

Application of staphylococcal enterotoxin B on normal and atopic skin induces up-regulation of T cells by a superantigen-mediated mechanism

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Background: The skin of patients with inflammatory skin diseases such as atopic dermatitis is frequently colonized with *Staphylococcus aureus*. Colonization with *S aureus* has been reported to exacerbate atopic dermatitis. Recent studies have demonstrated that *S aureus* isolated from the skin of patients with atopic dermatitis releases bacterial toxins that act as superantigens. We have previously applied the staphylococcal superantigen staphylococcal enterotoxin B (SEB) on intact human skin and found that the application led to induction of dermatitis.

Objective: The purpose of the study was to determine whether superantigen-induced dermatitis is primarily due to a T cell–superantigen–mediated reaction or represents nonspecific cytokine-driven inflammation.

Methods: We applied SEB, vehicle, and sodium lauryl sulfate on normal skin in healthy (n = 6) and atopic subjects (n = 6) and biopsy specimens were taken from all treated areas. The biopsy specimens from all subjects and peripheral blood from the atopic subjects were analyzed for the T-cell receptor (TCR) V β repertoire with mAbs against TCR V β 2, 3, 8.1, 12, 14, and 17.

Results: From all subjects, both healthy and patients with atopic dermatitis, skin biopsy specimens from SEB-treated areas demonstrated selective accumulation of T cells expressing SEB-reactive TCR V β 12 and 17 ($P < .05$). This selective up-regulation was not found in the sodium lauryl sulfate–treated areas.

Conclusion: Our data strongly support that superantigen-induced T-cell activation is involved in the dermatitis seen after experimental application of SEB on intact skin. (J Allergy Clin Immunol 2000;105:820-6.)

Key words: Superantigen, staphylococcal enterotoxin B, T-cell receptor V β , atopic dermatitis

Abbreviations used

AD:	Atopic dermatitis
PE:	Phycoerythrin
SEB:	Staphylococcal enterotoxin B
SLS:	Sodium lauryl sulfate
TCR:	T-cell receptor

Lesional skin in patients with inflammatory skin diseases such as atopic dermatitis (AD) is often colonized with *Staphylococcus aureus*.¹ A link between the degree of colonization with *S aureus* and the intensity of skin inflammation has been described.^{2,3} Most strains of *S aureus* isolated from atopic skin have been shown to produce enterotoxins with superantigen properties.^{4,5} When bound to MHC class II molecules, superantigens stimulate T cells expressing particular T-cell receptor (TCR) β -chain.⁶ In addition, superantigens can also activate the production and secretion of proinflammatory cytokines from MHC class II–expressing cells, such as Langerhans cells, activated keratinocytes, and macrophages, independent of T cells. Thus superantigens can induce skin inflammation by several different mechanisms.

We have previously demonstrated that the superantigen staphylococcal enterotoxin B (SEB) induces eczema when applied on intact healthy and atopic skin.⁷ The mechanism responsible for induction of the eczema is, however, not known. The objective of this study was to determine whether application of a staphylococcal superantigen on normal and atopic skin leads to accumulation of T cells in the skin by a superantigen-mediated mechanism.

METHODS

Subjects

The protocol was approved by the ethics committee, Copenhagen, Denmark (95222), and all subjects gave informed consent. Six adults without skin disease and 7 adults with AD of minimal activity and no eczema on their arms volunteered for the study. The diagnosis of AD was established as described by Hanifin and Rajka.⁸ No subjects were receiving oral medication and the subjects with AD did not receive any topical medication other than emollients.

Application of SEB

The SEB used in the study was purified by Patrick M. Schlievert as previously described.^{9,10} SEB and sodium lauryl sulfate (SLS) (Sigma, St Louis, Mo) were dissolved in PBS (vehicle). Vehicle,

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SEB, and SLS were placed in 12-mm diameter Finn chamber (Epitest, Tuusula, Finland) and applied on the upper inner arm. Vehicle, SEB 10 $\mu\text{g}/\text{cm}^2$, and 2% SLS were applied on all subjects. The Finn chambers remained in place for 24 hours. Seventy-two hours after application the reactions were graded with a clinical score. The clinical score was as follows: 0, healthy skin; 1, follicular redness but no induration; 2, even redness or slight induration; 3, redness and induration. Before application and 72 hours after application skinfold thickness was measured with a micrometer (Mitutoyo, Tokyo, Japan) as the mean of 3 measurements. The increase in skinfold thickness was calculated for each site by subtracting the preapplication thickness from the 72-hour postapplication thickness. After the clinical reactions were measured, 4-mm punch biopsies were taken from all tested sites. In one patient with AD application of SEB had a strong clinical reaction 24 hours after application, with edema and erythema on the major part of the upper arm. The patient was excluded from the study and immediately treated with a potent topical steroid. The reaction disappeared after a few days of treatment.

mAbs

mAbs Leu4 (CD3, unconjugated or peridinin chlorophyll-a protein [ie, PerCP]), Leu3a (CD4; FITC, phycoerythrin [PE]), Leu2a (CD8; PE), G1CL (mouse IgG1 control; unconjugated, FITC, PE), and G2GL (mouse IgG2 control; unconjugated, FITC, PE) were obtained from Becton-Dickinson Immunocytometry Systems (San Jose, Calif). Biotinylated or unconjugated mAbs specific for TCR V β 2, 3, 8.1, 12, 14, and 17 were obtained from Immunotech (Westbrook, Me). Monoclonal anti-SEB antibody was obtained from OEM Concepts (Toms River, NJ).

Assessment of peripheral blood T-cell repertoire

From all patients with AD and 2 of the healthy subjects blood samples were taken before SEB application. PBMCs were isolated from heparinized blood by Ficoll-Hypaque density gradient centrifugation. TCR V β repertoires on freshly isolated PBMCs were assessed by 2- or 3-color immunofluorescence on a FACScan flow cytometer (Becton-Dickinson Immunocytometry Systems) with FITC, PE, and PerCP as the 3 fluorescent parameters, as previously described.^{11,12} All antibodies were used at 1 $\mu\text{g}/\text{mL}$. List mode multiparameter data files (each file with forward scatter, side scatter, and 3 fluorescent parameters) were analyzed with use of the LYSIS II Windows program (Becton-Dickinson, San Jose, Calif). Negative control reagents were used to verify the staining specificity of antibodies. The percentage of T cells bearing each TCR V β phenotype was expressed as a percentage of total T (CD3⁺) cells.

Immunohistochemistry

Skin biopsy specimens placed in Tissue Tek ornithine carbamyl transferase compound (Miles, Elkhart, Ind) were flash-frozen in liquid nitrogen and stored at -80°C . Four micrometer cryostat sections were cut, dehydrated in acetone for 10 minutes, and air dried. Sections were incubated for 30 to 60 minutes at room temperature with the respective anti-V β mAb, anti-CD mAb, or anti-SEB mAb. All antibodies were used at 2 $\mu\text{g}/\text{mL}$. Sections were then washed and stained by the labeled avidin-biotin method as described previously^{13,14} with peroxidase-labeled streptavidin (Dako, Carpinteria, Calif). Afterward, sections were counterstained with hematoxylin and mounted. Control antibodies were tested on step sections of the same tissue specimens. The negative-staining controls were isotype-specific mouse IgG with irrelevant specificity. Normal human tonsil sections were used as positive controls for V β T-cell staining and CD antibodies. All lymphocyte counts of stained sections were per-

formed without knowledge of the subject's identity or condition. All T cells in epidermis and dermis were counted, excluding the hair follicles. The V β staining results are expressed as a percentage of the CD3-reactive T cells counted over 10 high-power fields.

Statistical analysis

For statistical analysis of the clinical appearance, the Fisher exact test was used. The Wilcoxon matched-pairs test was used to compare the increase in skinfold thickness, the number of T cells, and the accumulation of V β -specific T cells in SEB- and vehicle-treated skin. Furthermore, the test was used to compare the accumulation of V β -specific T cells in SEB-treated skin relative to SLS-treated skin and peripheral blood. *P* values of $<.05$ were considered statistically significant.

RESULTS

Application of SEB induces dermatitis on both healthy and atopic skin

SEB and SLS applied under occlusion for 24 hours induced a clinical reaction at day 3 in 6 of 6 healthy subjects and 6 of 6 patients with AD. In contrast, no reactions were seen after vehicle application (Table I). The skin changes induced by SEB were erythema and induration. In accordance with the clinical skin changes, SEB induced a significant increase in the skinfold thickness compared with vehicle-treated skin ($P < .05$) (Table I). In the group of healthy subjects SLS application induced a significant increase in the skinfold thickness compared with vehicle (Table I). However, in the group of patients with AD the increase in skinfold thickness after SLS application did not reach significant levels.

Application of SEB to normal skin in healthy subjects and patients with AD induces up-regulation of T cells in the skin

To obtain an adequate degree of skin reaction, SEB was applied at different concentrations and biopsy specimens were taken at different time points up to 168 hours. The number of T cells in the skin was maximal 72 hours after application of SEB and therefore 72 hours was determined to be the optimal time point for taking skin biopsy specimens and 10 $\mu\text{g}/\text{cm}^2$ of SEB as the optimal concentration.

With use of an mAb to SEB, SEB reactivity in skin biopsy specimens of SEB, but not SLS, patch test sites was demonstrated. SEB reactivity was localized in the epidermis to keratinocytes and hair follicles and in the dermis to endothelium and perivascular areas, as well as to spindle cells in the dermis, which had morphologic characteristics of fibroblasts (Fig 1).

To determine whether application of SLS and SEB led to an increased number of T cells in the skin, biopsy specimens from vehicle-, SLS-, and SEB-treated skin were analyzed for the total number of CD3⁺ T cells. Both SLS and SEB application led to a significantly increased number of T cells in the skin. With use of anti-CD3 mAb, as well as mAbs to V β subsets, T cells were usually

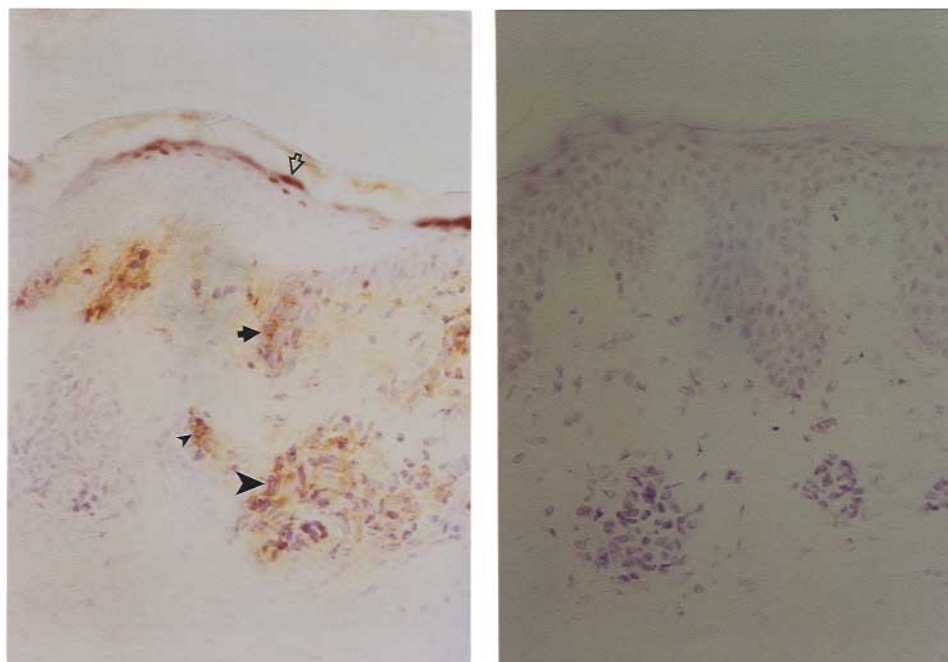


FIG 1. Skin section showing detection of SEB with mAb to SEB. *Left*, Skin from SEB patch test site showing positive staining of stratum corneum (*open arrow*) and deeper epidermis (*solid arrow*), endothelium (*small arrowhead*), and perivascular areas (*large arrowhead*), with mAb to SEB. *Right*, Skin from vehicle (control) patch test site showing lack of reactivity with mAb to SEB.

TABLE I. SEB applied to healthy and atopic skin leads to clinical reactions and increased skinfold thickness

Parameter	Subject	Application	Reaction
Clinical rating*	Healthy	Vehicle	0 (6/6)
		SLS 2%	2 (5/6), 3 (1/6)†
		SEB 10 µg/cm ²	1 (2/6), 2 (2/6), 3 (2/6)†
	Atopic dermatitis	Vehicle	0 (6/6)
		SLS 2%	2 (5/6), 3 (1/6)†
		SEB 10 µg/cm ²	1 (1/6), 2 (3/6), 3 (2/6)†
Skinfold thickness‡	Healthy	Vehicle	0.09 ± 0.16
		SLS 2%	0.52 ± 0.55§
		SEB 10 µg/cm ²	0.46 ± 0.22§
	Atopic dermatitis	Vehicle	0.10 ± 0.20
		SLS 2%	0.26 ± 0.27
		SEB 10 µg/cm ²	0.80 ± 0.39§

*Clinical rating is presented as the score followed by the frequency of the score in parentheses.

†All subjects react to SLS and SEB, none to vehicle, $P < .003$.

‡Skinfold thickness is presented as increase (in millimeters) compared with the preapplication value; data are means ± SD.

§Significance in comparison with the vehicle control, $P < .05$.

mainly confined to the dermis but were also localized in small numbers in the epidermis in some specimens. Within the dermis these cells localized to perivascular or interstitial areas. In interstitial areas the cells were diffusely infiltrating and did not form clusters, whereas the perivascular infiltrate was composed of clusters of variable size. The number of T cells in the skin sections correlated with the degree of clinical reaction and the increase in skinfold thickness. In the skin sections from healthy subjects the mean numbers of CD3⁺ T cells were

385 ± 233 in SLS-treated skin (mean ± SD, $n = 6$) and 491 ± 246 in SEB-treated skin (mean ± SD, $n = 6$) compared with 118 ± 32 in vehicle-treated skin (mean ± SD, $n = 6$) ($P < .03$ for SLS and SEB compared with vehicle). In the skin sections from patients with AD the numbers of CD3⁺ T cells were 354 ± 222 in SLS-treated skin (mean ± SD, $n = 6$) and 567 ± 307 in SEB-treated skin (mean ± SD, $n = 6$) compared with 108 ± 40 in vehicle-treated skin (mean ± SD, $n = 6$) ($P < .03$ for SLS and SEB compared with vehicle).

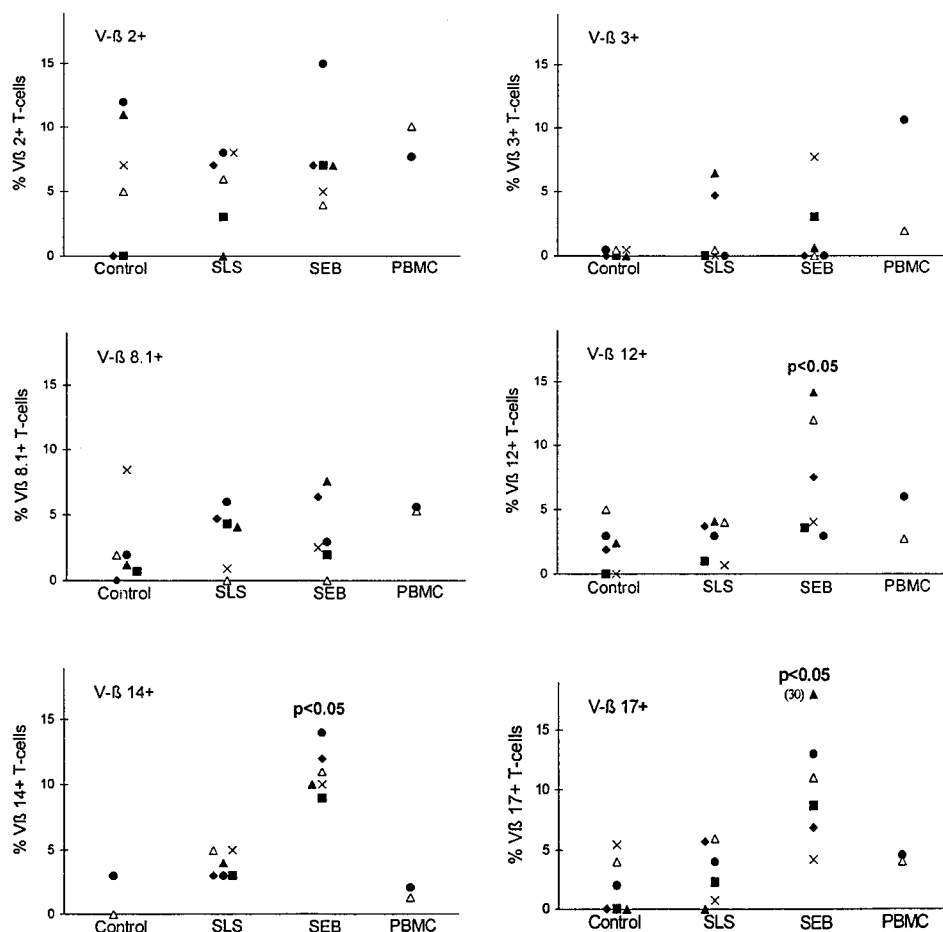


FIG 2. Application of SEB on normal human skin leads to selective accumulation of Vβ 12 and 17+ T cells in the skin. TCR Vβ expression in biopsy specimens and blood from healthy subjects. A comparison of TCR Vβ 2, 3, 8.1, 12, 14, and 17 expression in biopsy specimens from vehicle- (control), SLS-, and SEB-treated skin from 6 healthy subjects and PBMCs from 2 of these. For Vβ 14 the control values are missing for the other 4. The same symbol is used throughout for the same person. A value of $P < .05$ indicates statistical significance for the comparison of the percent Vβ+ T cells in SEB-treated skin with the percent Vβ+ T cells in corresponding vehicle and SLS-treated skin; for Vβ 14 only between SEB- and SLS-treated skin.

Application of SEB on both healthy and atopic skin induces up-regulation of T cells by a superantigen-mediated mechanism

To determine whether the T-cell up-regulation in SEB-treated skin was specific for the superantigen, T cells in the skin sections were analyzed for reactivity with mAb specific for Vβ 2, 3, 8.1, 12, 14, and 17. A marked accumulation of Vβ 12 and 17+ T cells was observed in biopsy specimens from SEB-treated skin compared with vehicle-treated skin in both healthy subjects and patients with AD (Figs 2 and 3). The up-regulation of Vβ 12 and 17+ T cells in the skin lesions was specific for SEB because biopsy specimens from SEB-induced skin lesions demonstrated a significantly higher percentage of Vβ 12 and 17+ T cells compared with biopsy specimens from SLS-induced irritant skin reactions (Figs 2 and 3).

We did not find any relationship between the percent of Vβ 12 or 17+ T cells in the skin and the severity of the clinical reaction after SEB application.

A significant accumulation of Vβ 14+ T cells was observed in atopic skin treated with SEB compared with vehicle-treated skin but not compared with SLS-treated skin (Fig 3). Similarly, in the group of healthy subjects a significant accumulation of Vβ 14+ T cells was observed in SEB-treated skin compared with SLS-treated skin. No up-regulation of Vβ 2, 3, and 8.1 was found in biopsy specimens from SEB-treated skin compared with control and SLS-treated skin.

In patients with AD the T-cell repertoire in peripheral blood was analyzed. Compared with the T-cell repertoire in peripheral blood, a significant accumulation of Vβ 12 and 17+ T cells was found in biopsy specimens from SEB-treated skin and not in SLS-treated skin (Fig 3).

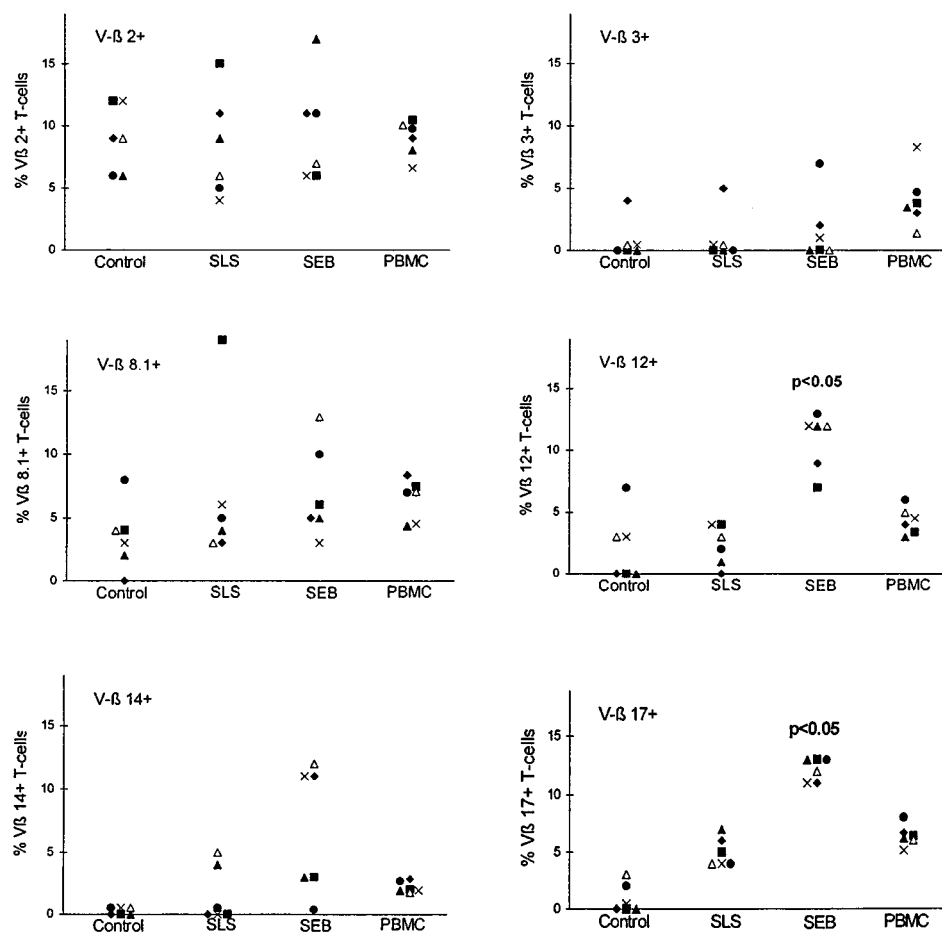


FIG 3. Application of SEB on atopic skin leads to selective accumulation of Vβ 12 and 17+ T cells in the skin. TCR Vβ expression in biopsy specimens and blood from patients with AD. A comparison of TCR Vβ 2, 3, 8.1, 12, 14, and 17 expression in biopsy specimens from vehicle- (control), SLS-, and SEB-treated skin and PBMCs. The same symbol is used throughout for the same person. A value of $P < .05$ indicates statistical significance for the comparison of the percent Vβ+ T cells in SEB-treated skin with the percent Vβ+ T cells in corresponding vehicle-treated skin, SLS-treated skin, and PBMCs.

DISCUSSION

Staphylococcal enterotoxin B induces inflammation when it is applied to human skin, with a significant T-cell accumulation in the skin. Biopsy specimens taken from the skin where SEB has been applied demonstrated a significant accumulation of T cells expressing SEB-reactive TCR Vβ 12 and 17. Choi et al¹⁵ have previously shown that SEB reacts with human T cells expressing TCR Vβ 3, 12, 14, and 17. However, we did not find the accumulation of Vβ 14+ T cells consistent and did not observe a significant accumulation of Vβ 3+ T cells in skin sections after SEB application. Of note, with use of the same SEB preparations to stimulate PBMCs from other donors, we found a significant stimulation of Vβ 3, 12, 14, and 17.¹¹ The reason for this discrepancy between skin and peripheral blood T-cell stimulation is unknown but nevertheless highlights the importance of examining the direct effects of superantigens on the skin immune response. In con-

trast to the current findings, no superantigen-induced Vβ-skewing of the T-cell repertoire occurs in uninvolved psoriasis skin after patch testing with a superantigen.¹⁶ The explanation for this discrepancy may be that HLA-DR-bearing keratinocytes found in psoriasis skin causes an acute inflammatory reaction by superantigens, leading to a T-cell repertoire that reflects the peripheral blood T-cell repertoire.¹⁶ These studies may, however, not be comparable because, in the latter, a much lower concentration of SEB was applied after tape stripping of the skin. However, a more likely explanation is that, in contrast to psoriatic skin, uninvolved atopic skin and healthy skin do not express HLA-DR.¹⁷

The SEB staining of biopsy specimens from skin treated with SEB demonstrated that SEB is widely distributed in both epidermis and dermis. There are multiple cellular targets for superantigens in the skin, including Langerhans cells, macrophages, keratinocytes, and fibroblasts, as well as T cells. It was therefore of interest to determine

whether the clinical reaction seen after application of SEB was primarily the result of a T cell–superantigen effect or a nonspecific cytokine-driven inflammation from the engagement of superantigen with MHC class II–bearing cells. Our data strongly support that superantigen-induced T-cell activation is involved in the dermatitis seen after experimental application of SEB to intact skin.

Besides activation of T cells, bacterial superantigens have been shown to induce expression of the skin-homing receptor, the cutaneous lymphocyte antigen, which may facilitate homing of T cells to the skin.¹¹ However, other mechanisms may be involved in the SEB-induced dermatitis. The first is direct binding of superantigen to antigen-presenting cells in the skin, such as Langerhans cells, leading to activation and cytokine release.^{18,19} The second is direct binding to MHC class II+ keratinocytes. In healthy skin MHC class II+ molecules are constitutively expressed on the acrosyringial epithelium^{20,21} and in most inflammatory skin conditions keratinocytes are induced to express MHC class II molecules, probably mediated by IFN- γ released from activated T cells.^{22,23} Direct binding of SEB to MHC class II+ keratinocytes has been shown to induce up-regulation of the adhesion molecule intracellular adhesion molecule-1 and release of TNF- α .^{24,25} The third mechanism that may be involved in SEB-induced dermatitis is induction of IgE-mediated immune responses. Up to half of patients with AD may have IgE directed against staphylococcal superantigens⁵; IgE specific for SEB may bind to mast cells or basophils and induce histamine release and thereby inflammation.

Whether our finding has any relevance to AD is at present not known. Several findings indicate a link between *S aureus* colonization and exacerbation of AD. *S aureus* is found in high numbers on the skin,¹ the number of bacteria correlates to the degree of inflammation,^{2,3} and treatment with steroids in combination with antibiotic demonstrates more efficacy than treatment with steroids alone.²⁶ One of the most likely mechanisms by which *S aureus* may lead to exacerbation of AD is through release of superantigens. *S aureus* isolated from patients with AD is shown to release superantigens,^{4,5} and superantigens applied to the skin induce dermatitis.⁷ Two groups have previously studied the TCR V β expression in lesional atopic skin. Ha et al²⁷ found in 4 of 14 patients with AD up-regulation of T cells with specific TCR V β ; however, it is not known whether the patients were colonized with *S aureus*. In a study with 6 AD patients colonized with *S aureus*, an up-regulation of T cells expressing TCR V β 3, 8, and 12 was demonstrated.²⁸ A final proof for the role of staphylococcal superantigens in the pathogenesis of the aggravation of AD seen after *S aureus* infection is still needed. The proof will require direct staining for superantigens in the skin and demonstration of accumulation of T cells specific for the superantigen in skin lesions from the same patient. However, this study may not be easy because several antigens, including aeroallergens such as house dust mite, may be involved in T-cell activation in atopic skin. Nevertheless,

our observation that TCR V β skewing can be seen at the site of superantigen application indicates the feasibility of this approach, although the T-cell repertoire expanded by superantigens in the skin appears more limited than in peripheral blood. The current study is therefore an important step to elucidate the role of staphylococcal superantigens as an aggravating factor in AD.

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