

Association of increased CD4⁺ T-cell infiltration with increased IL-16 gene expression in atopic dermatitis

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Background: The mechanisms involved in the initiation and the maintenance of skin inflammation in atopic dermatitis (AD) are poorly understood. Previous studies have demonstrated increased numbers of infiltrating CD4⁺ T cells in acute lesions compared with normal control skin. IL-16 is a cytokine that has selective chemotactic activity for CD4⁺ cells.

Objective: We sought to examine whether IL-16 expression might be upregulated in acute versus chronic AD.

Methods: We investigated the expression of IL-16 mRNA in skin biopsy specimens from acute and chronic skin lesions, as well as from the uninvolved skin of patients with AD and normal skin. Cryostat sections from 4% paraformaldehyde-fixed skin biopsy specimens were processed for in situ hybridization by using cRNA coding for IL-16 mRNA. Numbers of infiltrating CD4⁺ and CD8⁺ cells were also determined by immunocytochemistry.

Results: There were positive signals for IL-16 mRNA both in the basal layer of the epidermis and in the dermis of AD skin biopsy specimens from all subjects studied. The numbers of epidermal and dermal IL-16 mRNA⁺ cells were significantly increased in acute skin lesions compared with chronic ($P < .01$) and uninvolved ($P < .001$) skin lesions and compared with normal skin ($P < .001$). The number of CD4⁺ cells was significantly increased in acute skin lesions compared with chronic ($P < .01$) skin lesions and uninvolved skin ($P < .01$) and compared with normal skin ($P < .01$). Significant correlations were found between the numbers of CD4⁺ cells and the numbers of epidermal ($r = 0.82$, $P < .001$) and dermal ($r = 0.71$, $P < .001$) IL-16 mRNA⁺ cells.

Conclusion: The results demonstrate that upregulation of IL-16 mRNA expression in acute AD is associated with increased numbers of CD4⁺ cells, suggesting that IL-16 may play a role in the initiation of skin inflammation, presumably through recruitment of CD4⁺ cells. (*J Allergy Clin Immunol* 1998;102:645-50.)

Key words: Atopic dermatitis, IL-16, allergic inflammation, chemotaxis

Atopic dermatitis (AD) is a chronic skin disease that affects over 10% of children and is manifested by

Abbreviation used

AD: Atopic dermatitis

eczematous skin lesions, which may cause considerable morbidity.^{1,2} Studies of skin biopsy specimens from patients with AD reveal marked tissue inflammation. The histologic features of AD depend on the acuity and the duration of the disease. The inflammatory infiltrate within acute skin lesions is characterized by a predominance of CD4⁺ T cells, which express CD25 and HLA-DR on their cell surface, whereas macrophages dominate the dermal mononuclear cell infiltrate in chronic AD lesions.^{3,4} CD4⁺ T cells are a major cellular source of cytokines, which are critical to IgE synthesis and eosinophil accumulation in skin lesions. We and others have shown that acute AD skin lesions are associated with an increase in the numbers of cells expressing mRNA for IL-4, IL-5, IL-10, and IL-13, but not IFN- γ , compared with uninvolved skin of subjects with AD and normal subjects.⁵⁻⁷ These findings suggest a predominant role of the so-called T_{H2}-type cytokines at the site of disease. Clinical improvement associated with a reduction in the numbers of activated skin-infiltrating T cells after therapy with cyclosporine A further supports the view that AD is based on a T cell-mediated skin inflammation.⁸⁻¹⁰ However, despite considerable advances in our understanding of the pathogenesis of AD, the mechanisms involved in the initiation and maintenance of skin inflammation in AD are yet incompletely understood.

Because of the regulatory role of CD4⁺ T cells in the pathogenesis of atopic dermatitis, the study of the mechanisms responsible for the recruitment of these cells in skin lesions is of primary importance. This process likely involves leukocyte-endothelial interactions through adhesion molecules and local generation of chemotactic agents that direct cell migration from the blood vessel into the dermal and epidermal tissues. The CD4⁺ cell chemoattractant IL-16 has biologic properties that may be pathologically relevant to AD. Apart from other cytokines and chemokines, IL-16 has the ability to selectively attract CD4⁺ cells, including CD4⁺ T cells and CD4-bearing eosinophils, in vitro.^{11,12} It is a potent chemotactic agent for CD4⁺ T cells with half the maximal effective dose of recombinant protein in the 10⁻¹¹ range.¹³ Previous studies have shown that IL-16 is produced by a variety of inflammatory cells, including lym-

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Supported by the Medical Research Council of Canada.

Received for publication Mar 30, 1998; revised May 27, 1998; accepted for publication May 27, 1998.

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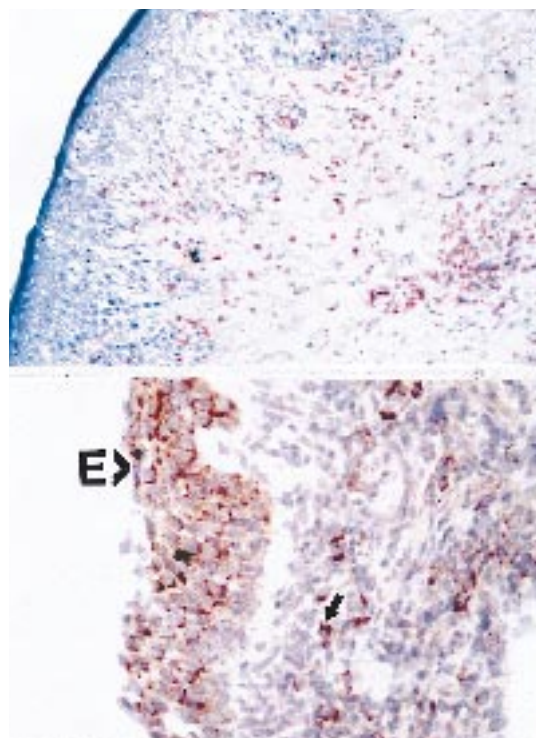


FIG 1. CD4 immunoreactivity and IL-16 mRNA expression in acute AD lesions. CD4⁺ cells were localized predominantly in dermis of acute lesions as shown by immunocytochemistry (top). In situ hybridization showed IL-16 mRNA⁺ cells mainly in epidermis (E) and, to a lesser extent, in dermis (arrow).

phocytes, eosinophils,¹⁴ mast cells,¹⁵ and airway epithelial cells.¹⁶ Moreover, IL-16 has been shown to be an important mediator of inflammatory responses in the mucosal pathology of asthma.¹⁷⁻¹⁸ In addition, we have previously shown that inhibition of antigen-induced late nasal response after topical glucocorticoid treatment was associated with inhibition of IL-16 expression *in vivo*.¹⁹

The current study was undertaken to investigate the expression of IL-16 mRNA in AD lesions and its relation to CD4⁺ cell infiltration. We hypothesized that IL-16 expression is upregulated in acute AD skin lesions and that IL-16 expression correlates with CD4⁺ cell infiltration in skin tissues. Because the numbers of infiltrating CD4⁺ cells are likely to be higher in acute lesions versus chronic lesions and uninvolved skin, we investigated and compared IL-16 mRNA expression in these different types of AD skin lesions.

METHODS

Skin biopsy specimens

A total of 17 punch skin biopsy specimens were obtained from 8 subjects, all of whom met the diagnostic criteria for AD²⁰; 6 specimens from acute erythematous AD lesions of less than 3 days' onset, 6 specimens from chronic lichenified AD lesions of greater than 2 weeks' duration, and 5 specimens from uninvolved skin.⁵ None of these subjects had other skin conditions, and none had previously received oral steroids. Topical steroids were withheld for at least 2 weeks before biopsies were performed. Control skin biopsy

TABLE I. CD4 and CD8 immunoreactivity in skin biopsy specimens

	Acute AD	Chronic AD	Uninvolved	Normal
CD4 ⁺ cell counts*	49.2 ± 21.8†‡	23.6 ± 9.5	7.0 ± 2.5	4.6 ± 1.4
CD8 ⁺ cell counts	11.5 ± 3.4§	14.0 ± 4.2‡	3.2 ± 1.3	1.4 ± 1.1

*Data are expressed as number of positive cells per field.

†*P* < .01 compared with chronic AD skin.

‡*P* < .001 compared with uninvolved and normal skin.

§*P* < .01 compared with uninvolved and normal skin.

specimens were also obtained from nonatopic normal volunteers (*n* = 5). Informed consent was obtained from all subjects before all procedures.

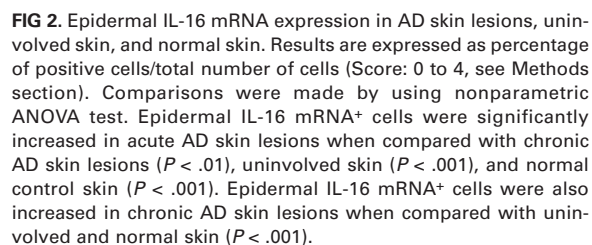
Skin biopsy specimens were fixed immediately in freshly prepared 4% paraformaldehyde/PBS solution for 2 hours, washed in 15% sucrose/PBS 3 times, embedded in OCT compound (Tissue-Tek, Miles Inc, Elkhart, Ind), and snap-frozen in isopentane cooled in liquid nitrogen. Cryostat sections 5 mm thick were cut on 0.1% poly-L-lysine-coated slides, air-dried overnight at 37°C, and stored at -80°C until used.

Immunocytochemistry

Anti-human CD4 and CD8 mAbs were obtained from Dako Canada Inc (Mississauga, Ontario, Canada). Immunoreactivity was assessed by using the alkaline phosphatase anti-alkaline phosphatase technique as previously described²¹ and by using an anti-CD4 (1:10 dilution) or an anti-CD8 (1:10 dilution) mAb. The reaction was visualized with Fast Red alkaline-phosphatase substrate. As controls, sections were processed in the absence of the primary antibody.

In situ hybridization

Nonradiolabelled IL-16-specific cRNA probe was produced from a 379 bp fragment of the human IL-16 cDNA corresponding to the 436 to 815 bp fragments of the originally published sequence.¹² The DNA template was inserted into an SPT19 vector and linearized with the appropriate enzymes before transcription. Linearized templates were labeled with digoxigenin-11-UTP by transcription with the RNA polymerases SP6 or T7 to generate antisense or sense probes, respectively. In situ hybridization was performed as previously described.²² Cryostat sections were first permeabilized by immersion in 0.3% Triton X-100 in PBS followed by exposure to proteinase K solution (1 µg/mL in 20 mmol/L Tris-HCl and 1 mmol/L EDTA, pH 7.2) for 30 minutes at 37°C. Concentrations of digoxigenin-labeled IL-16 probe were adjusted to 250 ng per section in hybridization buffer, which contains 50% formamide, 5× Denhart's solution, 5× standard saline citrate buffer, and 500 µg/mL denatured salmon sperm DNA. The sections were incubated with the hybridization mixture overnight at 40°C. Posthybridization washings were performed with standard saline citrate solutions, with increasing stringency at 45°C followed by RNase treatment. After a brief wash in Tris-buffered saline, sections were incubated with 3% BSA-Tris buffered saline for 10 minutes to reduce nonspecific background and then incubated with 1:1000 dilution of digoxigenin-alkaline-phosphatase conjugate. Color development was achieved with nitro-blue tetrazolium salt in equalization buffer. Sections were counterstained with hematoxylin. The corresponding sense probe and RNase pretreatment were used as controls, and in all cases this gave negative results.



Slides were encoded, and evaluations of immunoreactivity and in situ hybridization signals were performed by a blinded assessor. Two slides (sections) were processed for each in situ hybridization analysis. IL-16 mRNA⁺ cells in the epidermis were counted by optical analysis and expressed as a semiquantitative score on the basis of the percentage of epithelium showing positive signal/total epithelium (0: no staining; 0.5: <12.5%; 1: 12.5% to 25%; 1.5: 25% to 37.5%; 2: 37.5% to 50%; 2.5: 50% to 62.5%; 3: 62.5% to 75%; 3.5: 75% to 87.5%; and 4: 87.5% to 100%). The epidermal IL-16 mRNA score was reported as the average of the individual scores obtained for each of the 2 sections. In the dermis, IL-16 mRNA⁺ cells, as well as CD4⁺ cells and CD8⁺ cells, were counted with an eyepiece graticule at 100 $\times$ magnification. The results were expressed as mean counts $\pm$ standard deviation (mean $\pm$ SD) per field. The number of fields per section counted was 2 to 6 depending on the size of the biopsy specimen and the pattern of alignment of the grid covering an intact area of epithelial and subepithelial tissue. The within-observer coefficient of variation for repeated counts was less than 5%.

FIG 3. Dermal IL-16 mRNA expression in AD skin lesions, uninvolved skin, and normal skin. Results are expressed as number of positive cells per field. Dermal IL-16 mRNA⁺ cells were significantly increased in acute AD skin lesions when compared with chronic AD skin lesions ($P < .001$), uninvolved skin ($P < .001$), and normal control skin ($P < .001$).

Immunocytochemical staining performed on the skin sections showed significantly increased numbers of CD4⁺ cells in the acute lesions compared with chronic lesions ($P < .01$), uninvolved skin ($P < .001$), and normal skin ($P < .001$) (Table I). In acute lesions CD4⁺ cells localized predominantly in the dermis (Fig 1). Numbers of CD4⁺ cells were higher in chronic lesions compared with uninvolved and normal skin, but this did not reach statistical significance. CD8⁺ cell counts were higher in chronic lesions compared with uninvolved skin from subjects with AD ($P < .001$) and normal skin ($P < .001$) (Table I). The numbers of CD8⁺ cells were slightly higher, although not significantly so, in chronic lesions compared with acute skin lesions.

By using the *in situ* hybridization technique, all biopsy specimens from acute and chronic skin lesions showed positive signals for IL-16 mRNA (Figs 2 and 3). Biopsy specimens from uninvolved skin obtained from subjects with AD and normal skin biopsy specimens demonstrated low numbers of IL-16 mRNA⁺ cells. IL-16 mRNA⁺ cells were identified both in the epidermal and in the dermal layers (Fig 1). Within the dermis, IL-16 mRNA⁺ cells were generally located around the blood vessels. No signal was observed from either the sections hybridized with the sense probe or sections pretreated with RNase, confirming the specificity of the IL-16 probe and signal.

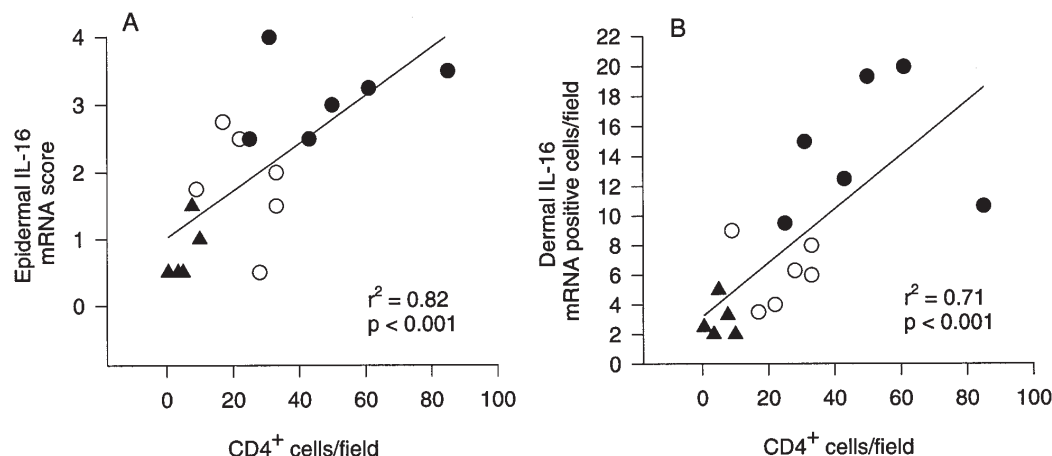


FIG 4. Relationships between IL-16 mRNA expression and CD4⁺ cell infiltration in AD skin lesions. There were significant correlations between epidermal IL-16 mRNA score (A), number of dermal IL-16 mRNA⁺ cells (B), and CD4⁺ cell infiltration. Triangles, Uninvolved skin; open circles, chronic lesions; closed circles, acute lesions.

Within the epithelium, the numbers of IL-16 mRNA⁺ cells were significantly higher in acute lesions compared with chronic lesions ($P < .01$), uninvolved skin ($P < .001$), and normal skin ($P < .001$) (Fig 2). The numbers of epidermal IL-16 mRNA⁺ cells in chronic skin lesions were significantly greater compared with uninvolved ($P < .01$) and normal skin ($P < .001$).

The numbers of dermal IL-16 mRNA⁺ cells were significantly higher in acute lesions compared with chronic lesions ($P < .001$), uninvolved skin ($P < .001$), and normal skin ($P < .001$) (Fig 3). The numbers of dermal IL-16 mRNA⁺ cells in chronic skin lesions were significantly greater compared with normal skin ($P < .01$).

In biopsy specimens obtained from subjects with AD, significant correlations were found between the numbers of CD4⁺ cells and the numbers of epidermal IL-16 mRNA⁺ cells ($r = 0.82$, $P < .001$) and the numbers of dermal IL-16 mRNA⁺ cells ($r = 0.71$, $P < .001$) (Fig 4).

DISCUSSION

We and others have reported that acute skin lesions in AD are characterized by a lymphocytic infiltrate that consists predominantly of cells bearing CD4 and CD45RO surface antigens.^{1,23} However, the precise mechanisms responsible for the recruitment of CD4⁺ cells in inflamed skin lesions remain unclear. To investigate the potential role of a novel cytokine, IL-16, which has specific chemotactic activity for CD4⁺ cells, we examined and compared the expression of IL-16 mRNA in skin biopsy specimens from patients with AD and from normal control subjects. We have demonstrated the presence of IL-16 mRNA⁺ cells in the skin lesions of subjects with AD and predominance of these cells in acute lesions compared with chronic lesions or uninvolved skin. Both acute and chronic AD lesions were associated with an increased expression of IL-16 mRNA compared with skin biopsy specimens from normal control subjects. Consistent with our previous observations, acute skin lesions are character-

ized by increased numbers of skin-infiltrating CD4⁺ cells.¹ In addition, we found evidence of a correlation between the numbers of infiltrating CD4⁺ cells and the numbers of IL-16 mRNA⁺ cells.

The highest number of IL-16 mRNA⁺ cells was observed in the acute lesions, and this number was significantly greater than that seen in chronic skin lesions, suggesting that IL-16 may play a role in the initiation of acute skin inflammation. The mechanisms by which IL-16 participates in the acute phase of skin inflammation in AD remain to be elucidated. IL-16 is mainly known as a potent chemoattractant factor for CD4⁺ cells in vitro. Whether IL-16 favors migration of a specific subset of CD4⁺ cells is not known. Although the contribution of IL-16 in mediating CD4⁺ cell migration in vivo remains to be fully established, our data show that increased expression of IL-16 is associated with increased numbers of infiltrating CD4⁺ cells in acute lesions. Interestingly, dermal IL-16 mRNA⁺ cells were localized to the perivascular infiltrate, suggesting that these cells may participate to the chemotactic gradient. Furthermore, the correlation between IL-16 mRNA expression and CD4⁺ cell counts supports, but does not prove, its potential functional significance in vivo. Because T cells are a known source of IL-16 mRNA, it can be argued that the association observed between IL-16 mRNA⁺ cells in the skin and the numbers of CD4⁺ cells could be attributed to the accumulation of IL-16-producing CD4⁺ cells. However, the association between IL-16 mRNA expression in the epidermal compartment and the numbers of infiltrating CD4⁺ cells rather suggest that these two phenomena are linked. It is therefore plausible that local mRNA expression of IL-16 both by keratinocytes and by resident/infiltrating inflammatory cells may, at least partly, contribute to the release of IL-16 and recruitment of CD4⁺ cells.

In the current experiments topical steroids were withheld for at least 2 weeks before skin biopsies were performed. We cannot exclude the possibility that previous

glucocorticoid treatment may have, in part, contributed to the lower expression of IL-16 mRNA and the associated lower numbers of CD4⁺ cells in chronic AD lesions compared with acute lesions. In this regard we have previously shown that topical glucocorticoid treatment prevented allergen-induced IL-16 expression and CD4⁺ cell infiltration in late nasal response in vivo.¹⁹ However, the idea that the differential cell profiles in acute versus chronic AD may be associated with differential expression of cytokines is not without precedent. In similar types of experiments with an identical study design, we have shown that chronic AD lesions are associated with a markedly elevated expression of IL-5 and GM-CSF mRNAs and the presence of elevated numbers of eosinophils and monocyte-macrophages compared with acute lesions.^{5,24} Studies in the nose, airways, and sinuses of allergic individuals have provided convincing evidence of the efficacy of the glucocorticoids in attenuating tissue eosinophilia and IL-5 gene expression.²⁵ Therefore although previous use of glucocorticoids could have affected our results in chronic AD lesions, we feel that this is unlikely.

The numbers of epidermal and dermal cells expressing mRNA for IL-16 were significantly lower in uninvolved skin compared with both acute and chronic skin lesions but did not differ from those observed in normal skin. Thus enhanced synthesis of IL-16 does not reflect an atopic disposition of the skin. These results are consistent with our previous observations that epithelial IL-16 expression is upregulated in bronchial biopsy specimens obtained from atopic asthmatic subjects, but not from atopic nonasthmatic subjects, compared with normal control subjects.¹⁷ In this study low constitutive IL-16 gene expression was found in skin biopsy specimens from normal volunteers. Constitutively produced IL-16 may play a role in cutaneous homeostatic responses and immune surveillance under normal conditions and may therefore explain the presence of small numbers of CD4⁺ cells identified in normal skin.

Several cell types have been found to express IL-16 mRNA in vitro, including lymphocytes (CD4⁺ and CD8⁺), eosinophils,¹⁴ airway epithelial cells,¹⁶ and mast cell lines.¹⁵ In this study IL-16 mRNA⁺ cells were identified both in the epidermis and the dermis. Although colocalization studies were not performed, the positive IL-16 mRNA hybridization signal in the epidermis (as shown in the microphotograph) was mainly associated with keratinocytes. Keratinocytes have become increasingly recognized for their ability to modulate immune reactions in the skin partly through their capacity to synthesize and release an array of mediators, including growth factors, eicosanoids, and cytokines. IL-16 thus joins the list of cytokine mRNAs known to be expressed by keratinocytes, including IL-1 α and β , IL-8, IL-10, GM-CSF, IL-12, and RANTES.²⁶⁻³⁰ Although some of these cytokines are constitutively produced, they are often released in increased amounts when epidermal cells are exposed to various stimuli. The epithelial cellular source of IL-16 in vivo has been demonstrated in

other types of tissues, such as the airways and the nose.^{17,31} The cellular source of dermal IL-16⁺ cells remains to be fully investigated. The morphology of the dermal IL-16⁺ cells is consistent with T lymphocytes, although the contribution of other inflammatory cells has not been excluded. In a recent study on bronchial biopsy specimens from subjects with allergic asthma, we have shown that CD3⁺ T cells were the predominant nonepithelial IL-16-producing cells.³²

In summary, we have demonstrated enhanced expression of mRNA for IL-16 in acute and, to a lesser extent, chronic skin AD lesions. Increased expression of IL-16 mRNA was associated with increased numbers of skin-infiltrating CD4⁺ cells, suggesting that IL-16 may play a role in the pathogenesis of AD and in mediating the infiltration of CD4⁺ cells in AD. Further studies of the regulation of IL-16 synthesis and release are indicated to determine whether new therapeutic approaches targeted at this cytokine will have beneficial effects in dermatologic settings.

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