Topical Superantigen Exposure Induces Epidermal Accumulation of CD8+ T Cells, a Mixed Th1/Th2-Type Dermatitis and Vigorous Production of IgE Antibodies in the Murine Model of Atopic Dermatitis

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Patients with atopic dermatitis (AD) have repeated cutaneous exposure to both environmental allergens and superantigen-producing strains of *Staphylococcus aureus*. We used a murine model of AD to investigate the role of staphylococcal enterotoxin B (SEB) in the modulation of allergen-induced skin inflammation. Mice were topically exposed to SEB, OVA, a combination of OVA and SEB (OVA/SEB), or PBS. Topical SEB and OVA/SEB exposure induced epidermal accumulation of CD8⁺ T cells and TCRVβ⁺ cells in contrast to OVA application, which induced a mainly dermal infiltration of CD4⁺ cells. SEB and OVA/SEB exposure elicited a mixed Th1/Th2-associated cytokine and chemokine expression profile within the skin. Restimulation of lymph node cells from OVA- and OVA/SEB-exposed mice with OVA elicited strong production of IL-13 protein, whereas substantial exposure elicited a mixed Th2/Th1 type dermatitis and vigorous production of specific IgE and IgG2a Abs, which can be related to the chronic phase of atopic skin inflammation. The Journal of Immunology, 2005, 175: 8320–8326.
which involves both the antigenic and superantigenic properties of SEB.

Materials and Methods

Mice and sensitization

BALB/c mice were obtained from Taconic M&B and kept under pathogen-free conditions. All procedures performed were in accordance with Social and Health Services of State Provincial Office of Southern Finland.

Six-week-old female mice (eight per group) were epicutaneously treated with OVA (OVA group), SEB (SEB group), combination of OVA and SEB (OVA/SEB group), or vehicle (PBS group) in isoflurane anesthesia (University 400 Anesthesia Unit; Abbott Laboratories). The back of the mice was shaved with an electronic razor and tape-striped by adhesive tape to introduce standardized skin injury. Stripping included adhering a piece of tape to the shaved skin four times, after which it was removed against the direction of the hair. Two different amounts of SEB (Sigma-Aldrich), 0.5 and 5 μg, were topically applied to a 1 cm × 1 cm patch of sterile gauze alone (in 100 μl of PBS) or with OVA. One hundred micrograms of OVA (grade V; Sigma-Aldrich) in 100 μl of PBS was used for epicutaneous sensitization. The gauze was secured to the shaved skin with transparent adhesive tape (Tegaderm; Owens and Minor) for 1 wk (first sensitization week).

Two weeks later (second sensitization week), mice were again tape-stripped, and an identical patch was reapplied to the same skin site. The last epicutaneous sensitization (third sensitization week) was similarly given 2 wk later. Mice got a total of three 1-wk patch exposures, separated from each other by 2-wk intervals, totaling 7 wk (16).

Histological analysis

Skin specimens were obtained from patched areas 24 h after the third sensitization week and fixed in 10% buffered formalin and embedded in paraffin. Multiple 4-μm sections were stained with H&E for eosinophil counting and with toluidine blue for mast cell counting. Individual inflammatory cell types were counted in 15 high-power fields at ×1000 and expressed as cells per high-power field, with mean and SEM calculated. Mast cell counting was made at ×400 magnification.

Immunohistochemistry

Skin specimens from patched areas were embedded in Tissue-Tek oxalacitrol compound (Sakura Finetek) on dry ice. Immunoperoxidase staining was used to detect CD11c, CD4+, and CD8+ lymphocytes in the sensitized skin. Briefly, 4-μm frozen sections were fixed with cold acetone and stained with anti-mouse CD3 Ab (clone 17A2), anti-mouse CD4 Ab (clone RM4-5), and anti-mouse CD8 Ab (clone 53-67). All primary Abs were purchased from BD Pharmingen. Biotin-conjugated secondary Ab anti-rat IgG (1/200, 1/600, 1/1800, and 1/5400) were used. Bound IgG2a was detected with biotin-conjugated rat anti-mouse IgG2a mAb (clone R19-15).

SEB-specific IgE and IgG2a were measured by using the same protocol as detecting OVA-specific IgE and OVA-specific IgG2a. Plates were coated (50 μl) with 1 μg/ml SEB in 0.05 M NaHCO₃ (pH 9.6).

RNA isolation and cDNA synthesis

Skin biopsies were taken 24 h after the third patch was applied. Skin was immediately frozen in dry ice and kept in −70°C until homogenized with Ultra-Turrax T8 (IKA Labortechnik) to TRizol (Invitrogen Life Technologies). Further RNA extraction was performed following the TRizol instructions. DNaseI (RNase-free; Invitrogen Life Technologies) treatment was used to remove contaminating genomic DNA. RNA was further extracted with phenol-chloroform-isooamylalcohol (25:24:1). One microgram of total RNA was reverse transcribed using MultiScribe reverse transcriptase and random hexamers (Applied Biosystems).

Quantitative real-time PCR analysis

Quantitative real-time PCR (Taqman) analysis was performed with Applied Biosystems (TaqMan 7700 Sequence detector) as described previously (16). Primers for cytokines and chemokine ligands and target-specific probes were purchased as predeveloped reagents (IL-4, IL-13, IL-12p40, CCL1, CCL3, CXCL9, CXCL10, and CXCL11) from Applied Biosystems or, for IFN-γ and CCL8, were designed (H11001) from Invitrogen Life Technologies. Further RNA extraction was performed following the TRizol instructions. DNaseI (RNase-free; Invitrogen Life Technologies) treatment was used to remove contaminating genomic DNA. RNA was further extracted with phenol-chloroform-isooamylalcohol (25:24:1). One microgram of total RNA was reverse transcribed using MultiScribe reverse transcriptase and random hexamers (Applied Biosystems).

Statistical analysis

Nonparametric Mann-Whitney U test was used to compare differences between mice groups, and a p value of <0.05 was considered statistically significant.

Results

Topical exposure to SEB induces skin inflammation and exacerbates allergic dermatitis

Skin-infiltrating eosinophils and mast cells are a characteristic feature of AD (2, 3, 17). The number of eosinophils was increased in a dose-dependent manner in the SEB-exposed skin sites (Fig. 1, B and G) compared with PBS-treated controls (Fig. 1F). However, the number of eosinophils was equal in both OVA (Fig. 1D) and OVA/SEB-exposed (Fig. 1E) skin sites. Cell counting also revealed a significant increase of mast cells in SEB-treated skin sites compared with controls. Unlike eosinophils, the number of mast cells was further increased in OVA/SEB-treated skin sites compared with OVA-sensitized skin (Fig. 1C). The majority of mast cells in SEB-, OVA-, and OVA/SEB-treated skin sites were degranulated (data not shown). Exacerbation of skin inflammation after SEB application was also seen as the dermal thickening (Fig. 1A).

Epidermal CD3+ and CD8+ cells are significantly increased after topical exposure to SEB

Infiltrating T cells play an important role in the pathogenesis of AD (1). Immunohistochemical staining revealed an increased number of CD3+ and CD8+ cells in the exposed skin sites of both
**FIGURE 1.** Comparison of inflammatory cells and thickness of the skin counted from H&E-stained skin sites after repeated epicutaneous exposure to SEB, OVA, OVA/SEB, or vehicle (PBS) in BALB/c mice. Two different amounts of SEB (0.5 and 5 μg) were used with PBS or OVA (100 μg/100 μl). A, Epidermal thickness is shown with white bars, and black bars represent dermal thickness. Statistics show differences in total thickness is shown with white bars, and black bars represent COVA/SEB, and vehicle exposed mice. B, Eosinophil counting of SEB, OVA, OVA/SEB, and vehicle. C, Mast cell counting from toluidine blue-stained skin from mice epicutaneously treated with SEB, OVA, OVA/SEB, and vehicle. D–G, Histological features in magnification ×200 of H&E-stained skin sites epicutaneously treated with OVA (D), OVA/SEB (E), vehicle (F), and SEB (G). Insets in D, E, and G represent ×1000 magnifications of the same skin site in which eosinophils can be detected. Bars represent mean ± SEM (n = 8 mice/group). *, p < 0.05; **, p < 0.01; and ***, p < 0.001.

SEB-treated groups (SEB and OVA/SEB) compared with controls (Fig. 2A). CD8+ T cells (Fig. 2, A and G–J) predominantly infiltrated the epidermal compartment after topical application of SEB (Fig. 2, A and H) or OVA/SEB (Fig. 2, A and J), whereas this T cell subset was almost absent in OVA-sensitized skin (Fig. 2F). The number of CD4+ cells was dose-dependently increased after topical SEB exposure only (Fig. 2A). However, no significant changes in the numbers of CD4+ cells were seen between OVA- and OVA/SEB-treated skin sites (Fig. 2A). Representative immunohistological staining of CD4+ (Fig. 2, C–F) and CD8+ (Fig. 2, G–J) cells in the skin of PBS-, SEB-, OVA-, and OVA/SEB-treated mice are shown in Fig. 2.

It has been shown in BALB/c mice that SEB can activate TCRVβ8+ cells (18). We found that TCRVβ8+ cells were dose-dependently increased in the skin after SEB treatment. As a control, we stained for TCRVβ6+ cells and found no significant difference between SEB (5 μg), OVA, or OVA/SEB-exposed skin sites. However, a modest but significant increase in the number of TCRVβ6+ cells was observed in the skin after topical exposure to SEB alone (Fig. 2B).

**SEB induced both Th1- and Th2-associated cytokines and chemokines in the skin**

Cytokine analysis revealed a mixed Th2- and Th1-type skin inflammation in SEB-treated skin. The expression of Th2-type cytokine IL-4 mRNA was significantly up-regulated in the exposed skin of SEB-treated mice compared with PBS controls. The expression of Th1 type cytokines, IL-12p40, and IFN-γ mRNAs were significantly induced at all SEB-treated skin sites (SEB- and OVA/SEB-exposed mice; Fig. 3). Recently, several chemokines have been associated with an atopic dermatitis phenotype (19, 20). A synergistic induction of proinflammatory chemokines CCL3 and CCL4 mRNAs was found in the skin after OVA/SEB treatment compared with OVA or SEB exposure alone (Fig. 4A). Furthermore, a significantly elevated mRNA expression of proinflammatory chemokine CCL8 was found in the skin of all SEB-exposed groups (SEB- and OVA/SEB-exposed mice compared with PBS or OVA-sensitized skin. Moreover, significant induction of Th2-associated chemokines CCL1, and CCL11 were found in the skin of all SEB-exposed groups (SEB- and OVA/SEB-treated mice) compared with PBS control or OVA-exposed mice. The Th2-associated chemokine CCL17 was markedly increased in the skin of SEB-treated mice, and a smaller effect was found between OVA- and OVA/SEB-exposed groups (Fig. 4B). Finally, IFN-γ-inducible chemokines CXCL9, CXCL10, and CXCL11 were significantly up-regulated in the skin of both SEB and OVA/SEB groups compared with PBS controls or OVA-exposed mice (Fig. 4C).

**Production of IL-13 and IFN-γ is increased after stimulation of draining LN cells with SEB**

To investigate allergen- and superantigen-induced production of major Th1 and Th2 cytokines in vitro, cells from skin draining LNs...
were harvested and stimulated with OVA or SEB. Stimulation of cells from OVA- and OVA/SEB-exposed mice with OVA elicited a strong production of IL-13 protein, whereas no IL-13 could be detected in PBS- or SEB-exposed mice after OVA stimulation. SEB stimulation elicited modest IL-13 production in SEB- and OVA-exposed mice but not in OVA/SEB- or PBS-treated mice. Moreover, the levels of IL-13 protein after SEB stimulation were lower compared with OVA stimulation (Fig. 5 A).

In contrast to IL-13 secretion, substantial amounts of IFN-γ protein were detected after SEB stimulation in cells derived from SEB- or OVA/SEB-exposed mice but not in PBS- or OVA-treated mice. Stimulation with OVA did not induce detectable amounts of IFN-γ protein in any of the study groups (Fig. 5B).

**Discussion**

Strong evidence exists that environmental allergens play an important role in the pathogenesis of AD (1, 22). Several studies also indicate that severe AD is associated with skin colonization of SEB-producing strains of *S. aureus* (5, 23, 24). To better understand the role of SEB in AD, we took advantage of the murine model of AD and investigated the effects of cutaneous SEB exposure in the modulation of allergen-induced skin inflammation. Our results show that topical exposure to SEB induces a mixed Th2/Th1-type dermatitis and vigorous production of specific IgE and IgG2a Abs. These results suggest an important role of staphylococcal infections in the pathogenesis and disease severity of AD.
It has been shown previously that CD4+ T cells predominate in acute skin lesions of AD patients, whereas only a few CD8+ T cells can be detected (25). This is consistent with our data, which shows CD4+ dominance in OVA-sensitized skin. However, in contrast to allergen exposure, topical SEB treatment elicited a marked infiltration of CD3+CD8+ cells into the skin, especially into the epidermis. Limited earlier evidence exists for the involvement of CD8+ T cells in protein-induced allergic inflammation. Previous reports have shown that few CD8+ T cells are found in the epidermis of AD patients but not in the epidermis of healthy controls (26). Furthermore, Huang et al. (27) reported recently that epicutaneous exposure to mite allergen induces allergic dermatitis and the infiltration of few CD8+ T cells into the epidermis. Similar with our OVA sensitization, both of these earlier observations show only few numbers of CD8+ T cells in the epidermis. The increased epidermal accumulation of CD8+ T cells after SEB exposure has not been reported to our knowledge in other animal experiments or in S. aureus-colonized atopic skin. However, it has been shown that CD8+ T cells cultured from lesional skin or CLA+ (cutaneous lymphocyte-associated Ag) CD8+ T cells from peripheral blood of AD patients efficiently proliferate after superantigenic stimulation (28). Furthermore, the restimulation of SEB-primed animals with SEB have been reported to lead to the clonal expansion of SEB-reactive CD8+ T cells (29). Our results suggest an increasing importance of CD8+ T cells in atopic skin, especially when colonized with SEB-producing strains of S. aureus.
In line with earlier reports (9, 15, 18, 30), we also found that cutaneous SEB exposure can trigger allergic inflammation. Laouini et al. (15) reported previously that repeated epicutaneous SEB exposure in the murine model of AD induces Th2-type skin inflammation in the skin. Similar to their results, we found increased levels of Th2-type cytokines in SEB-treated mouse skin. However, in contrast, our analysis also revealed a substantially increased Th1 cytokine mRNA expression at all SEB-treated skin sites, featuring a mixed Th2- and Th1-type immune response. These discrepant findings may due to differences in PCR methodology used for mRNA measurements. In the present study, mRNA expression was analyzed by a highly sensitive and quantitative real-time PCR using Taqman technology, whereas Laouini et al. (15) used a conventional PCR with multispecific internal plasmid control for their competitive PCR measurements. It should be noted that although the overall study by Laouini et al. (15) is reporting Th2 dominance in the SEB-exposed skin sites, they also observed a weak IgG2a Ab response to SEB supporting simultaneous induction of Th1 response. The enhanced IFN-γ production in SEB- and OVA/SEB-treated skin sites in our present study is likely derived from CD8+ cells rather than CD4+ cells since the number of epidermal CD8+ T cells was significantly increased in SEB- and OVA/SEB-treated mice, whereas the increase of CD4+ T cells was observed only in SEB-treated mice.

To elucidate mechanisms underlying the recruitment of inflammatory cells into SEB-treated skin sites, several chemokines were analyzed. Mechanical skin injury observed in patients with AD, subsequent to the intense itching, is known to induce the production of proinflammatory cytokines (1). Expression of proinflammatory cytokine regulated chemokines, CCL3 and CCL4, was strongly up-regulated in the exposed skin of OVA/SEB-treated mice, suggesting synergistic effect of topical SEB and allergen exposure. In addition, Th2-associated chemokines CCL1, CCL11, and CCL17 were significantly induced in the OVA/SEB-treated group. This data support the recruitment of Th2 cells to sites of inflammation. Furthermore, CXCR3 ligands, CXCL9, CXCL10, and CXCL11, were all enhanced after topical SEB or OVA/SEB treatment. Because chemokine receptor CXCR3 is expressed predominantly on Th1-type cells (31), cutaneous exposure to SEB recruits Th1 cells to the site of skin inflammation. Taken together, the present study shows a mixed production of proinflammatory Th1- and Th2-regulated chemokines in response to topical SEB exposure, which can be related to a chronic phase of atopic skin inflammation.

It is known that Vβ8+ T cells are the major SEB-responsive T cell population in BALB/c mice (32). According to Saloga et al. (18), cutaneous SEB exposure can cause inflammatory reactions, which are Vβ8+ T cell dependent. We found a dose-dependent increase in SEB-responsive Vβ8+ T cell population in the skin after repeated topical application of OVA/SEB or SEB. In contrast, SEB also induced the production of anti-SEB IgE Abs and also enhanced secretion of OVA-specific IgE. These results indicate a dual role of SEB in AD: 1) SEB can act as a superantigen, but 2) also as a conventional allergen by inducing specific IgE. SEB-induced enhancement of OVA-specific IgE may be partly explained by enhanced surface expression of MHC II molecules after topical allergen exposure. Although a major site of superantigen binding to MHC II lies outside the Ag-binding groove, the binding of certain Ag peptide to MHC II can affect binding of superantigen, leading to enhanced superantigen binding to MHC II (33).

**FIGURE 6.** Epicutaneous exposure to SEB elevates both IgE and IgG2a Ab production. A, Total, OVA- and SEB-specific IgE levels were detected from serum after topical exposure to SEB, OVA, OVA/SEB, and vehicle. B, Total, OVA-, and SEB-specific IgG2a Abs were measured in the serum after epicutaneous exposure to SEB, OVA, OVA/SEB, or vehicle. Bars represent mean ± SEM (n = 8 mice/group). *p < 0.05; **p < 0.01; and ***p < 0.001.

**FIGURE 5.** OVA- and SEB-induced production of major Th1 and Th2 cytokines in vitro. Production of IL-13 (A) and IFN-γ (B) after stimulation of skin draining LN cells from mice epicutaneously exposed to SEB, OVA, OVA/SEB, and vehicle. White bars represent IL-13 production after stimulation of LN cells with OVA, and black bars represent IL-13 and IFN-γ production after stimulation of LN cells with SEB (pooled results from four mice per group).
Present results demonstrate that SEB-stimulated LN cells from mice exposed to SEB in vivo produced high amounts of IFN-γ. This may due to SEB-specific T cells in LNs because LN cells from OVA- or PBS-sensitized mice did not produce IFN-γ. It is likely that these IFN-γ-producing cells are predominantly CD8+ T cells because the number of CD8+ cells, as well as Vβ8+ cells, is highly increased in SEB-exposed skin. Supporting our data, it has been earlier shown that a strong induction of IFN-γ in splenocytes from SEB-injected mice was detected in CD8+ Vβ8+ subset (34). Contrary to IFN-γ production in our experiment, LN cells from OVA-sensitized mice produced IL-13 after OVA stimulation, but a minor production of IL-13 was observed after stimulation of these cells with SEB. These findings are also in line with mRNA measurements demonstrating that OVA stimulation of LN cells from OVA- and OVA/SEB-sensitized mice elicits substantially higher levels of IL-13 mRNA compared with SEB stimulation (data not shown). IL-13 is likely derived from OVA-specific CD4+ T cells since only a few CD8+ Vβ8+ cells could be detected within the skin of OVA-sensitized mice. Furthermore, the absence of IFN-γ or IL-13 production in LN cells of PBS-sensitized mice after stimulation with SEB or OVA can be explained by the absence of inflammatory stimuli, and therefore, no or very few mature dendritic cells bearing MHC II migrate to draining LNs.

Increasing evidence exists that CD8+ T cells may play an important role in the pathogenesis of AD (25, 28). The present study shows that repeated SEB exposure induces the recruitment of CD8+ T cells into the epidermis. In addition to the CD4+ T cell subset, our results might provide increasing importance of CD8+ T cells in atopic skin colonized with SEB-producing strains of S. aureus. Therefore, the control of S. aureus colonization is an important aspect in the treatment of atopic dermatitis.

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Disclosures
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