and visualize bound primary antibodies. Samples were mounted in Aquamount and analyzed by light microscopy.

As controls sections from all samples were stained using the above procedures but omitting primary or secondary antibodies.

Semiquantitative evaluation of in situ data

To evaluate the signals resulting from ISH, in situ RT-PCR and immunohistochemistry, the positively stained cells were counted in two high-power visual fields (HPF; × 400) of different compartments of each tissue sample. The degree of positivity was graded semiquantitatively as follows: absence of positivity (fewer than 5 positive cells/HPF), moderate positivity (5 to 20 positive cells/HPF), high positivity (more than 20 positive cells/HPF).

Results

Histomorphology in LP

The lower subepithelial connective tissue revealed moderate numbers of lymphocytes surrounding capillaries and postcapillary venules. The upper subepithelial connective tissue showed intense band-like mononuclear infiltrates mostly composed of lymphocytes attacking the epithelium. The basal epithelial layer of skin/oral mucosa embraced a few apoptotic keratinocytes. In three cases this epithelial layer was partially obscured by an intense mononuclear infiltrate.

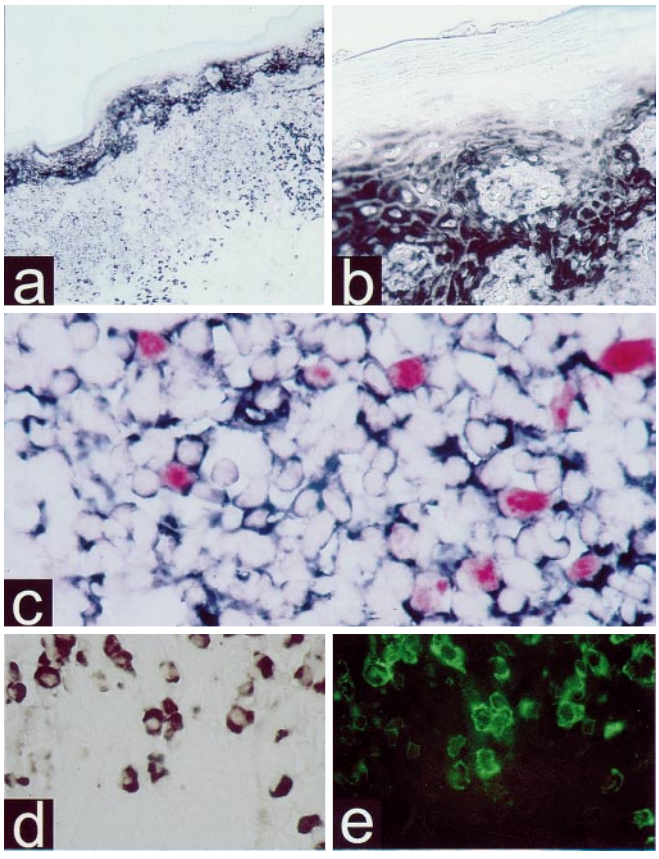
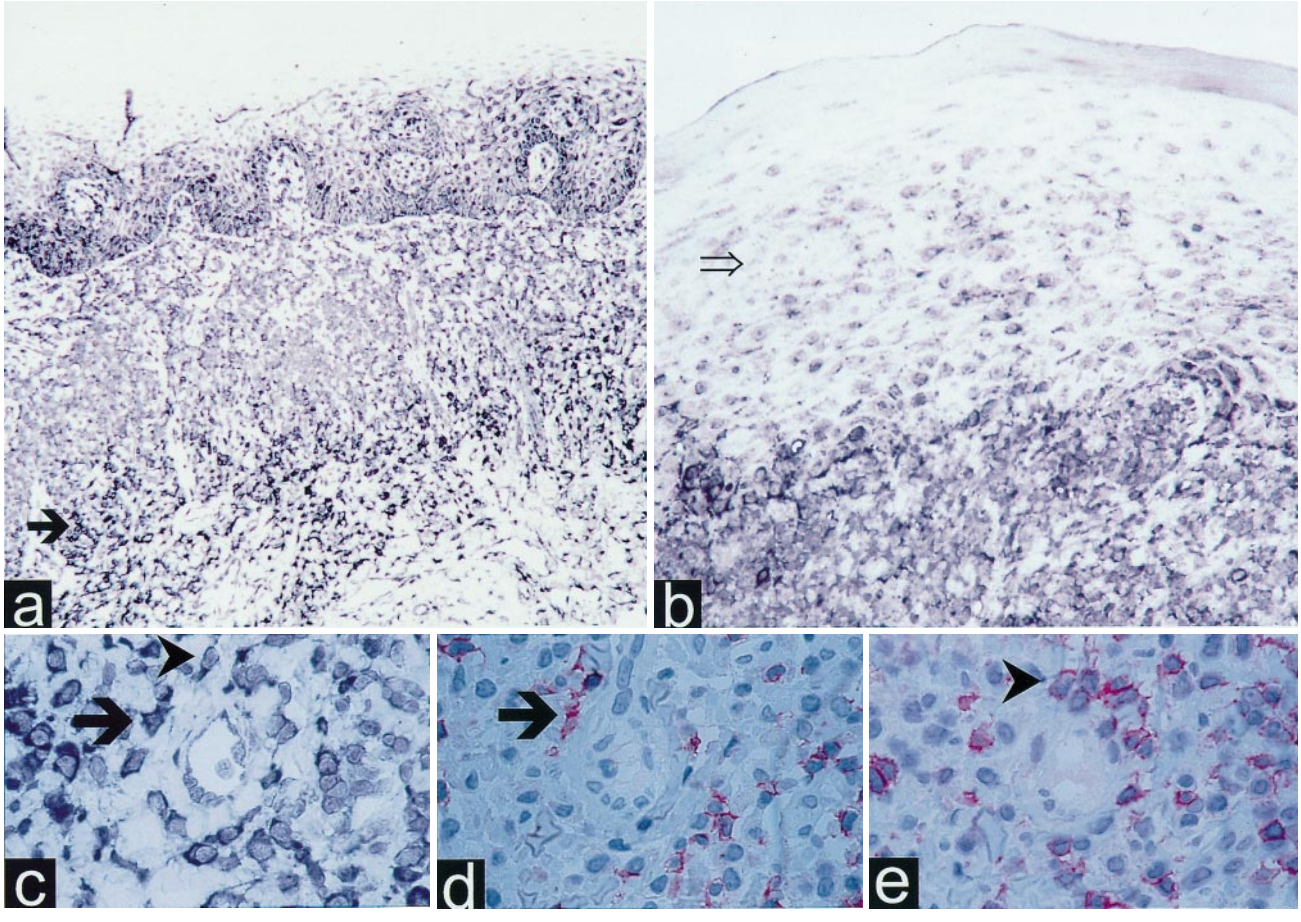


Fig. 1 a–d Expression of IFNγ mRNA in lichen planus. IFNγ mRNA was detected by in situ hybridization combined with immunohistochemistry for Ki-67 or indirect immunofluorescence for CD3 on the same sections. IFNγ is expressed in the epidermis as well as in the upper subepithelial connective tissue (**a–d**; *black cytoplasmic signals*). In the epidermis basal and suprabasal keratinocytes reveal IFNγ mRNA (**b**), whereas superficially localized keratinocytes do not exhibit any IFNγ expression (**b**). In subepithelial connective tissue numerous leukocytes expressing different amounts of IFNγ mRNA can be seen (**a**, **c**, **d**). Leukocytes with the strongest staining for IFNγ mRNA are situated at the inferior border of the lesion (**a**). Some IFNγ-expressing leukocytes exhibit the proliferation-associated antigen Ki-67 (**c**; *red nuclear signals*). All $IFN\gamma^+$ leukocytes (**d**) express the T cell marker CD3 (**e**; *green membranous signals*). Original magnification: **a** × 100; **b** × 630; **c** × 400; **d**, **e** × 250

Fig. 2 a–e Expression of IL-6 mRNA in lichen planus. IL-6 mRNA was detected by in situ RT-PCR followed by immunohistochemistry for T cell subsets CD4 and CD8 in serial sections. IL-6 expression can be seen in the epidermis as well as in the subepithelial connective tissue (**a–c**; *black cytoplasmic signals*). In the epidermis basal and suprabasal keratinocytes reveal IL-6 mRNA (**b**), whereas superficially localized keratinocytes do not exhibit any IL-6 expression (**b**; *open arrow*). In the upper subepithelial connective tissue high numbers of leukocytes with positive staining for IL-6 mRNA can be seen (**a**, **c**). Leukocytes with the strongest staining for IL-6 mRNA are situated at the inferior border of the lesion (**a**; *filled arrow*). The majority of IL-6-expressing leukocytes are $CD4^+$ (**c** and **d**; *filled arrows*) or $CD8^+$ lymphocytes (**c** and **e**; *arrowheads*). Original magnification: **a** × 100; **b** × 200; **c–e** × 400



Expression of IFNγ mRNA in lichen planus

In the upper subepithelial connective tissue of affected skin/oral mucosa moderate to high numbers of leukocytes with strong positive cytoplasmic staining for IFNγ mRNA were noted. Leukocytes with the strongest staining for IFNγ mRNA were mainly situated at the inferior border of lesion (Fig. 1). The combination of ISH and immunohistochemistry revealed that some IFNγ-expressing leukocytes exhibited the proliferation-associated antigen Ki-67 (Fig. 1). Combined ISH and immunofluorescence showed that all IFNγ-expressing leukocytes were $CD3^+$ T lymphocytes (Fig. 1). Surprisingly, not only T lymphocytes but also keratinocytes of the basal and suprabasal cell layers of affected skin/oral mucosa exhibited a strong positive cytoplasmic staining for IFNγ mRNA (Fig. 1).

By applying sense cRNA probes, no positive staining could be registered in tissues from LP (not shown).

Expression of IL-6 mRNA in LP

In the upper subepithelial connective tissue of affected skin/oral mucosa high numbers of leukocytes with positive cytoplasmic staining for IL-6 mRNA were noted. Leukocytes with the strongest positive staining for IL-6 mRNA were mainly situated at the inferior border of the lesion (Fig. 2). In serial sections, the vast majority of IL-6-expressing leukocytes were characterized as $CD4^+$ or $CD8^+$ lymphocytes (Fig. 2). In addition to lymphocytes, few to moderate numbers of macrophages also expressed IL-6 mRNA. Furthermore, basal and suprabasal keratinocytes of skin/oral mucosa exhibited strong PCR signals indicating IL-6 mRNA expression in the epithelium as well (Fig. 2).

PCR-signals could not be detected in control LP sections run without primers (not shown).

Expression of IFNγ mRNA and IL-6 mRNA and immunohistochemistry in normal skin

Expression of IFNγ mRNA or IL-6 mRNA was not observed in any compartment or cells of the normal skin, although a few $CD4^+$ and $CD8^+$ T lymphocytes and macrophages were noted (not shown).

Discussion

In the present study we localized the site of IFNγ mRNA and IL-6 mRNA expression in biopsy specimens of LP.

By ISH moderate to high numbers of $IFN\gamma^+$ inflammatory cells were observed in the upper subepithelial connective tissue. It is difficult to reconcile this finding with recently published in situ data suggesting that IFNγ mRNA is expressed only in a small set of leukocytes infiltrating the subepithelial connective tissue of oral LP lesions (Simark Mattsson et al. 1998). To address this discrepancy, one may speculate that the application of a long IFNγ cRNA probe in the present study (instead of the short synthetic oligonucleotide cDNA probes used by Simark Mattsson et al. 1998) provided more stability in probe-target hybridization and thereby more sensitivity in detection of cells expressing low copies of IFNγ mRNA (Komminoth 1996; Wetmur et al. 1981). To prove the stringency of the hybridization conditions used in the present work we combined ISH with indirect immunofluorescence. Thereby all $IFN\gamma^+$ inflammatory cells were identified as $CD3^+$ T lymphocytes, which represent the main mononuclear cell population infiltrating the subepithelial connective tissue of LP lesions and are known to be capable of expressing IFNγ (Gajewski and Fitsch 1988; Schmitt et al. 1994). Interestingly some $IFN\gamma^+$ lymphocytes exhibited positive immunoreactivity for the cell proliferation-associated antigen Ki-67, a nonhistone nuclear protein present in the G1, S and G2 phases but not in the G0 phase of the cell cycle (Gerdes et al. 1991).

The evidence for IFNγ-producing $CD3^+$ T lymphocytes with proliferative activity confirms the hypothesis that a delayed-type hypersensitivity reaction (Th1 immune response) is responsible for the development of LP, because IFNγ acts as an autocrine growth factor for the proliferation and differentiation of $CD3^+$ T cells involved in Th1 responses (Gajewski and Fitsch 1988; Schmitt et al. 1994). In the course of T cell differentiation, IFNγ may also induce the production of IL-6 mRNA (Faggioli et al. 1997), which we observed in many $CD4^+$ and $CD8^+$ T cells within the subepithelial connective tissue. On the one hand, IL-6 is known to promote the proliferation activity of lymphocytes (Lotz et al. 1988; Noma et al. 1987). On the other hand, IL-6 serves as a cytotoxic differentiation factor, because it upregulates the expression of the genes for pore-forming protein (perforin) and serine protease (granzymes) in CTL (Okada et al. 1988; Takai et al. 1988). Perforin and granzymes are stored in cytolytic granules (Takai et al. 1986) that are released and fuse with the target cell membrane within minutes of target cell recognition by CTL (Cernetti et al. 1987; Cernetti et al. 1988). Following secretion perforin forms holes in the plasma membrane of target cells, which act as conduits for the granzymes to enter and initiate apoptosis of the target cells (Takai et al. 1986; Takai et al. 1988). Evidence for the involvment of perforin and granzymes in the apoptosis of keratinocytes affected by LP has recently been reported by Shimizu et al. (Shimizu et al. 1997), who demonstrated that tissue-infiltrating $CD8^+$ lymphocytes express perforin and granzyme B and induce apoptosis in basal keratinocytes throughout granzyme application into the target cells.

The expression of IFNγ and IL-6 mRNA, however, was not restricted to the inflammatory cells. Transcripts of both cytokines were also found in basal and suprabasal keratinocytes of LP specimens but not in specimens of normal skin. Previously Nickoloff and Naidu (1994) have observed upregulation of both epidermal and dermal IFNγ mRNA production following repeated tape stripping. In

that study the cellular localization of IFNγ mRNA expression was not further investigated. Using a combination of ISH and immunohistochemistry, Howie et al. (1996) have shown for the first time that epidermal keratinocytes from patients with allergic contact dermatitis challenged in vivo with allergen, but not irrelevant antigen, produce IFNγ. It is tempting to speculate that the expression of IFNγ by stimulated keratinocytes augments the clinical course of LP because IFNγ may cause the expression of the lymphocyte-attractant chemokine MIG (monokine induced by IFNγ) in affected keratinocytes and thereby promote the infiltration of the epidermis by lymphocytes (Spandau et al. 1998).

IFNγ can also induce MHC class II and upregulate MHC class I gene expression in keratinocytes, and these events are essential for the recognition of keratinocytes by helper T cells and CTL (Accola et al. 1991; Basham et al. 1984; Farthing and Cruchley 1989). Furthermore, IFNγ is able to upregulate the expression of intercellular adhesion molecule-1 (ICAM-1), which plays an important role in the induction of lymphocyte-dependent damage of keratinocytes (Bennion et al. 1995). Finally, it is known that IFNγ can induce IL-6 production in both oral and skin keratinocytes (Fujisawa et al. 1997; Li et al. 1996) and therefore might be responsible for the IL-6 expression in basal/suprabasal keratinocytes affected by LP. On the one hand, IL-6 may promote the proliferation of keratinocytes (Krueger et al. 1991) resulting in the process of epithelial hyperplasia seen in LP. On the other hand, IL-6 may amplify apoptosis of basal/suprabasal keratinocytes, because it induces the expression of "tissue" transglutaminase (tTG) in keratinocytes (Suto et al. 1993). tTG is a Ca^{2+}-dependent enzyme which crosslinks cellular proteins during the terminal steps of apoptosis (Piacentini et al. 1991). Whereas tTG is undetectable in normal epidermis, this enzyme is markedly expressed in apoptotic keratinocytes affected by LP (Bianchi et al. 1994).

Based on mRNA expression profiles, our findings suggest that LP represents a delayed-type hypersensitivity reaction triggered and amplified by cytokines such as IFNγ and IL-6, the expression of which is not only due to tissue-infiltrating lymphocytes but also to stimulated keratinocytes. Further studies are needed to demonstrate whether IFNγ and IL-6 transcripts are translated into functionally active proteins promoting the trafficking of mononuclear cells to the interface area of LP lesions and augmenting apoptosis of basal keratinocytes.

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