

Epicutaneous sensitization with superantigen induces allergic skin inflammation

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Background: Atopic dermatitis (AD) is characterized by skin infiltration with eosinophils and lymphocytes and expression of Th₂ cytokines in acute skin lesions. The skin of patients with AD is frequently colonized with enterotoxin-secreting strains of *Staphylococcus aureus*. Staphylococcal enterotoxins have been implicated in the exacerbations of the inflammatory skin lesions in patients with AD.

Objective: We sought to determine whether epicutaneous (EC) sensitization of mice with staphylococcal enterotoxin B (SEB) results in allergic skin inflammation.

Methods: BALB/c mice were EC-sensitized with SEB. Their skin was examined for allergic inflammation and cytokine expression, and their splenocytes were examined for cytokine secretion in response to SEB.

Results: EC sensitization with SEB elicited a local, cutaneous, inflammatory response characterized by dermal infiltration with eosinophils and mononuclear cells and increased mRNA expression of the Th₂ cytokine IL-4 but not of the Th₁ cytokine IFN- γ . EC-sensitized mice mounted a systemic Th₂ response to SEB evidenced by elevated total and SEB-specific IgG1 and IgE. Although EC sensitization with SEB resulted in selective depletion of SEB-specific T-cell receptor V β 8⁺ cells from the spleen and sensitized skin, splenocytes from SEB-sensitized mice secreted relatively more IL-4 and less IFN- γ than did saline-sensitized controls, consistent with Th₂ skewing of the systemic immune response to the superantigen.

Conclusion: These results suggest that EC exposure to superantigens skews the immune response toward Th₂ cells, leading to allergic skin inflammation and increased IgE synthesis that are characteristic of AD. (*J Allergy Clin Immunol* 2003;112:981-7.)

Key words: Atopic dermatitis, superantigens, skin inflammation, cytokines, staphylococcal enterotoxin B, in vivo animal models

Staphylococcus aureus and *Streptococcus* produce a large family of proteins known as superantigens, which

Abbreviations used

AD: Atopic dermatitis
EC: Epicutaneous
H&E: Hematoxylin and eosin
OVA: Ovalbumin
SEB: Staphylococcal enterotoxin B
TCR: T cell receptor

include the staphylococcal enterotoxins A (SEA) and B (SEB). Superantigens bind directly to MHC class II molecules and to a subset of T-cell receptor (TCR) V β chains.^{1,2} Unlike conventional antigens, superantigens do not require processing by antigen-presenting cells.³ Administration of superantigen results in initial selective expansion of T cells that bear specific V β chains that recognize the superantigen, followed by their deletion.⁴

Atopic dermatitis (AD) frequently affects individuals with a personal or family history of atopic disease and is often characterized by elevated serum IgE. AD skin lesions display a marked inflammatory cell infiltrate, which consists of eosinophils; lymphocytes, which are predominantly CD3⁺; CD4⁺ T cells; monocytes/macrophages, and Langerhans cells.⁵ The Th₂ cytokines IL-4, IL-5, and IL-13 are expressed in acute skin lesions of AD, whereas the Th₁ cytokine IFN- γ is found in the skin lesions at later stages of the disease.⁶

The skin of up to 100% of patients with AD is colonized with *S aureus*.⁷ Of all *S aureus* strains isolated from lesional skin, up to 65% produce exotoxins with superantigenic properties.⁸ Superantigens might act as additional trigger factors in the development of skin inflammation in patients with AD.⁹ A subset of patients with AD mounts an IgE response to staphylococcal superantigens grown from their skin.¹⁰ Furthermore, superantigen stimulation of human PBMCs upregulates the expression of cutaneous lymphocyte antigen on the appropriate V β -bearing T cells in both CD4⁺ and CD8⁺ T-cell subsets.¹¹ More important, cutaneous lymphocyte antigen⁺ cells isolated from patients with AD secrete Th₂ cytokines in response to superantigens.¹² Moreover, *S aureus* colonization can cause severe exacerbation of AD in many patients.⁹ Finally, some but not all studies have shown that antimicrobial treatment leads to improvement of AD.^{7,13,14}

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We have developed a mouse model of AD using repeated epicutaneous (EC) sensitization with ovalbumin (OVA) to tape-stripped skin. This model displays many of the features of human AD.^{15,16} In this study, we investigated whether EC sensitization of mice with superantigen results in allergic skin inflammation.

METHODS

Mice and sensitization

BALB/c mice were obtained from The Jackson Laboratory (Bar Harbor, Me) and were kept in a pathogen-free environment. All procedures were in accordance with the Animal Care and Use Committee of the Children's Hospital. For EC sensitization, the skin of anesthetized mice was shaved and tape-stripped 6 times by transparent intravenous dressing (Tegaderm). SEB (Toxin Technology, Inc, Sarasota, Fla), 10 µg in 100 µL of normal saline, or placebo (100 µL of normal saline) was placed on a patch of sterile gauze (1 × 1 cm), which was secured to the skin with a transparent bio-occlusive dressing. Each mouse had a total of three 1-week exposures to the patch, separated from each other by 2-week intervals.

Histologic analysis

Specimens obtained 24 hours after the end of the third sensitization were fixed in 10% buffered formalin and embedded in paraffin, and multiple 4-µm sections were stained with hematoxylin and eosin (H&E). Cells were counted in a blinded fashion in 15 to 20 high-power fields at 1000×.

Cytokine mRNA expression in skin

Skin biopsy samples were immediately frozen in dry ice. RNA preparation for PCR amplification of reverse-transcribed cDNA and quantification of cytokine mRNA were performed as previously described.¹⁵

ELISA

The standard PharMingen ELISA protocol was used to quantify total serum IgE, IgG1, and IgG2a. Specific anti-SEB antibodies were determined by following the procedures that we have previously described for the determination of antibodies to OVA,¹⁵ except that SEB (1 µg/mL) instead of OVA was used to coat the plates used for IgG1 and IgG2a antibody determination, and biotinylated SEB, instead of biotinylated OVA, was used for IgE antibody determination.

In vitro IL-4 and IFN-γ synthesis

Single-cell suspensions of spleen cells were prepared in complete RPMI-1640 (JRH Biosciences, Lenexa, Kan) supplemented by 10% FCS, 1 mmol/L sodium pyruvate, 2 mmol/L L-glutamine, 0.05 mmol/L 2-mercaptoethanol, 100 U/mL penicillin, and 100 µg/mL streptomycin. Cells were cultured in the aforementioned medium at 2×10^6 /mL in 24-well plates in the presence of SEB (10 to 1000 pg/mL) or in plates coated with anti-CD3ε monoclonal antibody (1 µg/mL, PharMingen). Supernatants were collected after 96 hours of culture, and IL-4 and IFN-γ were determined by ELISA, following the manufacturer's instructions (PharMingen).

Flow cytometry analysis

Splenocytes were stained with FITC-conjugated anti-mouse rat Vβ6-FITC or mouse Vβ8-FITC or conjugated anti-mouse hamster CD3-PE antibodies (BD PharMingen) in 2% FCS serum PBS containing Fc-block, fixed in 2% formaldehyde, and analyzed on an FACSCalibur cytometer (BD PharMingen).

Proliferation of splenocytes to SEB

Splenocytes at 4×10^5 cells in 20 µL were plated in flat-bottom microtiter plates and cultured in triplicate at 37°C in 5% CO₂ in the presence of varying concentrations of SEB. After 3 days, cultures were pulsed for 16 hours with [³H]thymidine, cells were harvested, and radioactivity was measured in a liquid scintillation β-counter.

Skin immunohistologic analysis

Skin sections were embedded in Tissue-Tek oxacalciotriol compound (Miles Inc, Elkhart, Ind) on dry ice. Four-micron sections were prepared and stored at -80°C and then stained for mouse Vβ8 and Vβ6 using anti-Vβ8 and anti-Vβ6 antibodies from PharMingen and an avidin-biotin complex method described previously.¹⁷

Statistical analysis

The nonparametric Mann-Whitney test was used to compare mice groups, because standard deviations varied widely between groups. A *P* value <.05 was considered statistically significant.

RESULTS

EC sensitization with SEB induces allergic skin inflammation

H&E analysis of skin of BALB/c mice revealed increased thickening in the dermis and epidermis at sites of EC sensitization with SEB (Fig 1, A). The epidermal layer was approximately 3 to 5 cell layers thick in SEB-sensitized sites compared with 2 to 3 cell layers thick in saline-sensitized sites and exhibited foci of acanthosis and mild spongiosis (Fig 1, A). The dermal layer of SEB-sensitized skin sites contained increased numbers of nucleated cells and eosinophils compared with saline-sensitized skin sites (Fig 1, A). SEB-sensitized skin sites exhibited a significant increase in the numbers of nucleated cells and eosinophils compared with saline-sensitized skin sites, which were comparable to unsensitized skin (Fig 1, B, and data not shown). Immunoperoxidase staining revealed that the majority of infiltrating cells in SEB-sensitized skin sites were CD3⁺, CD4⁺ cells, with very few CD8⁺ cells. Major basic protein (MBP) staining was readily detectable in SEB-sensitized skin sites but virtually absent in saline-sensitized skin sites (data not shown). These findings suggest that EC sensitization with superantigen causes allergic skin inflammation.

EC sensitization with SEB induces T_H2 cytokine expression in the skin

Semiquantitative RT-PCR revealed that IL-4 and IFN-γ were minimally expressed in saline-sensitized skin. Expression of IL-4 but not IFN-γ significantly increased after sensitization with SEB (Fig 2). These results indicate that EC sensitization with superantigen results in local expression of the T_H2 cytokine IL-4.

EC sensitization with SEB induces elevated serum levels of total and SEB-specific IgE and IgG1

To investigate whether EC sensitization with SEB triggered a systemic T_H2 response, we measured total and

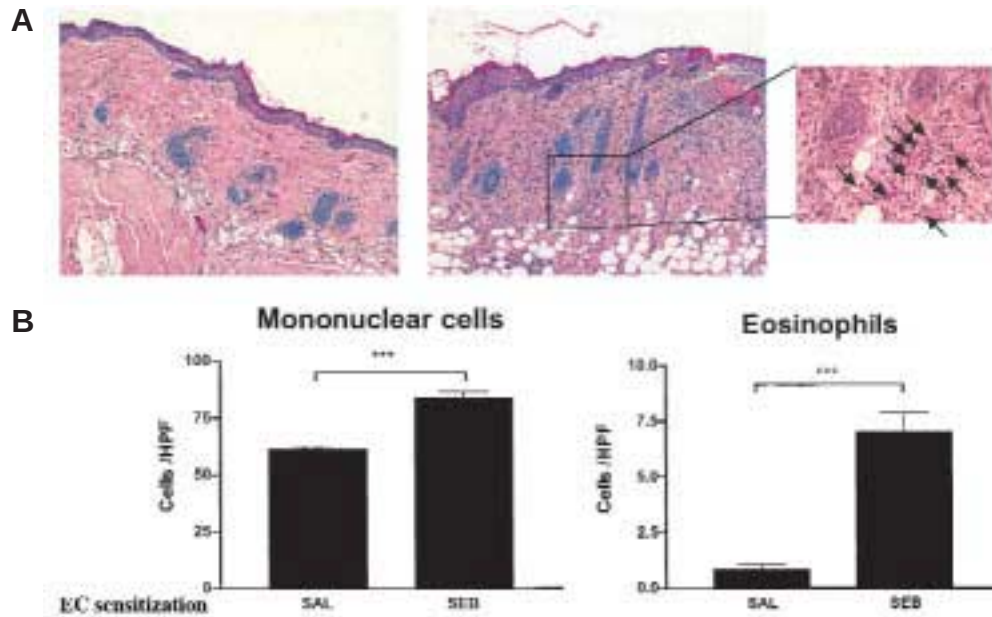


FIG 1. Histologic features of SEB and saline-sensitized skin sites in BALB/c mice. **A**, Skin sections were stained with H&E and examined at 200 $\times$ and 400 $\times$ magnification (*bold-bordered box*) and show the presence of eosinophils (*arrows*). **B**, Nucleated cell and eosinophil counts in SEB- and saline-sensitized skin sites. The *columns* and *error bars* represent the mean + SEM ($n = 6$ animals per group). *** $P < .001$.

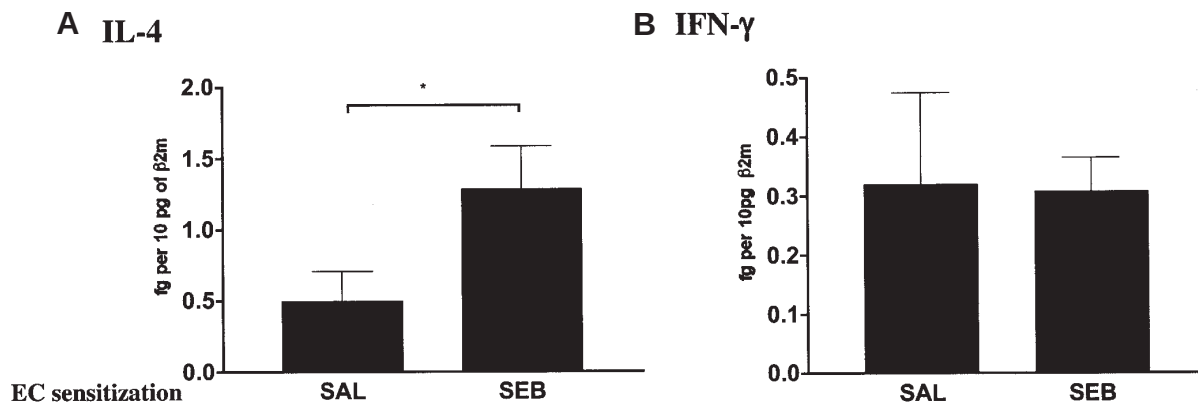


FIG 2. Expression of IL-4 (**A**) and IFN- γ (**B**) mRNA in SEB- and saline-sensitized skin sites. The *columns* and *error bars* represent the mean + SEM ($n = 6$ animals per group). * $P < .05$.

SEB-specific IgE and IgG1 in serum. **Fig 3, A** shows that EC sensitization with SEB resulted in a significant and marked increase in total serum IgE and IgG1, but not IgG2a, compared with sensitization with saline. Furthermore, it induced strong IgE and IgG1 antibody responses but only a relatively weak IgG2a antibody response to SEB (**Fig 3, B**).

Response of splenic T cells to SEB in EC-sensitized mice

Fig 4 shows that splenocytes from saline-sensitized mice secreted both IL-4 and IFN- γ in response to SEB in a dose-dependent manner. This is consistent with the known ability of SEB to stimulate naive T cells to secrete both cytokines.^{18,19} Splenocytes from SEB-sensitized mice secreted equivalent amounts of IL-4 in

response to SEB as did splenocytes from saline-sensitized controls (**Fig 4, A**). However, they were severely impaired in their ability to secrete IFN- γ (**Fig 4, A**). This was not because of a general impairment in the ability of these cells to secrete IFN- γ because splenocytes from SEB-sensitized mice secreted normal amounts of IFN- γ in response to anti-CD3 stimulation (data not shown). These results suggest that EC sensitization with SEB skews the cytokine profile of SEB-responding T cells toward T_H2 .

EC sensitization with SEB causes selective depletion of TCR V $\beta 8^+$ cells from the spleen

Administration of superantigen by the intraperitoneal, intravenous, or intrathymic route induces initial expansion of superantigen-reactive T cells, followed by their

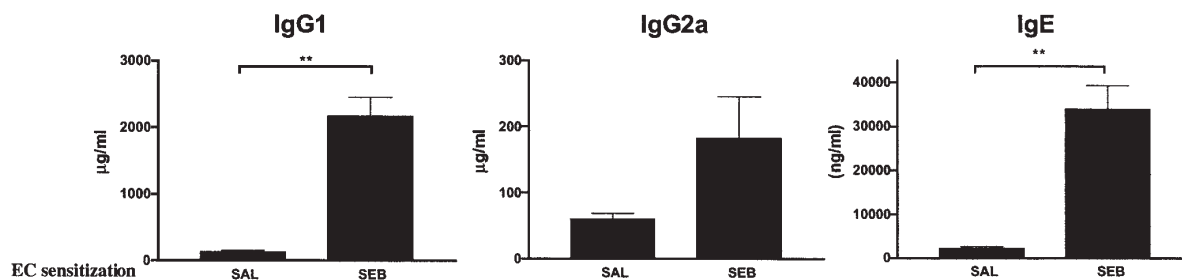
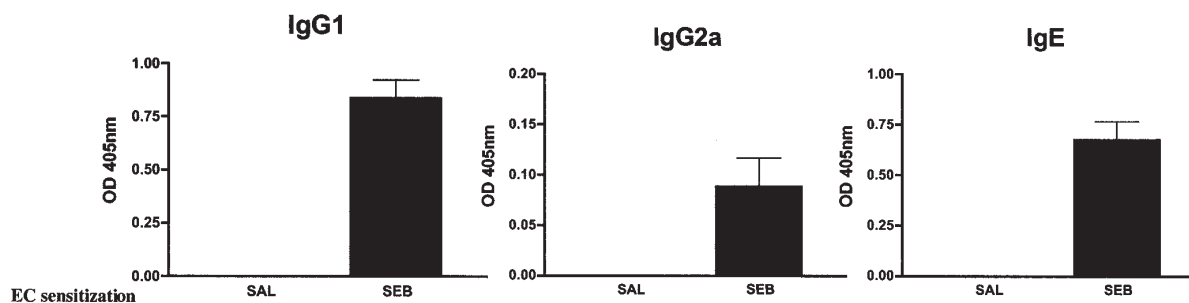
A Total Ig**B****SEB specific Ig**

FIG 3. Levels of total (A) and SEB-specific (B) IgG1, IgG2a, and IgE in mice EC-sensitized with SEB or saline, as determined by ELISA. The columns and error bars represent the mean $\pm$ SEM (n = 6 animals per group). ** $P < .01$.

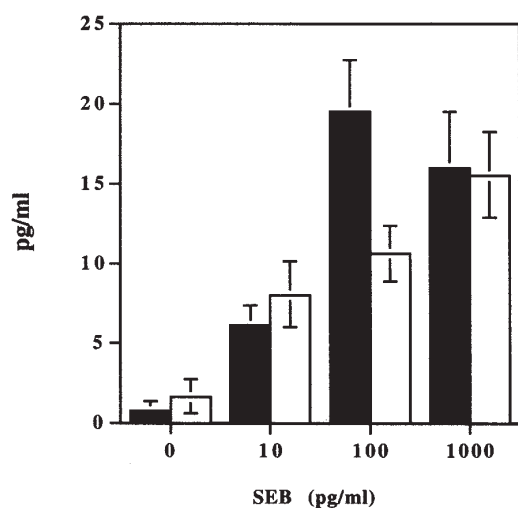
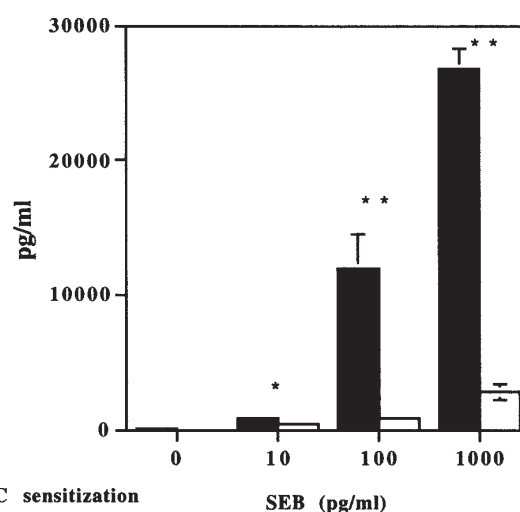
A
IL-4**B**
IFN- γ 

FIG 4. IL-4 (A) and IFN- γ (B) in supernatants of splenocytes from mice EC-sensitized with SEB or saline after their in vitro stimulation with SEB. The columns and error bars represent the mean $\pm$ SEM (n = 6 animals per group). * $P < .05$, ** $P < .01$.

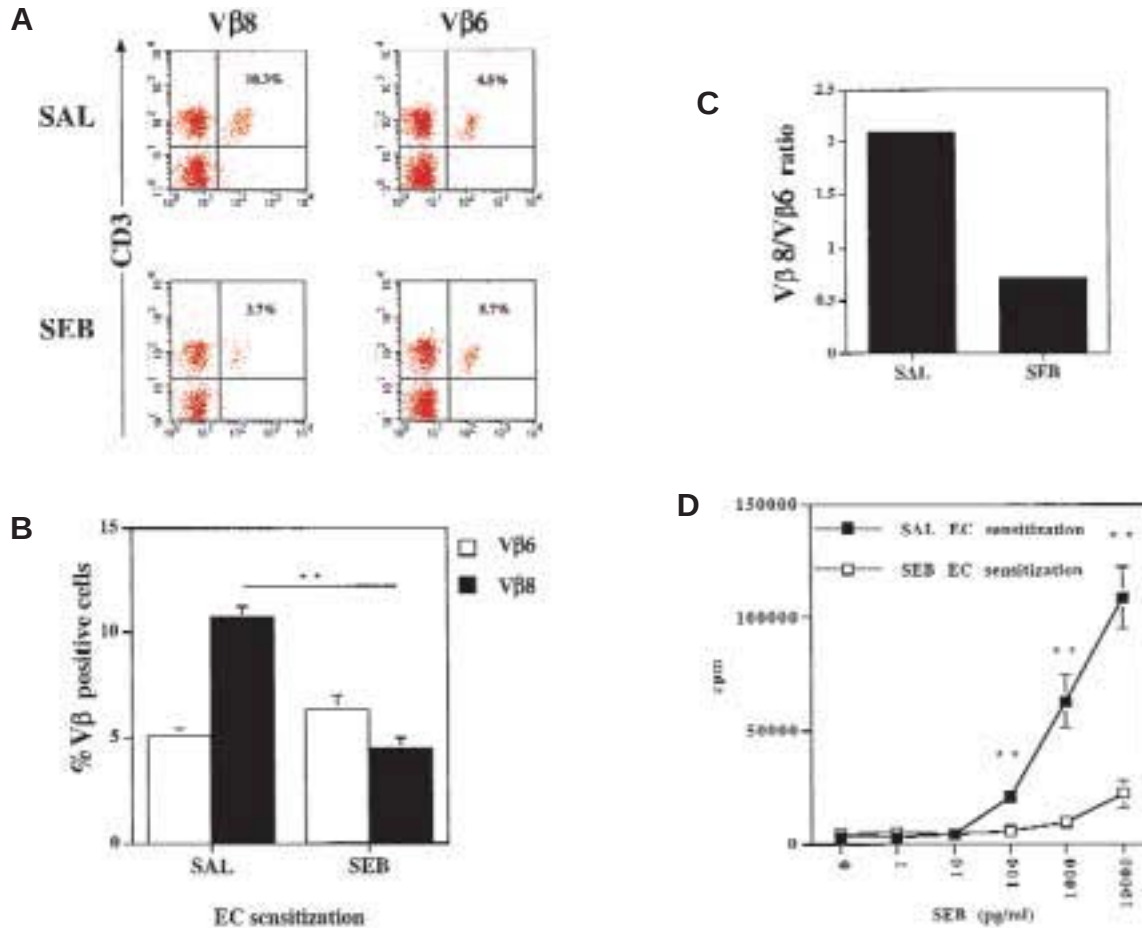


FIG 5. **A**, Representative fluorescence-activated cell sorting analysis of CD3+Vβ8+ and CD3+Vβ6+ spleen cells. **B**, Mean+SEM percentage of CD3+Vβ8+ and CD3+Vβ6+ cells. **C**, Ratio of CD3+Vβ8+/CD3+Vβ6+ cells in spleens. **D**, [3H]thymidine incorporation by spleen cells of SEB- and saline-sensitized mice stimulated with SEB. The columns and error bars in B and D represent the mean + SEM (n = 6 animals per group). **P < .01.

deletion.²⁰ SEB primarily engages TCR Vβ8⁺ cells in BALB/c mice. We examined the effect of EC sensitization on the number of Vβ8⁺ T cells in the spleen of BALB/c mice. As control, we enumerated T cells that express Vβ6. Fig 5, A shows a representative fluorescence-activated cell sorting analysis of splenocytes from BALB/c mice EC-sensitized with SEB or saline. There was depletion of CD3⁺ Vβ8⁺ but not of CD3⁺ Vβ6⁺ cells in SEB-sensitized mice compared with controls. EC sensitization with SEB caused a 3-fold decrease in the percentage of Vβ8⁺ cells in the spleen but no detectable change in the percentage of Vβ6⁺ cells (Fig 5, B). This resulted in a 3-fold decrease in the ratio of Vβ8⁺ to Vβ6⁺ T cells, from 2.1 to 0.7 (Fig 5, C). Fig 5, D shows that splenocytes from SEB-sensitized mice were impaired in their capacity to proliferate to SEB. This impairment was specific, because splenocytes from SEB-sensitized mice and saline-sensitized mice proliferated equally well to anti-CD3 (data not shown). These results suggest that EC sensitization causes selective depletion of Vβ8⁺ T cells from the spleen.

EC sensitization with SEB causes selective depletion of TCR Vβ8⁺ cells from sensitized skin sites. We next examined the status of Vβ8⁺ T cells in SEB-sensitized skin sites. Immunohistochemical analysis of skin for Vβ8⁺ and Vβ6⁺ cells was performed at the beginning and end of each of the 3 rounds of EC sensitization, ie, on days 0, 7, 21, 28, 42, and 49. Fig 6 shows that there were virtually no detectable Vβ8⁺ or Vβ6⁺ cells in tape-stripped skin sites biopsied immediately after application of SEB or saline on day 0. Saline-sensitized skin sites exhibited a small increase in the number of infiltrating Vβ8⁺ and Vβ6⁺ cells at the end of each round of sensitization (Fig 6, A). In contrast, the number of infiltrating Vβ8⁺ cells in SEB-sensitized skin sites significantly increased at the end of each round of sensitization compared with day 0 (P < .001). However, the number of these cells was significantly less at the end of the third round (day 49) than at the ends of the first 2 rounds, ie, days 7 and 28 (P < .001; Fig 6, B). The number of Vβ6⁺ cells in SEB-sensitized skin sites remained very low at

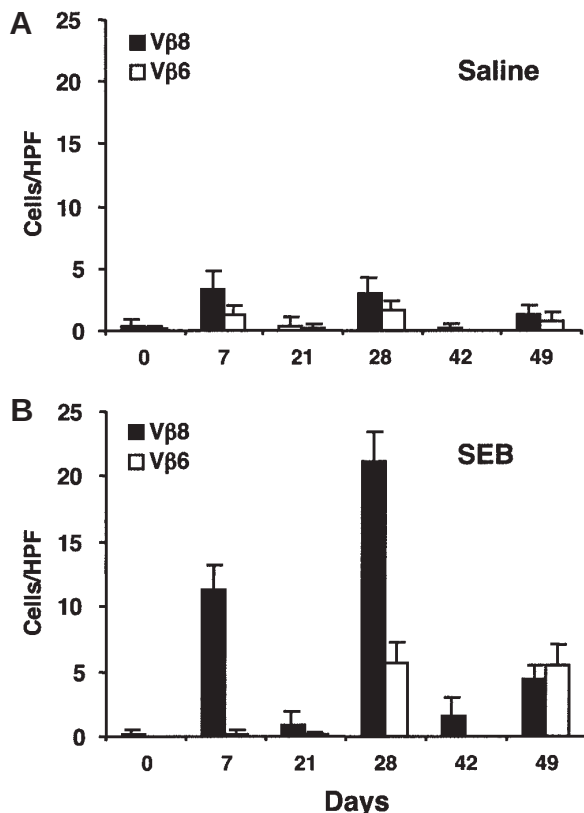


FIG 6. Numbers of Vβ8⁺ cells and of Vβ6⁺ cells in skin sites sensitized with saline (A) and SEB (B). The columns and error bars represent the mean ± SEM (n = 4 animals per group).

day 7 but showed a significant increase at the end of rounds 2 and 3 of sensitization, ie, on days 28 and 49 ($P < .05$; Fig 6, B). Two weeks after the end of each of the first 2 rounds of sensitization (days 21 and 42), there was a marked reduction in the number of Vβ8⁺ cells compared with immediately after sensitization and negligible numbers of Vβ6⁺ cells. Taken together, these results suggest that SEB sensitization causes a transient, selective, early infiltration of Vβ8⁺ cells in the dermis, followed by their relative depletion, and a late but sustained influx of nonspecific T cells.

DISCUSSION

The present work shows that EC immunization with SEB elicits allergic skin inflammation accompanied by a systemic T_H2 response to the superantigen. This suggests that superantigens might play a role in the pathogenesis of AD.

EC exposure to SEB elicited a local, cutaneous, inflammatory response characterized by dermal infiltration with eosinophils and mononuclear cells and increased mRNA expression of the T_H2 cytokine IL-4 (Figs 1 and 2). Thus, the inflammatory lesion in the skin of mice EC-sensitized with SEB shares several features with skin lesions in AD and in the mouse model of allergen-driven skin inflammation. It is likely that the mechanism of eosinophil infiltration in SEB-sensitized skin is

similar to that described in antigen-sensitized skin that involves IL-5 and eotaxin.^{16,21} EC sensitization with SEB also induced a systemic T_H2 immune response, evidenced by the presence in the serum of significantly elevated serum concentrations of total IgG1 (20-fold rise) and IgE (30-fold rise), but not IgG2a, and by a vigorous induction of anti-SEB IgG1 and IgE antibodies (Fig 3). Repeated intraperitoneal administration of SEB (twice a week for 3 weeks) has been reported to result in marked hypergammaglobulinemia that affects IgG1 (8-fold rise), IgE (16-fold rise), and IgG2a (5-fold rise).²² The fact that EC sensitization did not cause a significant rise in serum IgG2a levels further indicates that this mode of sensitization results in a skewed T_H2 response to the superantigen.

Direct evidence for T_H2 skewing of the response to SEB after EC sensitization was provided by examining the profile of cytokine production by splenocytes after in vitro stimulation with SEB. Consistent with the results of previous studies,^{23,24} splenocytes from saline-sensitized control mice secreted IL-4 and copious amounts of IFN-γ in response to SEB stimulation. Splenocytes from EC-sensitized mice made equivalent amounts of IL-4 in response to SEB stimulation as splenocytes from saline-sensitized controls. In contrast, the former were severely impaired in their capacity to secrete IFN-γ (Fig 4). There are precedents for skewing of the T_H response to superantigens. It has been shown that different conditions of primary SEB stimulation of resting CD4⁺ T cells could selectively induce production of either IFN-γ or IL-4.^{23,25} Our results are the first to show that the route of sensitization influences the T_H response to SEB. We propose that factors released in response to mechanical skin injury skew the response to EC-introduced SEB toward T_H2, possibly by targeting MHC class II⁺ cells that carry the superantigen to the draining lymph nodes, where they present it to T cells.

Vβ8⁺ T cells were selectively depleted from spleens of mice EC-sensitized with SEB (Fig 5, A). This was accompanied by reduced proliferation to SEB (Fig 5, B). Depletion of Vβ8⁺ T cells and reduction of proliferation to SEB in EC-sensitized mice were comparable to those reported after intraperitoneal, intravenous, or intrathymic administration of superantigen.²⁰ Despite the depletion of approximately two thirds of SEB-reactive Vβ8⁺ T cells, splenocytes of SEB-sensitized mice secreted equivalent amounts of IL-4 but 10-fold less IFN-γ in response to SEB as splenocytes from saline-sensitized controls, which did not delete Vβ8⁺ T cells. These results suggest that the residual Vβ8⁺ T cells are strongly skewed toward T_H2.

EC introduction of SEB caused a selective infiltration of Vβ8⁺ cells in the dermis at the end of each of the 3 rounds of EC sensitization. However, there were significantly fewer Vβ8⁺ cells at the end of the third round of SEB sensitization compared with the end of the first and second rounds of EC sensitization. Nevertheless, at that time point, there was increased mRNA expression for IL-4 but not IFN-γ. This suggests that the residual Vβ8⁺ cells in SEB-sensitized skin are skewed toward T_H2, as in the spleen, and might play an important role in the persistent allergic skin inflammation induced by repeated

EC sensitization with SEB. Interestingly, 2 weeks after the end of each of the first 2 rounds of sensitization (days 21 and 42), there was a marked reduction in the number of V β 8⁺ cells compared with immediately after sensitization, suggesting that persistent exposure to SEB is important for sustaining dermal T-cell infiltration.

There was a relatively late influx of V β 6⁺ cells in SEB-sensitized skin sites that became evident at week 4 and persisted at week 7. This suggests that skin inflammation induced by SEB results in nonspecific recruitment of T cells. This could be caused by increased expression of endothelial adhesion molecules and of proinflammatory chemokines in sensitized skin sites, as we have shown in the OVA sensitization model,^{15,16} and as has been shown in AD skin lesions.^{14,26}

Solaga et al²⁷ reported that acute 1-time intradermal injection or EC application of SEB causes infiltration of T cells and eosinophils in the skin. Our model of chronic EC sensitization with SEB differs from theirs in that we observed depletion of V β 8⁺ cells from spleen and skin, whereas they observed no deletion of V β 8⁺ cells from draining lymph nodes. This might be caused by differences in sensitization protocols, SEB dose, and/or organs examined. The nature of the Th response to SEB was not characterized in the study of Solaga et al, whereas in our work we demonstrate that EC sensitization with SEB gives rise to a vigorous T_H2 response.

Mechanical injury inflicted by scratching in AD likely results in EC sensitization to superantigens. On stimulation with SEB or staphylococcal enterotoxin A, PBMCs from patients with AD secrete significantly more IL-4 and IL-5 and markedly less IFN- γ than those from healthy control subjects.²⁸⁻³⁰ Our observations indicate that EC exposure skews the response to superantigen toward T_H2 cells, leading to allergic skin inflammation and increased IgE synthesis, which are characteristic of AD. Furthermore, they suggest that therapies that effectively decrease skin colonization with superantigen-producing organisms might have a beneficial effect in AD.

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