Clinical and Experimental Allergy, 2000, Volume 30, pages 994–1000

Colonization with superantigen-producing *Staphylococcus aureus* is associated with increased severity of atopic dermatitis

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Summary

Background Atopic dermatitis (AD) is a chronic inflammatory skin disease associated with colonization of the skin with *Staphylococcus aureus* known to produce toxins with superantigen (SAg) activity. Besides T-cell activation these toxins induce T-cell skin homing *in vitro*. This may contribute to the observed induction or enhancement of skin inflammation.

Objective The aim of this study was to determine whether colonization with SAg-producing *S. aureus* isolates modulates the intensity of AD. If so, it was of interest whether this may be primarily due to the toxins' effects as SAgs or as allergens.

Methods In AD patients, healthy controls, and atopic controls SAg production by *S. aureus* isolated from skin or mucous membranes was investigated and correlated to the severity of the disease. Total IgE, SAg-specific IgE, and T-cell activation and recirculation markers were analysed and correlated with SAg production.

Results Fifty-seven percent of *S. aureus* strains isolated from AD patients produced SAgs. This frequency was higher compared to healthy controls (33%). SAg production by *S. aureus* was correlated with a significantly higher scoring of AD (SCORAD index, 58 ± 19 in SAg-producing vs 41 ± 7 in non-SAg-producing germs; $P < 0.05$). However, the severity of the disease was not associated with sensitization against the SAgs staphylococcal enterotoxin A (SEA) and staphylococcal enterotoxin B (SEB). Furthermore, SAg production by *S. aureus* was inversely correlated with total IgE concentration ($P < 0.05$) and positively correlated with T-cell activation (as measured by HLA-DR and CD69 expression) and the expression of the T-cell skin homing phenotype cutaneous lymphocyte-associated antigen.

Conclusion SAg production by *S. aureus* is suggested to be associated with an increased severity of atopic dermatitis. Since SAg production was found neither exclusively in AD patients nor in all patients, other pathogenic factors may be additionally effective.

Keywords: Atopic dermatitis, cutaneous lymphocyte-associated antigen, *Staphylococcus aureus*, SCORAD, specific IgE against SEA/SEB, staphylococcal enterotoxins, superantigen, T cell skin homing.

Clinical and Experimental Allergy, Vol. 30, pp. 994–1000. Submitted 2 July 1999; revised 15 October 1999; accepted 18 October 1999.

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Introduction

Although an association of atopic dermatitis (AD) with *Staphylococcus aureus* skin colonization has been repeatedly

reported for over more than 20 years, the role of bacterial superantigens (SAg) in the pathogenesis of AD is discussed controversially [1,2]. SAgs have been shown to induce eczematoid skin reactions when applied to intact normal or uninvolved skin of AD patients [3]. Since epicutaneous application of staphylococcal enterotoxin B (SEB) elicits a strong inflammatory response in the skin of BALB/c mice, but not T-cell-deficient SCID mice it is suggested that SAg-induced skin inflammation is T-cell dependent [4]. Furthermore, SAgs enhance skin inflammation in allergen-sensitized SCID mice and trigger dermal T cell infiltration [5]. This is probably due to induction of the skin homing phenotype in T cells by SAgs which has been observed *in vitro* [6–8]. Direct *in vivo* evidence for SAg involvement in skin homing of T cells in AD was recently shown [9]. Finally, 80% of AD patients exhibit elevated serum IgE levels. Interestingly, several authors demonstrated that SAgs influence IgE production *in vitro* and *in vivo* which seems to be dose-dependent [5,10–14]. Despite these studies the potential pathogenic role of SAgs in AD is doubted by some authors because only about 50% of *S. aureus* isolates are SAg-producing and SAg-producing germs are also found in healthy controls [15]. However, the genetic background is of major importance for the induction of T-cell-mediated dermatoses [16–18] and was not considered in the report by Jappe *et al*. [19].

The aim of this study was to determine the prevalence and role of SAg-producing *S. aureus* isolates in adults with AD. For this purpose SAg production was correlated with the total IgE concentration, presence of specific SAg IgE antibodies, peripheral blood T cell activation and homing markers and finally with the severity of AD as measured by the SCORAD index. We show that colonization of skin or mucous membranes with SAg-producing *S. aureus* is associated with an increased severity of adult AD.

Materials and Methods

Study populations

Sixty-five patients with moderate to severe AD (38 females, 27 males; mean age: 41 years; mean disease duration: 7 years), 65 healthy controls (35 females, 30 males; mean age: 46 years), and 21 atopic controls (13 females, eight males; mean age: 39 years) were included into the study. AD was diagnosed according to the criteria summarized by Hanifin and Rajka [20]. Severity of AD was determined according to the SCORAD index [21]. Atopic controls were suffering either from allergic rhinoconjunctivitis or asthma and had elevated total IgE (> 140 kU/L) and positive-specific IgE for airborne allergens or positive skin prick test reactions to several airborne allergens. Healthy controls never suffered from atopic disease, had normal total IgE (< 140 kU/L), negative-specific IgE for airborne allergens, and negative skin prick test reactions for airborne allergens such as mites and pollen.

Study inclusion and exclusion criteria

Inclusion criteria consisted of informed consent and aggravation of atopic dermatitis within the last 4 weeks with a 20% rise of involved area (for AD patients), age > 17 years. Exclusion criteria included stable disease and antibiotic or systemic immunosuppressive therapy within the last 4 weeks before the study, age < 18 years.

Isolation of S. aureus *and detection of* S. aureus*-derived superantigens*

During their first visit the patients' healthy skin of elbows (controls) or involved skin (AD patients) and mucous membranes (nose and throat) were scrubbed with sterilized swabs that had been soaked in sterilized saline solution [22,23]. Thereafter, bacterial samples were plated onto nonselective blood agar and in addition onto mannitol agar plates containing 7.5% sodium chloride to improve detection of *S. aureus*. Colonies recovered from nonselective media and appearing to be *S. aureus* were tested for rapid agglutination using the Staphslide kit (bioMerieux, Marcy l'etoile, France). To detect the production of enterotoxins and toxic shock syndrome toxin-1 (TSST-1) by *S. aureus,* five colonies were incubated in tryptine soya broth and brain heart infusion, respectively, for 24 h at 37 °C. Supernatants were tested for the presence of exotoxins using Oxoid toxin detection kits TST-RPLA and SET-RPLA for detection of TSST-1 and staphylococcal enterotoxins A-D, respectively (Unipath Limited, Hampshire, England) according to the manufacturer's instructions. This technique, which is based on the reverse passive latex agglutination, enables soluble antigens, such as bacterial toxins, to be detected in an agglutination assay. The sensitivity of the tests for undiluted samples is reported to be 0.5 ng/mL (SET-RPLA) and 2 ng/mL (TST-RPLA), respectively.

Reagents

The following fluoresceine isothiocyanate (FITC)- or phycoerythrin (PE)-conjugated monoclonal antibodies (mAb) or antibody pairs (FITC/PE) were obtained from Becton Dickinson, Heidelberg, Germany: CD3/CD4, CD3/CD8, CD3/CD19, CD3/CD16 + CD56, CD3/HLA-DR, CD8/HLA-DR, CD3/CD38, CD3/CD69, CD8/CD69, isotype controls (FITC/PE), CD15s (anti sialyl Lewis^x). Monoclonal antibodies HECA-452 recognizing cutaneous lymphocyte-associated antigen (CLA) was a kind gift of Dr Adrian M. Duijvestijn, University of Maastricht, The Netherlands.

CD103-FITC (mAb BerAct8) was obtained from DAKO, Hamburg, Germany, goat antimouse IgM-PE and goat antirat IgM-PE from Dianova and Hamburg, respectively. Total IgE assays (Abbott, Wiesbaden, Germany) were performed according to the manufacturer's instructions. Specific IgE for airborne allergens (mites, grass and tree pollen) and for the *S. aureus* superantigens staphylococcal enterotoxin A (SEA) and SEB was determined by use of ALASTAT assay (DPC Biermann, Bad Nauheim, Germany) according to the manufacturer's instructions. Specificity for SEA and SEB was demonstrated by inhibition experiments with SEA, SEB, and TSST-1 (personal communication, DPC Los Angeles, CA, USA).

Lymphocyte isolation and lymphocyte phenotype analysis

Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll-Paque density centrifugation. Staining was then performed in lysed-heparinized blood by standard direct or indirect labelling techniques with goat antimouse IgG-, goat antimouse IgM-, or goat antirat IgM-F(ab)2-FITC or -PE as secondary antibodies. In each sample, irrelevant mAb of the appropriate isotype were used as controls. Fluorocytometer analysis was performed on a flourescence-activated cell sorter (FACS) Calibur (Becton Dickinson) with CellQuest (Becton Dickinson) computer program. Lymphocytes were detected in a gate set on lymphocyte-sized cells.

Statistical analysis

Data are expressed as the mean ± SD or median (for total and specific IgE) for each group as indicated. Statistical comparisons in case of parametric values were made by use of Welch's *t*-tests for unpaired samples with the BiAS 5.01 computer program (H. Ackermann, Frankfurt, Germany). Differences between groups were considered significant at $P < 0.05$.

Results

The presence of serum IgE antibodies to SEA and SEB was analysed in 65 healthy controls, 21 atopic controls, and in 65 AD patients. In all controls, SEA- or SEB-specific IgE was less than 0.7 kU/L (range in healthy controls: SEA < 0.2–0.62 kU/l, median, 0.2 kU/l; SEB < 0.2–0.67 kU/l, median, 0.2 kU/l; atopic controls: SEA < 0.2–0.61, median, 0.2 kU/l; SEB < 0.2–0.68, median, 0.2 kU/l;). However, 22/65 AD patients (33.8%) were sensitized against SEA and/or SEB (12/22 to SEA and SEB, 7/22 to SEB, and 3/22 to SEA) with a median SEA-specific IgE of 1.9 kU/L (range, 0.7–8.7 kU/l) and of 1.6 kU/L (range, 0.7–37.3 kU/L for anti-SEB IgE). As reported before [24], IgE concentration in SAg-sensitized patients was significantly higher as compared to nonsensitized patients (Fig. 1).

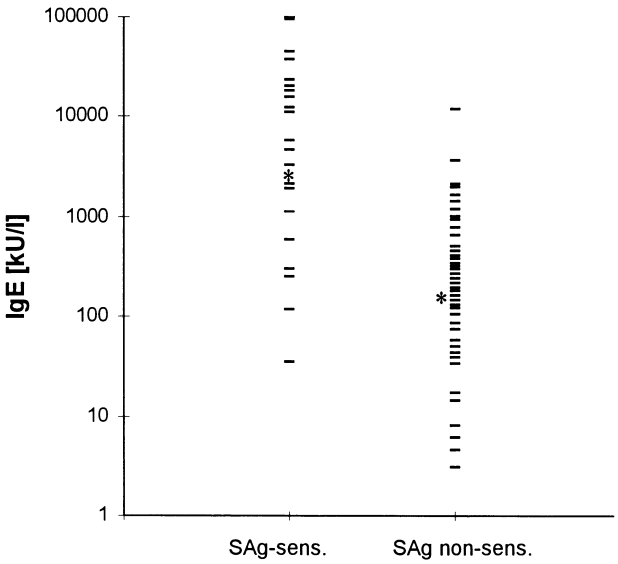

Fig. 1. The total IgE concentration in SAg-sensitized AD patients is significantly increased as compared to nonsensitized AD patients. *Represents the median IgE serum concentration. SAg sens., AD patients sensitized to SAg as determined by detection of antiSAg-specific IgE antibodies (specific IgE ≥ 0.7 kU/L); SAg nonsens., AD patients in which no significant levels of anti-SAg specific IgE antibodies have been detected (specific IgE < 0.7 kU/L).

In a subgroup of 104 individuals (50 healthy controls, 21 atopic controls and 33 AD patients) three swabs were taken according to standard procedures [22,23] (one from affected skin in AD patients or unaffected skin of elbows in controls, one each from nose and throat) for isolation of *S. aureus*. In 15/50 healthy controls, *S. aureus* was isolated and 5/15 (33%) isolates produced staphylococcal SAgs (three TSST-1, one SEB and one SEA and TSST-1). In 9/21 atopic controls, *S. aureus* was isolated of which 3/9 (33%) isolates produced SAgs (one SEA, one SEB, one SEA and TSST-1). In AD patients, SAg production by *S. aureus* isolates was markedly higher. In 23/33 AD patients, *S. aureus* colonization was detectable. Thirteen from twenty-three isolates (57%) produced SAgs (five SEB, three TSST-1, one SEC, one SED, and three isolates produced more than one SAg) compared to healthy controls (5/15; 33%). In contrast to an earlier report on childhood AD [24], in this study the rate of SAg-producing germs isolated from SAg-sensitized patients did not increased as compared to nonsensitized patients (40% in SAg-sensitized compared to 69% in nonsensitized patients). Furthermore, in adult AD, SAg-sensitization occurred in both patients with moderate and severe AD. There was no association between SAg sensitization and the SCORAD index in adult patients suffering from AD

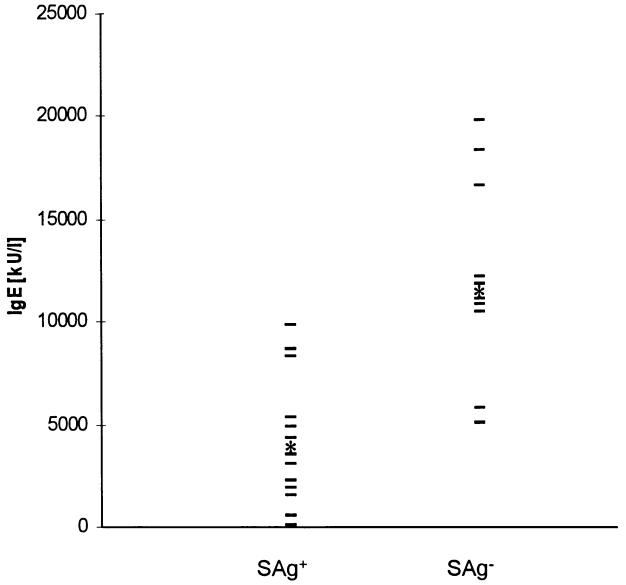

Fig. 2. The total IgE concentration inversely correlates with colonization with SAg-producing *S. aureus* isolates. *Represents the median of IgE concentration. SAg^+, AD patients in which colonization with SAg-producing *S. aureus* isolates has been detected; SAg^-, AD patients with SAg nonproducing *S. aureus* isolates.

(SCORAD in SAg-sensitized patients 53 ± 18 vs 48 ± 17 in SAg-nonsensitized).

Since several reports demonstrated an impact of SAgs on IgE production [5,10–14], IgE concentration was analysed in individuals colonized by SAg-producing *S. aureus* isolates as compared to SAg-nonproducing germs. In both control groups, IgE concentration tended to be lower in patients colonized by SAg producers (healthy controls: median 81.5 kU/L (range, 8–760 kU/L) in the SAg positives vs 113.0 kU/L in SAg negatives (range, 53–631); atopic controls: 412 KU/L (range, 134–631 kU/L) in SAg positives vs 800.0 kU/L in SAg negatives (range, 60.2–2134 kU/L). In AD patients, this trend was again observable and reached statistical significance. Colonization with SAg-producing *S. aureus* was correlated with a significantly lower total IgE than colonization with SAg-nonproducing isolates (median: 3534 kU/L (range, 72–8708 kU/L) vs 11176 kU/L (range, 5438–19735 kU/l; $P < 0.05$; Fig. 2).

SAgs have been recently shown to deteriorate skin inflammation in response to allergen in allergen-sensitized SCID mice [5]. We therefore assessed the influence of *S. aureus* SAgs by comparing the SCORAD index in AD patients colonized with SAg-producing isolates with patients colonized with SAg-nonproducing germs. In the former group, the SCORAD index was significantly higher compared to the latter group (58 ± 19 vs 41 ± 7; $P < 0.05$; Fig. 3).

Since SAgs have a strong influence on T cell activation and homing receptor expression [6–8], we analysed these parameters in peripheral blood T cells to see whether colonization of the skin or mucous membranes with

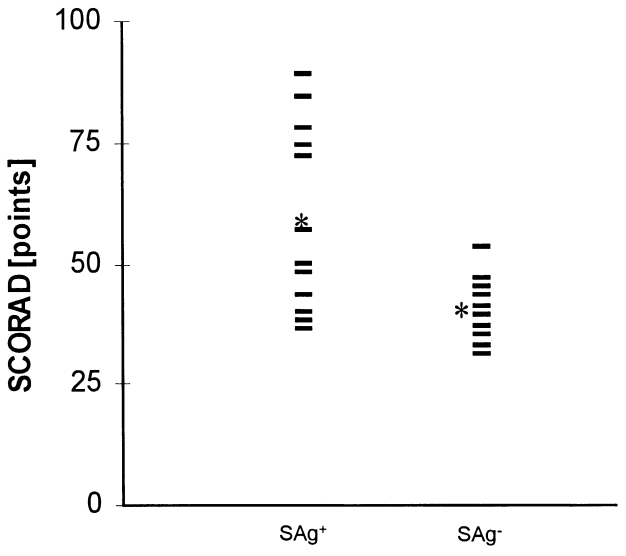

Fig. 3. The severity of AD as determined by the SCORAD index correlates with SAg-production of *S. aureus* isolates. *represent the mean SCORAD indices.

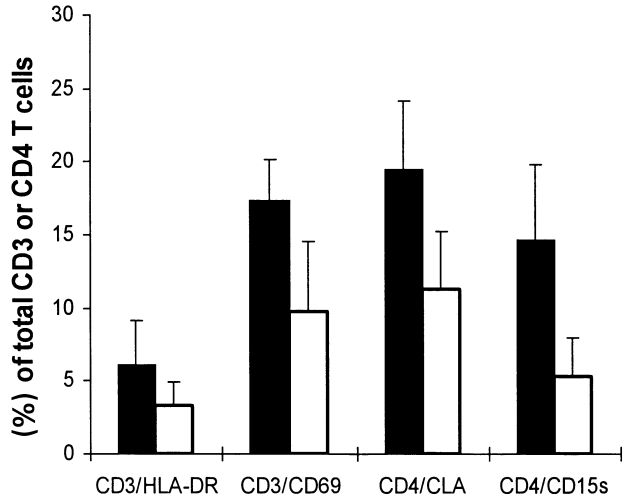

Fig. 4. HLA-DR, CD69, CLA, and CD15s expression by T cells. PBMC were obtained from patients suffering from atopic dermatitis. Black bars (SAg^+) represent patients in which SAg-producing *S. aureus* were detectable, whereas open bars (SAg^-) represent patients in which no SAg production was detectable from *S. aureus* isolates. Data show mean ± SD. SAg^+; $n = 13$, SAg^-, $n = 10$. $P < 0.05$ CD3/HLA-DR expression SAg^+ vs SAg^-; $P < 0.05$ CD3/CD69 expression SAg^+ vs SAg^-; $P < 0.05$ CD4/CLA expression SAg^+ vs SAg^-; $P < 0.05$ CD4/CD15s expression SAg^+ vs SAg^-.

SAg-producing germs may have a general impact on the cellular and humoral immune system. The total and relative numbers of lymphocyte subsets such as $CD3^+$, $CD3^+/CD4^+$, $CD3^+/CD8^+$, $CD19^+$, and $CD16^+ + CD56^+$ remained unchanged between the two groups (AD patients colonized with SAg-producing isolates as compared to SAg-nonproducing isolates). However, in AD patients colonized with SAg-producing isolates as compared to nonSAg-producing ones, activation markers such as CD69 and HLA-DR were significantly upregulated by $CD3^+$ and $CD3^+/CD8^+$ lymphocytes, but not by $CD3^+/CD4^+$ lymphocytes ($P < 0.05$ using the Welch's test; Fig. 4, data for $CD8^+/CD69^+$ and for $CD8^+/HLA\text{-}DR^+$ cells not shown). Furthermore, CLA and CD15s expression by CD4+ lymphocytes was significantly induced in these patients ($P < 0.01$; Fig. 4). However, BerACT-8 expression recognizing gut-homing T cells was not differentially expressed among the two groups (data not shown).

Discussion

This study demonstrated that AD patients colonized with SAg-producing *S. aureus* had a significantly increased severity of the disease as measured by the SCORAD index compared to patients colonized with *S. aureus* isolates without SAg production. *S. aureus* isolated from AD skin secrete various SAgs in about 57% to 65% of patients [25–27]. In this study a prevalence of 54% was observed in AD patients. Strange *et al*. were the first to provide direct evidence that SAgs applied onto uninvolved skin of AD patients or skin of healthy individuals induced erythema and induration [3]. Additionally, survivors of toxic shock syndrome show an increased frequency of (atopic) dermatitis as compared to survivors of septic shock [28]. In SCID mice reconstituted intraperitoneally and subcutaneously with PBMC from Der p allergic AD patients, a skin infiltrate, acanthosis, parakeratosis, and spongiosis was observed after application of a SAgs onto transplanted human skin. An even stronger skin inflammation was observed when SAgs and the allergen Der p were applied simultaneously [5]. These data provide evidence that *S. aureus*-derived SAgs trigger inflammatory immune responses in different models of AD and in AD patients.

To study the mechanism(s) by which *S. aureus* might be responsible for an increased severity of AD, total IgE concentrations were measured in this study because (1) IgE is known to be elevated in 80% of AD patients. These levels correlate with the activity of skin disease [1] and (2) IgE concentrations are known to be affected by SAgs. If bacterial SAgs contribute to allergic inflammation in AD patients, enhanced IgE production in response to SAgs is expected. This has been reported *in vitro* and occurs if low SAg concentrations (e.g. 0.01 pg/mL TSST-1) are effective [10,12,13]. IgE production in response to bacterial SAgs is secondary to T-cell-dependent B-cell activation after bridging of T and B cells by SAgs and does not occur in the absence of T cells [10]. In contrast to low SAg concentrations, high concentrations (e.g. 1 pg/mL TSST-1) inhibited IgE production *in vitro* from both healthy individuals and AD patients. This inhibition of IgE production is probably due to IFNγ and/or IFNα which is released in response to high but not low concentrations of bacterial SAgs [10–12]. Our study revealed the unexpected finding that total IgE was significantly lower in AD patients colonized with SAg-producing *S. aureus* than in patients colonized with *S. aureus* who did not produce SAgs. Suppressed IgE production associated with enhanced skin inflammation by SAgs has been recently observed by Herz *et al*. in a SCID mouse model [5]. SEB treatment reduced allergen-induced IgE synthesis in SCID mice which were intraperitoneally reconstituted with PBMC from Der p allergic atopics. IgE suppression was explained by an increased IFNγ production after *in vivo* treatment with SEB [5]. A negative correlation between IFNγ levels and SAg-mediated IgE production was also recently observed [11]. Finally, suppression of IgE production by SAgs in IL-4-treated PBMC cultures from AD patients has also been reported by König *et al*. [29] and Jabara *et al*. [14]. Our observation of a suppressed IgE production in the presence of SAg-producing *S. aureus* indicates that in the local skin milieu high concentrations of bacterial SAgs are effective which may induce a pronounced local IFNγ production. This could be responsible for the reduced IgE production in the subgroup of patients with SAg-producing *S. aureus*. Induction of IFNγ is also a hallmark in chronic AD lesions [30,31] and locally-produced IFNγ in response to SAgs is likely to contribute to an aggravation of chronic skin inflammation. Furthermore, a high ratio of T cell:professional antigen presenting cell within the skin compartment may be responsible for the suppression of IgE production by SAgs [14].

Alternatively, the patients colonized with SAg might be intrinsically less atopic and may therefore have lower IgE serum concentrations. If this would be true it would even more underscore the importance of SAg-producing germs for the severity of AD. Thus, the lower IgE serum concentration might reflect a lesser atopic tendency rather than be causally related to the presence of SAgs themselves.

Besides their action as SAgs, staphylococcal exotoxins can act as allergens in AD patients. Thirty-four to 96% of sera from AD patients contain IgE antibodies against SEA, SEB and/or TSST-1 whereas relevant levels of those antibodies are extremely rare in healthy controls or psoriasis patients although the skin of these individuals is also colonized with *S. aureus* capable of SAg production [24,25,32,33]. The prevalence of antiSAg IgE antibodies of 33.8% as reported in this study is in the lower range as the

above mentioned reports. Methodological reasons for this can be excluded because in the study by Tada *et al*. (high prevalence of 80.2%), and in that of Bunikowski *et al*. and in our study (low prevalence of 34% and 33.8%, respectively) the same reagents have been used. IgE antibodies against SAgs are also of functional importance because basophils from SAg-sensitized AD patients released 5% to 59% of total histamine in response to SAgs [25]. Histamine release is, however, only detectable in about two-thirds of patients sensitized to SAgs [33]. It was hypothesized that Sags–as allergens–may exacerbate AD by activating mast cells, basophils, and/or other Fcϵ-receptor-bearing cells [25] which may then lead to the continuation of the itch-scratch cycle. In good agreement with the reported *in vitro* data, a correlation of antiSAg-antibodies and the SCORAD index has been found in children with AD [24], which has not been observed in adult AD patients [32]. In our study performed in adults we did not find an association of SAg sensitization with the clinical outcome as determined by the SCORAD index.

Besides the polyclonal activation of B cells by binding to MHC class II molecules, SAgs are the most potent activators of T cells acting in a T-cell-receptor Vβ-specific manner [34]. Therefore, we have analysed the *in vivo* influence of SAgs on T-cell activation and recirculation by comparing the activation and homing markers expressed by peripheral blood T cells from AD patients colonized with SAg-producing *S. aureus* germs with AD patients colonized with SAg-nonproducing isolates. Most interestingly, AD patients of the former group had significant higher relative and absolute counts of activated $CD3^+$ T cells as compared to the latter group. Furthermore, expression of the skin-selective homing receptor cutaneous lymphocyte-associated antigen CLA was significantly higher in the SAg-producing group as compared to the nonproducing group. Induction of HLA-DR has been shown previously to be correlated with the severity of AD [35,36]. Increased CLA expression by T cells was also observed in the peripheral blood of AD patients compared to healthy controls [36]. Induction of CLA by SAgs *in vitro* has been reported previously [6–8] and is probably due to induction of FucTVII mRNA expression by SAgs [37]. This may lead to the invasion of highly activated T cells into the skin with potential release of their cytokines and activation of cells other than lymphocytes such as keratinocytes and cells of the dermal monocyte/macrophage system which aggravates the skin inflammation. Additionally, by acting as allergens, superantigens induce histamine release from mast cells which results in another round in the itch-scratch cycle.

However, *S. aureus* was not detected in all patients. This might be due to the fact that approximately 60% of individuals are colonized only intermittently by *S. aureus* [38] and therefore *S. aureus* was not detected at the time of examination. Furthermore, SAgs are not produced by all *S. aureus* isolates and other bacterial products such as α- or β-toxins may account for induction of inflammation in these AD patients. Additionally, other triggering factors such as food allergens are also responsible for the exacerbation of AD and may be especially effective in the group in which SAg-producing S. *aureus* could not be isolated [39].

Our results suggest that SAgs are responsible for an increased severity of AD at least in a relevant subset of patients. The activation of T cells and induction of the T-cell skin homing phenotype by SAgs rather than the increase of total IgE production or induction of a SAg-specific sensitization seems to be accountable for the this observation.

Acknowledgements

T. M. Zollner acknowledges support from the August Scheidel-Stiftung and the Paul und Ursula Klein-Stiftung. We thank Sandra Diehl, Gitla Asmus, and Denia Franck for perfect technical assistance.

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