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SEB-induced T cell apoptosis in atopic patients – correlation to clinical status and skin colonization by *Staphylococcus aureus*

Authors' Contribution:

- A** Study Design
- B** Data Collection
- C** Statistical Analysis
- D** Data Interpretation
- E** Manuscript Preparation
- F** Literature Search
- G** Funds Collection

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Summary

Introduction:

We asked whether in atopic dermatitis (AD) increased T cell apoptosis in staphylococcal enterotoxin B (SEB)-activated cultures of peripheral blood mononuclear cells (PBMCs) is characteristic of the exacerbation of the disease or connected with skin colonization by *Staphylococcus aureus*.

Materials and Methods:

The clinical status of the patients was evaluated using the SCORAD index. The number of bacteria colonizing patients' skin lesions was determined by the cfu method. Mononuclear cells isolated from peripheral blood were stimulated by SEB and the apoptosis of CD3⁺ cells in culture was determined by flow cytometry using the monoclonal antibody APO2.7. The cytokine production in the culture supernatants was determined by ELISA and Cytometric Bead Array kits.

Results:

T cell apoptosis was increased, while the production of interferon (IFN)- γ was reduced in cultures of PBMCs of AD patients during exacerbation. The proportion of CD3⁺APO2.7⁺ cells positively correlated with the density of *S. aureus* recovered from skin lesions, but not with SCORAD index. By contrast, SCORAD index, but not *S. aureus* density, negatively correlated with IFN- γ production. Furthermore it was found that the presence of *S. aureus* on uninvolved skin distinguishes a group of severe cases with high serum IgE level, increased T cell apoptosis, and reduced production of tumor necrosis factor α in SEB-stimulated cultures.

Conclusions:

Among AD patients the increased activation-induced T cell apoptosis observed in SEB-stimulated cultures is related to skin colonization by *S. aureus*. The presence of bacteria on uninvolved skin is a feature of a distinct group of AD patients.

Key word:

atopic dermatitis • apoptosis • T cells • *Staphylococcus aureus* colonization

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INTRODUCTION

Atopic dermatitis (AD) is a chronic inflammatory skin disease with complex immune dysregulation^{2, 4}. Besides genetic background (familial occurrence) and environmental and food allergens, various other factors are believed to be involved in the pathogenesis of the disease^{2, 27}. Among these local skin infections, especially skin colonization/infection by *Staphylococcus aureus* has received much attention³⁰. Colonization of skin lesions by *S. aureus* has been observed in 90% of AD patients^{18, 34} and available data strongly suggest that the presence of the bacteria exacerbate AD and perpetuate the chronicity of this skin disease¹⁷. Staphylococcal exotoxins were isolated from the skin of AD patients¹⁸ and were shown to induce skin inflammation in a patch-tests²⁹. Furthermore, IgE specific to staphylococcal exotoxins are present in the serum of AD patients^{1, 5, 23}. Finally, staphylococcal exotoxins may play a role in the dysregulated apoptosis recently described in AD patients^{4, 25, 31}. The intralesional microenvironment in the skin of AD patients is responsible for increased eosinophil and T cell survival, the latter despite high CD95 and Fas ligand expression on T cells. This contrasts with the increased activation-induced apoptosis of T cells (AICD) isolated from the periphery^{28, 36}. Interestingly, staphylococcal exotoxins inhibit apoptosis of eosinophils with an effectiveness comparable to interleukin (IL)-3³³, adding new meaning to the exacerbation of the disease observed during skin colonization by *S. aureus*. Sohn et al.²⁸ reported that staphylococcal enterotoxin B (SEB) might upregulate CD95 expression on peripheral blood T cells and is responsible for AICD of these Fas-expressing cells. Furthermore, Yoshino et al.³⁶ have shown that SEB-induced AICD is more pronounced during an exacerbation of the disease and is connected with the reduced T cell proliferation in response to SEB and T cell mitogens. Although an exacerbation of AD and skin colonization by *S. aureus* are usually connected, it seemed reasonable for us to ask whether reduced T cell responses and AICD are primarily related to the clinical status or to the skin colonization by *S. aureus*. Therefore, we analyzed T cell apoptosis and cytokine production by peripheral blood mononuclear cells (PBMCs) in AD patients in relation to the severity of clinical symptoms and also to *S. aureus* skin colonization. Here we report that reduced T cell response is correlated to clinical condition, while AICD reflects colonization of the skin by *S. aureus*. Furthermore, we present data suggesting that the presence of *S. aureus* on uninvolved skin is characteristic of a distinct group of AD patients.

MATERIALS AND METHODS

Patients and controls

Thirty-seven patients suffering from AD were enrolled for the study. All patients suffered from chronic AD that fulfilled the criteria of Hanifin and Rajka¹². Their characteristics are shown in Table 1. Severity of the disease of all patients was assessed by the same physician using the SCORAD index⁹. Most of the patients (70%) had an elevation in their total serum IgE concentrations (mean 1896±756 kU/l), with 10 patients having a marked elevation over 1000 kU/l. Patients included in the study did not receive systemic corticosteroid therapy and for at least 1 week before examination were not treated with topical corticosteroids nor received oral antihistamine or drugs that block the release of mediators from activated mast cells. Skin colonization by *S. aureus* (see below) was determined in all the patients enrolled; other measurements (cell apoptosis and cytokine production) for various reasons could be completed in only 29 patients (Table 1).

Table 1. Characteristics of patients and controls

	All patients (mean±SE)	PBMC patients ^a (mean±SE)	Controls (mean±SE)
Number	37	29	11
Age, years	20.3±1.9	22.1±1.5	39.4±2.4
Sex (F/M)	17/20	15/14	6/5
Duration of AD, years	12.6±1.5	13.8±2.1	–
Total serum IgE (kU/l)	1896±756	1664±740	42.6±8.3
SCORAD index (points)	50.3±2.5	49.8±2.9	–
Serum ECP (µg/l) ^b	36.8±4.7	32.0±3.8	n.d.

^a Characteristic of 29 patients whose blood was used for PBMC isolation and culture, ^b eosinophil cationic protein, n.d. – not done.

The control group consisted of 11 patients undergoing routine blood examination prior to various elective medical procedures. This group comprised 5 males and 6 females (mean age 39.4±2.4 years) with no personal or family history of AD, allergic rhinitis, or asthma. Total serum IgE concentration of the controls averaged 42.6±8.3 kU/l, and in no case exceeded 100 kU/l.

All patients and control subjects gave informed consent before the examination and the study was approved by the local ethics committee.

Isolation procedure of S. aureus strains from patients' skin

Specimens for bacteriological examination were taken from 4 different regions of eczematous skin

and from 2 unaffected skin areas using a modified Williamson and Kligman method³⁴. Briefly, sterile glass cylinders (2.5 cm wide) were placed firmly on the surface of the skin and 2 ml of sterile 0.9% NaCl solution was applied. The surface of the skin was then rubbed with a glass rod and the fluid was aspirated. These washings were subsequently serially diluted and poured over 5% blood agar plates (Oxoid, UK) and incubated for 24 h at 37°C. *S. aureus* colonies were identified by testing for coagulase activity and confirmed by a Slidex Staph kit (BioMerieux S.A., France). The density of skin colonization was determined by counting the number of colony-forming units (cfu)/cm² and expressed as a logarithm.

Peripheral blood mononuclear cells

PBMCs were isolated from 10 ml of heparinized blood of 29 patients and of all 11 controls by a standard Ficoll-Paque (Pharmacia Biotech, Sweden) density gradient. The PBMCs were washed with RPMI medium and adjusted to 1×10^6 /ml in RPMI 1640 culture medium supplemented with L-glutamine and 10% fetal calf serum (all reagents from Gibco, USA).

Cell cultures

PBMCs (0.2 ml aliquots, 1.0×10^6 /ml) were seeded in triplicate into 96-well culture plates and incubated at 37°C in a humidified atmosphere containing 5% CO₂ in the presence of 0.1 µg/ml of SEB (Sigma, USA; 24 h for measurement of AICD). Parallel cultures activated with SEB or phytohemagglutinin (PHA; 2.5 µg/ml; Sigma) or pokeweed mitogen (PWM; Invitrogen, UK; final dilution 1/100) were cultured for 48–96 h and served for the determination of cytokine production.

Evaluation of T cell apoptosis

T cell apoptosis was detected by intracellular staining of the 38-kDa mitochondrial membrane protein (7A6 antigen)^{14, 37} as described by Yoshino et al.³⁶. The cells, cultured for 24 h, were harvested, washed once in phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide, and stained with mouse anti-human CD3 FITC-conjugated monoclonal antibody (Becton Dickinson, USA) at 4°C for 20 min. Thereafter, the cells were washed once with FACS medium and incubated for 30 min with 100 µl of FACS permeabilizing solution (Becton Dickinson) at room temperature. After washing they were incubated with APO2.7 phycoerythrin-conjugated monoclonal antibody (Immunotech, Coulter Co., France) or the isotype control at 4°C for 30 min. After final washing, the samples were analyzed using a FACSscan

flow cytometer and CellQuest software (Becton Dickinson Immunocytometry Systems, USA). The expression of APO2.7 was determined within the population of CD3⁺ cells with the exclusion of dead cells based on light scatter.

Cytokine production

Interferon (IFN)-γ production was measured in the supernatants of 96-h cultures stimulated with SEB, PHA, and PWM using OptEIA Human IFN-γ Set kits (Becton Dickinson Biosciences Pharmingen, USA) according to the procedure recommended by the manufacturer. The assay was sensitive to 10 pg/ml. SEB-activated 48-h culture supernatants were analyzed for interleukin (IL)-2, IL-4, IL-5, IL-10, and tumor necrosis factor (TNF)-α using Human Th1/Th2 Cytokine Cytometric Bead Array kits (Becton Dickinson Biosciences Pharmingen, USA) according to the manufacturer's instructions. The assay sensitivity was 15–10 pg/ml. The time of culture for the measurement of AICD, IFN-γ, and other cytokines was that chosen as suggested by Yoshino et al.³⁶.

Statistical analysis

Differences in the data sets were measured with the Student's *t*-test for independent means. Correlation coefficients were determined by Spearman's rank correlation. Statistical significance was considered achieved when $p < 0.05$.

RESULTS

Skin colonization by S. aureus correlates with the severity of AD

S. aureus was washed from the skin of 34 patients (86.5%), and the mean log of viable bacteria washed from skin lesions was 6.53 ± 0.29 /cm². In 23 patients, bacteria could also be washed from areas of uninvolved skin (mean log density: 5.59 ± 0.33 /cm²). The clinical score of patients ranged 21–84 (mean: 50.3 ± 2.5) and the density of bacterial colonization (infection) was higher among patients with higher index. The density of bacteria washed from the skin lesions also correlated with the clinical status expressed by the SCORAD index (Fig. 1).

For further analysis the patients were arbitrarily divided into groups according to the severity of symptoms and to the density of skin colonization by *S. aureus*. Based on SCORAD index, the patients were divided into mild (21–48), moderate (49–60), and severe (61–84) cases, and according to the density of bacte-

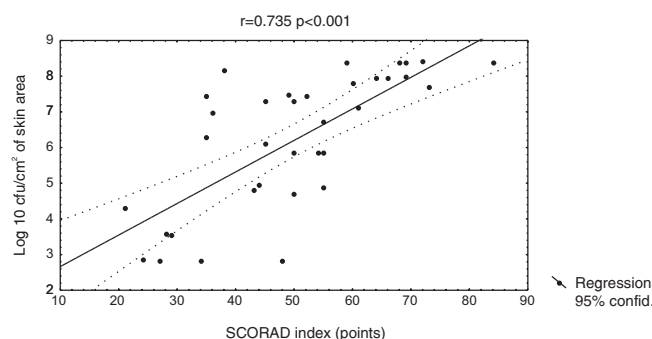


Figure 1. Correlation of patient clinical status and *S. aureus* skin colonization. Clinical status is expressed as the SCORAD index and skin colonization by *S. aureus* as $\log_{10}$ of the number of viable bacteria washed from 1 cm² of patient skin (mean of samples taken from 4 different eczematous lesions).

ria into low, medium, and highly *S. aureus* colonized (log density accordingly: <5.8, 5.9–7.8, and 7.9–8.4).

Increased SEB-activation-induced apoptosis and altered cytokine production by peripheral blood T cells of AD patients

The proportion of CD3⁺ lymphocytes labeled by APO2.7 antibody was significantly higher in cultures of PBMCs isolated from AD patients than parallel cultures from controls (Fig. 2). Furthermore, the increased proportion of APO2.7⁺ cells was seen only in the group of patients with more severe disease or more intense skin colonization by *S. aureus*. In contrast, the production of IFN- γ in SEB- and PHA- but not in PWM-stimulated cultures was significantly lower among AD patients. The reduced IFN- γ production in SEB-stimulated cultures was irrespective of severity of the disease and density of *S. aureus* colonization (Fig. 3A and B). IL-2 production in cultures of patients with severe AD was significantly lower than that of controls (Fig. 3A). Also, patients with a high density of *S. aureus* on the skin produced less IL-2 than patients with a moderate number of bacteria (Fig. 3B). TNF- α production was significantly lower in cultures of AD patients and particularly among those with high colonization by *S. aureus*. The concentrations of IL-10 (Fig. 3), IL-4, and IL-5 (not shown) did not differ between patients and controls.

SEB-induced apoptosis of patients T cells correlates with S. aureus colonization of the skin lesions

The positive correlation of the SCORAD index and the density of skin lesion colonization by *S. aureus* (Fig. 1) does not exclude the possibility that their relationship to other measured parameters may be different. Indeed, as shown in Table 2, T cell apoptosis positively correlated with the density of *S.*

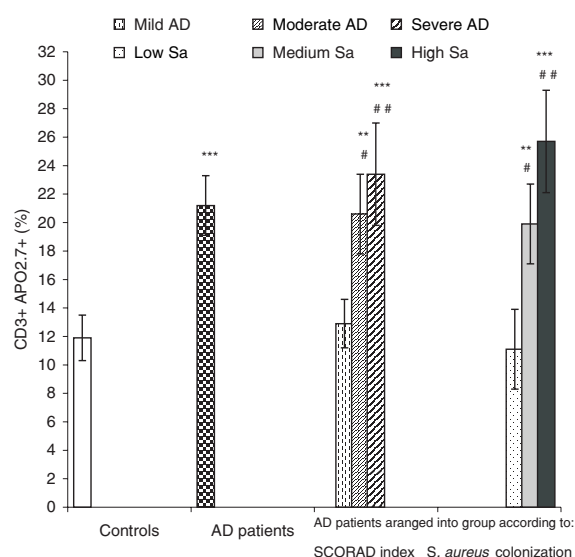


Figure 2. SEB-induced T cell apoptosis in cultures of PBMCs. Histograms show proportion (mean $\pm$ SE) of CD3⁺APO2.7⁺ cells measured by flow cytometry in cells of 24-h cultures of control and AD patient PBMCs. Data were also analyzed separately in groups with different severity of symptoms (SCORAD index) or density of *S. aureus* (Sa) skin colonization as indicated. Statistical significance was calculated by Student's *t*-test. **p*<0.05, ***p*<0.01, and ****p*<0.001 in comparison with the control group and #*p*<0.05, ##*p*<0.01 in comparison with patients with mild symptoms or low bacterial colonization.

aureus colonization of the skin but not SCORAD index. By contrast, a significant negative correlation was found between SCORAD index and the production of IFN- γ . This finding indicates that although bacterial colonization correlates with SCORAD index, *S. aureus* skin colonization is an independent factor that correlates with SEB-induced T cell apoptosis.

The importance of skin colonization by *S. aureus* was further explored. Twenty-six patients had bacteria present not only on eczematous, but also on uninvolved skin. Patients with both severe and mild AD belonged to this group. Furthermore, the densities of non-lesional skin colonization were comparable in patients with high and low SCORAD indexes. Therefore we asked whether the presence or absence of bacteria on uninvolved skin may distinguish a separate group of patients. To answer this question the measured parameters were compared in groups of patients with and without the presence of *S. aureus* on uninvolved skin (Table 3). As shown, the presence of bacteria on uninvolved skin characterized a group of patients with high SCORAD index and high serum IgE level whose PBMCs in culture showed increased SEB-activation-induced T cell apoptosis and reduced TNF- α production. By contrast, the two groups did not differ in IFN- γ production.

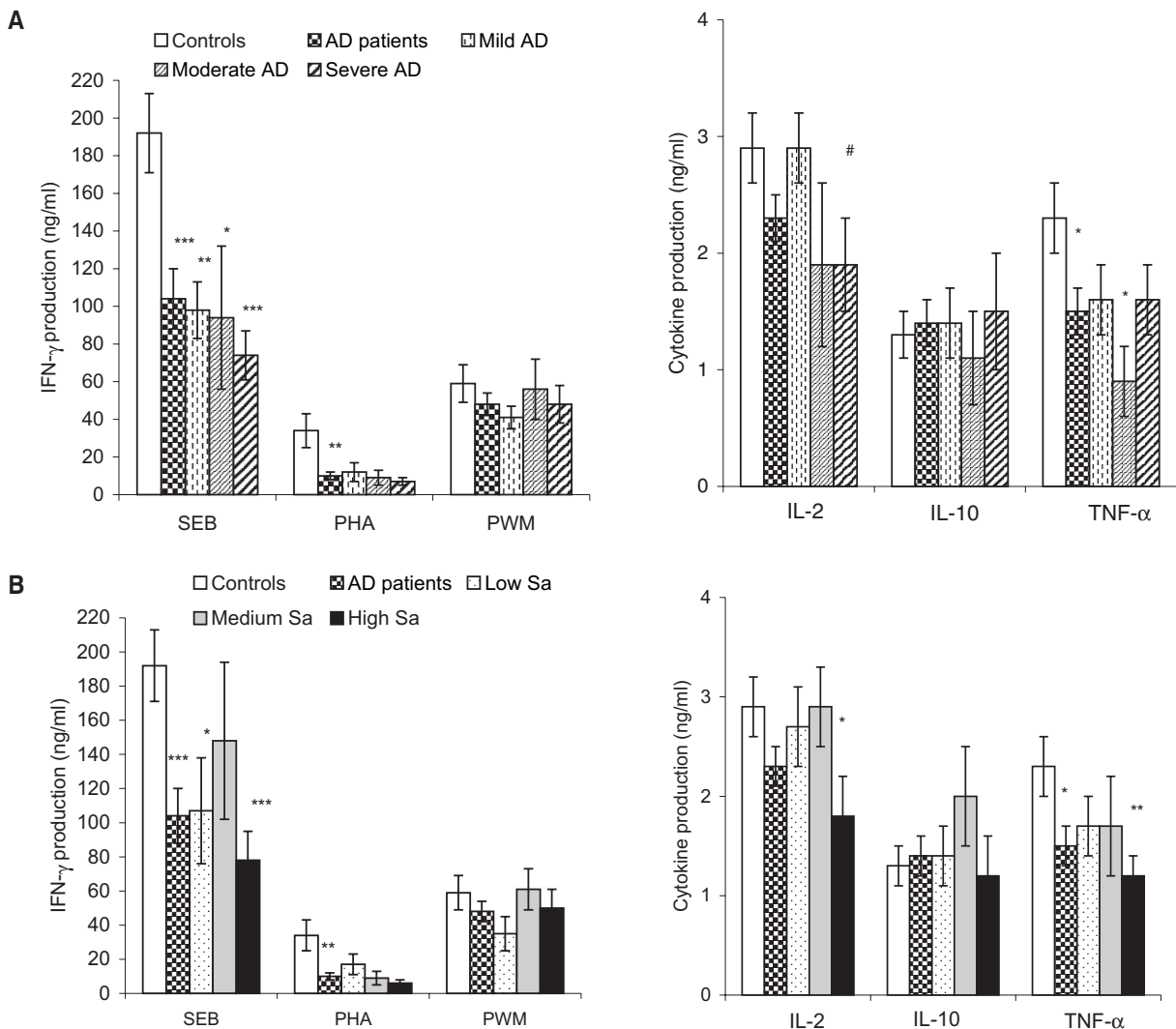


Figure 3. Cytokine production by control and patient PBMCs. IFN- γ production was measured in 96-h cultures of PBMCs stimulated with SEB, PHA, and PWM by ELISA method as described in Materials and Methods. In addition, the concentrations of IL-2, IL-10, and TNF- α measured by the Cytometric Bead Array kit in 48-h culture supernatants activated with SEB are shown. Histograms show a comparison of cytokine production (mean $\pm$ SE) in cultures of control subjects and: **A** – patients who were subdivided into groups according to the severity of symptoms and **B** – the density of skin colonization by *S. aureus* (Sa). Statistical analysis as in the legend to Figure 2.

Table 2. Correlation of SCORAD index and density of skin lesion *S. aureus* colonization with SEB-induced T cell apoptosis and production of IFN- γ

Correlation with:	SCORAD index ^a	Density of <i>S. aureus</i>
SEB-induced AICD	R=0.11 (p>0.05)	R=0.47 (p<0.01)
IFN- γ production	R= -0.46 (p<0.01)	R= -0.25 (p>0.05)

^a Correlation coefficient determined by Spearman's rank correlation. Statistically significant R-values are printed in bold.

DISCUSSION

There is much evidence that suggests that *S. aureus* and staphylococcal enterotoxins, including SEB, play an important role in the course of AD and that colonization of patients' skin lesions by *S. aureus* exacer-

Table 3. Comparison of selected clinical data and of measured parameters among AD patients with and without *S. aureus* colonization of uninvolved skin

Colonization of uninvolved skin by <i>S. aureus</i>	Absent	Present	Significance
Number of patients	10	19	
SCORAD index	38.0 $\pm$ 3.0*	57.5 $\pm$ 3.0	p<0.001
IgE (U/ml)	274.7 $\pm$ 20.0	1840 $\pm$ 557	p<0.03
AP02.7	13.3 $\pm$ 3.0	23.4 $\pm$ 2.6	p<0.03
IFN- γ	103.9 $\pm$ 26.0	104.7 $\pm$ 21.0	Ns
TNF- α	2336 $\pm$ 618	1216 $\pm$ 191	p<0.05
IL-10	1919 $\pm$ 598	1674 $\pm$ 369	Ns
IL-5	142 $\pm$ 45	131 $\pm$ 19	Ns
IL-4	48 $\pm$ 10	48 $\pm$ 7	Ns

AP02.7 (%). IFN- γ – ng/ml in 96-h, other cytokines as pg/ml of 48-h cultures of PBMCs activated with 0.1 μ g/ml of SEB. * Mean and p-values are printed in bold when differences are statistically significant. Ns – not significant.

bates and perpetuates the disease¹⁷. In cultures of PBMCs, SEB induces a vigorous proliferative response, cytokine production, and AICD of T cells^{6, 8, 19, 28}. The initial triggering of T cell activation by superantigens is restricted to some T cell receptor (TCR)V β isoforms of both CD4 and CD8 T lymphocytes^{3, 7, 13, 26, 32}. T cells with TCRV β isoforms that respond to SEB comprise about 20% of both CD4⁺ and CD8⁺ T cells²¹. Recent data of Lin et al.¹⁹ show that about 80% of CD4⁺ T cells with responding isoforms become activated by SEB and that upon stimulation with superantigen about 60% of them undergo apoptosis.

Recently, Yoshino et al.³⁶ found increased SEB-induced T cell apoptosis and reduced T cell responsiveness in AD patients during exacerbation of the disease. As *S. aureus* skin colonization and the numbers of bacteria on the skin are much higher during exacerbation than during remission of AD²², the main purpose of our study was to find out whether SEB-induced apoptosis is related to the severity of clinical symptoms or to the skin colonization by *S. aureus*. To make a direct comparison with the data reported by Yoshino et al.³⁶ possible we used the same method of apoptotic cell detection as well as culture conditions. Our data confirmed the studies of Yoshino et al.³⁶ by showing that during an exacerbation of the disease, SEB-induced peripheral blood CD3⁺ lymphocyte apoptosis is significantly greater, while IFN- γ production is lower. Similarly to other reports^{28, 36}, in some patients we found the proportion of apoptotic T cells higher than expected from the estimated number of SEB-responding TCRV β isoforms. This discrepancy was most likely due to the “fratricidal death” described in SEB-activated cultures¹¹.

Our data extend the observations of Yoshino et al.³⁶ by showing that the increased apoptosis of T cells correlates with the density of *S. aureus* colonization of skin lesions but not with severity of symptoms. By contrast, the reduced T cell responsiveness to SEB, namely the lower production of IFN- γ , correlated with the SCORAD index but not with the density of skin colonization by bacteria. The presence of superantigen-producing *S. aureus* on patients’ skin was shown to correlate with higher SCORAD in some³⁸, but not in other²⁰ studies. In our group of patients only 55% of skin-colonizing staphylococcal strains had genes coding superantigens, and the presence of these strains on the skin did not correlate with SCORAD (Wójcik et al., in preparation). Although this does not exclude the importance of patients’ previous exposure to superantigen-producing strains, it does show that massive colonization of patients’ skin by

S. aureus rather than the presence of exotoxin-producing bacteria are correlated with increased SEB-induced AICD.

We also noticed that increased T cell apoptosis was seen in the group of patients who in addition to skin lesions had *S. aureus* present in high numbers on uninvolved skin. To our knowledge such a group of patients has never been analyzed before. This group of patients had high serum IgE, suggesting that it had a characteristic of extrinsic AD. By contrast, other patients had significantly lower serum IgE and were presumably closer to the group of the non-allergic form of AD^{4, 35}. In addition, reduced production of TNF was observed in patients who had *S. aureus* on uninvolved skin. It is conceivable that the spreading of staphylococcal colonization on uninvolved skin is caused by a profound impairment of the skin defense system due to exposure of epidermal and dermal laminin and fibronectin receptors that may interact with staphylococcal adhesins¹⁰. Furthermore, it is very likely that the epidermis of these patients produces less antibacterial peptides, β -defensin, and cathelicidin that possess a bactericidal effect on *S. aureus*²⁴. The topical use of antibiotics has been reported to improve the clinical status of AD patients; however, except in patients with signs of infection, this type of therapy is not generally accepted³⁰. It is tempting to speculate that these patients represent a group where the topical use of antibiotics or other antibacterial treatment should be considered.

The lower production of IFN- γ by the PBMCs of AD patients with high SCORAD index may indicate a shift of the response towards the Th2 population². In our study the only other difference that suggested a shift between the Th1/Th2 populations was the reduced TNF- α production in the cultures of PBMCs of patients with a high density of skin colonization by *S. aureus*, including those who had *S. aureus* on uninvolved skin. The production of other cytokines in response to SEB was not much changed among AD patients. Lin et al.¹⁹ have recently shown evidence for a clear-cut difference between healthy controls and AD patients in SEB-triggered cytokine production by CD4⁺ T cells. Using cytoplasmic staining for IFN- γ and IL-4 they showed that in response to SEB, AD patients’ CD4⁺ T cells produce IL-4 instead of IFN- γ . Increased IL-4 and IL-5 production was also documented in skin-homing T cells^{2, 4}. In our studies and in the studies of Yoshino et al.³⁶ as well, the production of IL-4 was not increased in SEB-stimulated cultures of patient PBMCs, although IFN- γ production was significantly reduced. These differences may not be contradictory, since phenomena seen at the

single-cell level may not be detectable when supernatants of bulk PBMC cultures are analyzed. The secretion of cytokines by superantigen-activated T cells is strongly dependent on T cell-monocyte interactions and, in addition to CD4⁺ T cells, other cell populations are involved¹⁶. For example, in SEB-activated cord blood lymphocytes, CD8⁺ T cells are the main producers of IFN- γ ²⁶. Reduced IFN- γ production was also seen in cultures activated by PHA, but not when PWM was used as the activator. The differences between PHA and PWM probably reflect the various cell requirements for T cell activation by these

stimuli¹⁵. The similarity in SEB- and PHA-induced IFN- γ production is difficult to explain at the moment, but was also recently observed by Rowe et al.²⁶.

In conclusion, our data indicate that the massive *S. aureus* skin colonization in AD patients seen usually during exacerbation of the disease correlates with increased *in vitro* T cell apoptosis in cultures of PBMCs activated with SEB. We also suggest that the generalized skin colonization by bacteria is characteristic of a group of patients who probably represent an extrinsic form of the disease.

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