

Isolation of α -toxin-producing *Staphylococcus aureus* from the skin of highly sensitized adult patients with severe atopic dermatitis

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Summary

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Accepted for publication

27 February 2009

Key words

α -toxin, atopic dermatitis, atopy, exotoxin, *Staphylococcus aureus*

Conflicts of interest

None declared.

DOI 10.1111/j.1365-2133.2009.09229.x

Background *Staphylococcus aureus* (*S. aureus*) is a well-known trigger factor of atopic dermatitis (AD). Besides staphylococcal superantigens, α -toxin may influence cutaneous inflammation via induction of T-cell proliferation and cytokine secretion.

Objectives To investigate the association between sensitization to inhalant allergens and skin colonization with α -toxin-producing *S. aureus* in AD.

Patients and methods We investigated 127 patients with AD, aged 14–65 years, who were on standard anti-inflammatory and antiseptic treatment before investigation. We evaluated skin colonization, medical history, severity of AD and sensitization to inhalant allergens.

Results Forty-eight of 127 patients were colonized with *S. aureus*, suffered from more severe AD, had asthma more often and showed higher sensitization levels to inhalant allergens. Thirty of 48 patients with *S. aureus* skin-colonizing strains produced α -toxin and had higher total IgE and specific IgE to birch pollen and timothy grass pollen.

Conclusions Under topical treatment with antiseptic and anti-inflammatory agents the colonization of lesional skin with *S. aureus* was clearly lower than commonly found in untreated patients with AD. Colonization with *S. aureus* was associated with a higher severity of AD, higher degree of sensitization, and a higher frequency of asthma. The proportion of patients whose skin was colonized with α -toxin-producing *S. aureus* was higher than expected from a former study. Cutaneous colonization with α -toxin-producing *S. aureus* was associated with a higher sensitization level to birch pollen allergen in AD. This may point to a higher susceptibility of patients with higher T-helper 2 polarization towards α -toxin-producing *S. aureus*.

Atopic dermatitis (AD) is a chronic inflammatory skin disease, which often begins in infancy and usually has a chronic relapsing course. In recent years, the incidence of AD has increased worldwide.^{1,2} AD is clinically well characterized. Different working groups have established criteria aiding the clinician in diagnosing AD.^{3,4} The pathogenesis of AD is very complex and not yet fully understood. Together with allergic bronchial asthma, allergic rhinoconjunctivitis and food allergies, AD belongs to the group of atopic diseases.

Allergen-specific T cells play a crucial role in the pathogenesis of AD at the cellular level. Proliferative responses can be elicited in blood lymphocytes by inhalant allergens, including

house dust mite, pollen and food allergens.^{5–10} *Staphylococcus aureus* has been identified as a further trigger factor.^{9,10} In an untreated population 80–100% of patients with AD are colonized with *Staphylococcus aureus* (*S. aureus*) without showing any clinical signs typical of a bacterial infection.^{11–13} A reduction of the colonization is possible with systemic antibacterial and/or topical antiseptic or antibacterial treatment, but after such therapy a rapid recolonization occurs in these patients.¹⁴ In comparison, only 5–30% of healthy individuals are carriers of this microorganism. *S. aureus* can be isolated from both clinically affected and unaffected skin of patients with AD; however, bacterial counts on the latter are lower than on lesional

skin. The anterior nares have been identified as a reservoir of *S. aureus*, from where the cutaneous colonization can spread. A downregulation of some antimicrobial peptides in keratinocytes of patients with AD has been discussed as a possible cause for the susceptibility to cutaneous colonization of AD with *S. aureus*.^{15–17} Thirty per cent to 60% of *S. aureus* strains have been found to secrete exotoxins with superantigenic properties.^{14,18–20} Different studies have demonstrated an association between superantigen-producing *S. aureus* strains and the severity of AD.^{21–23} Superantigens can augment an antigen-specific T-helper (Th) 1 response via the induction of interleukin-12 in antigen-presenting cells.^{24,25} This could be a possible mechanism contributing to the chronification of AD. On the other hand, treatment with antimicrobial substances may lead to clinical improvement of AD in patients who were colonized with nontoxicogenic *S. aureus* strains.¹⁴ Therefore pathogenetic factors other than superantigens derived from *S. aureus* might be involved.

Some *S. aureus* strains are able to produce α -toxin.²⁶ In a previous study we found that 30% of *S. aureus* strains isolated from patients with AD produce α -toxin.²⁷ In higher concentrations, α -toxin may lead to necrosis of the skin and nerves and may induce apoptosis in lymphocytes. In the former study we showed that sublytic concentrations of α -toxin were able to effectively activate T lymphocytes, which led to proliferation and induction of IFN- γ on both the RNA and the protein level. This indicates that sublytic concentrations of α -toxin may serve as a pathogenetic factor in AD.²⁷

In the present study, we investigated skin colonization with *S. aureus* and its toxin production in adolescent and adult patients with mild to very severe AD who were on regular antiseptic and anti-inflammatory treatment before the investigation. We compared the clinical disease activity and sensitization to inhalant allergens between subgroups of patients colonized with α -toxin-producing *S. aureus*, and those who were not colonized.

Patients and methods

After approval by the local ethics committee of the Hannover Medical School and obtaining informed consent 127 adolescent and adult patients with AD [age range 14·17–71·5 years, 30·17 $\pm$ 23·35–41·57, median $\pm$ interquartile range (IQR)], defined according to the criteria of Hanifin and Rajka,³ were enrolled into the study. All patients were recruited from the outpatient clinic of the Department of Dermatology, Venerology and Allergology, Hannover Medical School, Hannover, Germany. Patients were on treatment with topical antiseptic (e.g. 2% triclosan) and anti-inflammatory (corticosteroids of mild to moderate potency and calcineurin inhibitors) substances. The clinical severity of AD was determined by the SCORAD, which is a scoring system combining extent, severity and subjective symptoms of AD. The SCORAD ranged between 1 and 83 (median 35, IQR 22–52). Patients were asked about their relevant medical history, especially allergic bronchial asthma and the disease activity of AD by questionnaires.

Isolation of *Staphylococcus aureus* and identification of α -toxin

Rayon-tipped swabs were taken from the lesional skin of 127 adolescent and adult patients with mild to very severe AD without any clinical signs of cutaneous infections. *S. aureus* isolates were screened for α -toxin production using the synergistic haemolysis (CAMP-like) test on Columbia 5% sheep blood agar plates (OXOID) with α -toxin-positive and -negative *S. aureus* strains as controls.

Determination of total IgE and specific IgE

The levels of total IgE and specific IgE to different inhalant allergens were determined in the sera of AD patients by CAP FEIA, according to the manufacturer's instructions (Phadia, Freiburg, Germany). Specific IgE concentrations of more than 0·35 kU L⁻¹ were judged to indicate sensitization.

Statistics

Statistical analysis was performed with the SAS statistical software package (Version 9.1; SAS Institute, Cary, NC, U.S.A.). Due to the skewed distribution of all variables considered here, the Mann–Whitney U-test was used as a nonparametric test. In view of the particularly small size of patient subgroups being compared, Monte Carlo estimation of the exact P-value with accompanying 99% confidence interval (CI) was employed. Categorical data were analysed with Fisher's exact test. $P < 0\cdot05$ indicated statistical significance. In view of the exploratory nature of the analyses, no adjustment for multiple testing was employed.

Results

Skin colonization with *Staphylococcus aureus* and detection of enterotoxins and α -toxin

Upon treatment with antiseptic and anti-inflammatory substances *S. aureus* was still detectable on lesional skin in 48 out of 127 patients. Thirty of the 48 colonized patients yielded α -toxin-producing *S. aureus* strains. In nine out of 30 patients who were colonized with α -toxin-producing *S. aureus* strains staphylococcal superantigens (SEA-D, TSST-1) were also detectable.

Disease severity in patients colonized with *Staphylococcus aureus*

Patients colonized with *S. aureus* showed a significantly higher disease activity measured by SCORAD (Fig. 1), exact P-value 0·0012 (99% CI 0·0003–0·0021). In total, 68 of 127 patients with AD (54%) also suffered from allergic asthma. Allergic asthma occurred more often in *S. aureus*-positive patients with AD (69%) compared with *S. aureus*-negative patients (44%) ($P = 0\cdot01$, Fisher's exact test). Comparing patients colonized with α -toxin-producing *S. aureus* strains and α -toxin-negative

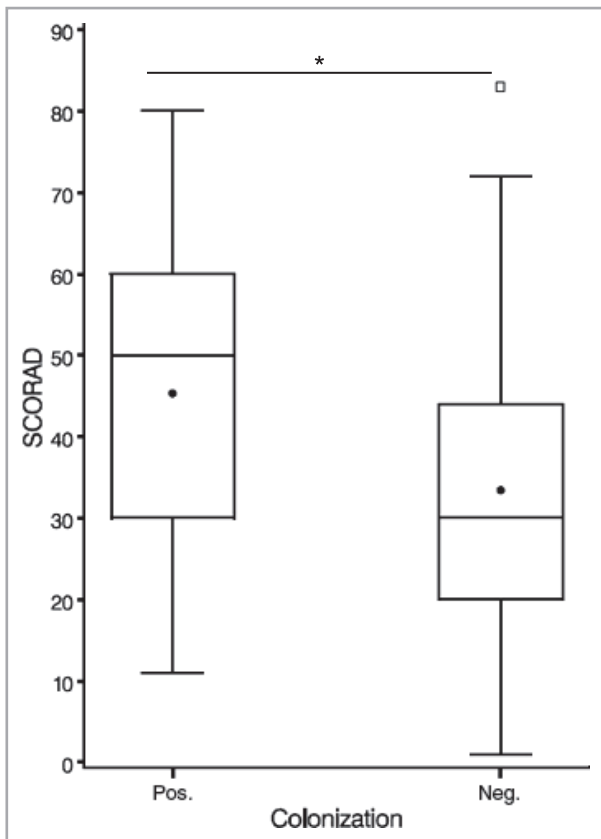


Fig 1. Severity of atopic dermatitis in *Staphylococcus aureus*-positive and -negative patients. Patients colonized with *S. aureus* had significantly higher disease severity measured by SCORAD (median $\pm$ IQR, $n = 127$, $*P < 0.001$, t -test).

strains a similar disease severity as measured by SCORAD was found in the two groups [median (IQR) 50 (30–60) vs. 46 (30–61)]; this difference was not significant: exact P -value = 0.948 (99% CI 0.943–0.954).

Total IgE and specific IgE to birch pollen and timothy grass pollen in patients with atopic dermatitis

Total IgE ranged from 4.44 to 55 050.00 kU L^{-1} (median 1151.00, IQR 207.25–7177.25 kU L^{-1}). Ninety-two of 127 patients were sensitized to birch pollen, and 97 of 127 showed a sensitization to timothy grass pollen. Specific IgE levels to birch pollen ranged from 0.37 to 100.00 kU L^{-1} (median 41.90, IQR 9.06–100.00 kU L^{-1}) in sensitized patients. Specific IgE levels to timothy grass pollen ranged from 0.37 to 100.00 kU L^{-1} (median 37.50, IQR 7.38–100.00 kU L^{-1}) in sensitized patients.

Total and specific IgE to birch pollen and timothy grass pollen in patients colonized with *Staphylococcus aureus* and patients not colonized with *Staphylococcus aureus*

Total IgE was significantly higher in patients who were colonized with *S. aureus* in comparison to *S. aureus*-negative patients

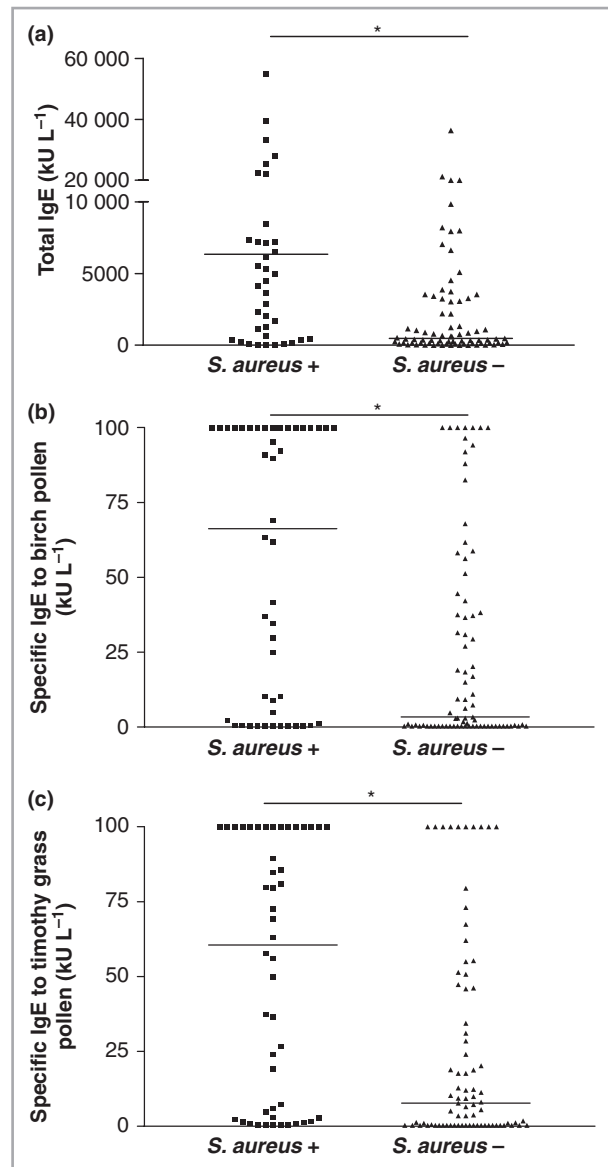


Fig 2. Total and specific IgE in *Staphylococcus aureus*-positive or -negative patients. *S. aureus*-colonized patients showed a significantly higher total IgE (a) and specific IgE to birch pollen (b) and timothy grass pollen (c) (median $\pm$ 25–75% quartile; $*P < 0.001$, Mann–Whitney U -test).

(Fig. 2a), exact P -value < 0.0001 (99% CI < 0.0001 – 0.0005). Forty-one of 48 patients colonized with *S. aureus* were sensitized to birch pollen, compared with 51 of 79 patients in the *S. aureus*-negative group ($P = 0.014$, Fisher's exact test). Sensitization to timothy grass pollen was observed in 44 of 48 patients colonized with *S. aureus* and in 53 of 79 patients without skin-colonizing *S. aureus* strains ($P = 0.0013$, Fisher's exact test). In accordance with this, we found significant differences for specific IgE serum levels to birch pollen (exact P -value 0.0001, 99% CI < 0.0001 – 0.0004) and to timothy grass pollen (exact P -value 0.0005, 99% CI < 0.0001 – 0.0011) between patients colonized with *S. aureus* and without skin colonization (Fig. 2b,c).

Total and specific IgE to birch pollen and timothy grass pollen in patients colonized with *Staphylococcus aureus* producing α -toxin compared with patients colonized with *Staphylococcus aureus* not producing α -toxin

We further compared total and specific IgE levels to common inhalant allergens in patients whose skin was colonized with α -toxin-producing *S. aureus* strains (Fig. 3). Patients colonized with α -toxin-producing *S. aureus* strains showed a trend to higher total IgE values (exact P-value 0.1331, 99% CI 0.1243–0.1419, Mann–Whitney U-test). Twenty-eight of 30 patients colonized with α -toxin-producing *S. aureus* strains were sensitized to birch pollen, compared with 13 of 18 in the α -toxin-

negative group. Specific IgE serum levels to birch pollen in the group of α -toxin-positive patients were significantly higher than in the negative patients (exact P-value 0.0280, 99% CI 0.0238–0.0322, Mann–Whitney U-test). Sensitization to timothy grass pollen was observed in 28 of 30 patients with α -toxin-producing *S. aureus* strains on their skin and in 16 of 18 patients with α -toxin-negative strains. Specific IgE serum levels to timothy grass pollen were also higher in the α -toxin-positive group, although this difference did not reach statistical significance (exact P-value 0.1419, 99% CI 0.1329–0.1509, Mann–Whitney U-test).

Discussion

S. aureus is a well-known trigger factor in AD. In untreated patients up to 90% have skin colonized with *S. aureus*.^{11,14,19,28–30} *S. aureus* is able to produce a variety of different exotoxins, among them staphylococcal superantigens (SEA, SEB) which can also act as an allergen.^{18–23,31,32} One reason for the high susceptibility to *S. aureus* colonization in patients with AD might be a deficiency in the production of antimicrobial peptides such as human defensins as a component of the innate immune system,^{16,17} although it has been shown that β -defensin 2 is induced in *S. aureus*-colonized AD lesions.¹⁵ The fact that antimicrobial treatment also leads to an improvement of AD in patients whose skin is colonized with superantigen-negative *S. aureus* strains¹⁴ led to the hypothesis that factors other than superantigens derived from *S. aureus* may trigger and maintain AD.

The cytotoxin α -toxin is produced by many *S. aureus* strains isolated from humans^{26,33} and does not belong to the group of exotoxins with superantigenic properties. In a former study we described that the skin of approximately 30% of adult patients with AD, who exhibited no obvious evidence of cutaneous infection, was colonized with α -toxin-secreting *S. aureus* strains.²⁷ Furthermore, immunohistochemical staining of skin sections derived from patients with AD colonized with α -toxin-producing *S. aureus* strains demonstrated α -toxin in the epidermis and the upper dermis in a pattern suggestive of penetration of α -toxin into the skin.²³

The incubation of α -toxin with rabbit erythrocytes leads to cell lysis, and intravenous injection of concentrations of α -toxin as low as 1 μ g is lethal for rabbits.^{26,33} It is known that α -toxin monomers have a molecular weight of 33 000 Da, are secreted by *S. aureus*, and integrate into the membrane of target cells, among them keratinocytes and lymphocytes,^{34,35} where they form cylindrical heptamers. These heptamers build 1- to 2-nm transmembrane pores and this allows rapid influx and efflux of different ions, which results, for example, in osmotic swelling and subsequent cell rupture.^{26,36,37} Pore-forming molecules (i.e. haemolysins, perforins, complement proteins) may activate cells in sublytic concentrations.^{38–41} Previously, we showed that sublytic concentrations of α -toxin were able to activate T lymphocytes in the absence of antigen-presenting cells with proliferation and IFN- γ production to a high degree.²⁷

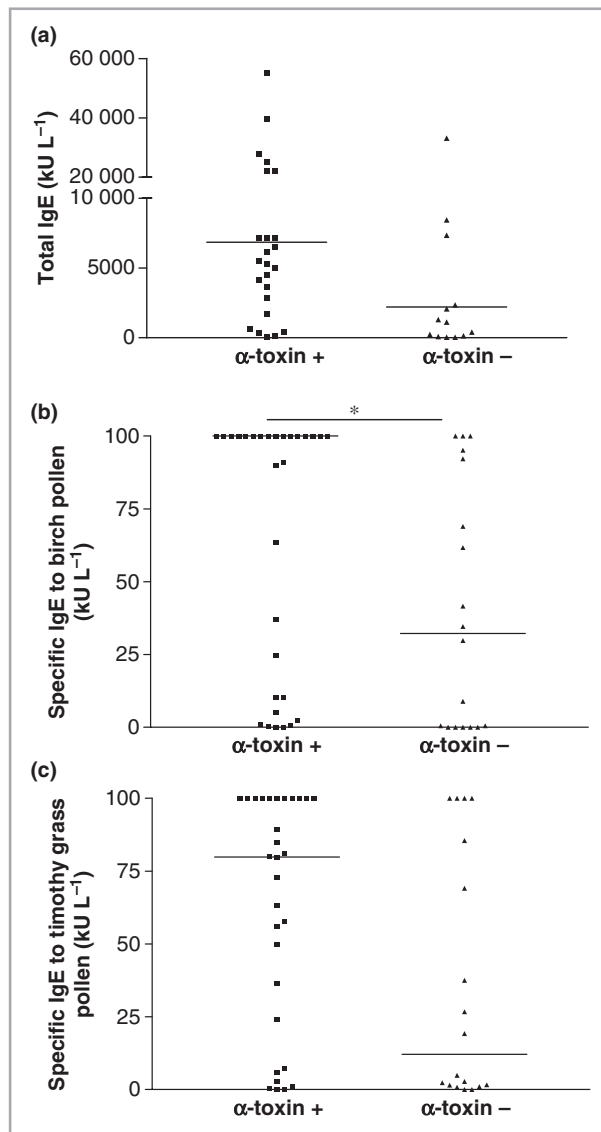


Fig 3. Total or specific IgE in patients with α -toxin-positive or -negative *Staphylococcus aureus* strains. α -toxin-positive patients showed a higher total IgE (a) and specific IgE to birch pollen (b) and timothy grass pollen (c) (median $\pm$ 25–75% quartile; *P < 0.001, Mann–Whitney U-test).

In the current study, we demonstrated that in patients with severe AD a high percentage (61.0%) was still colonized with *S. aureus* despite topical treatment with antiseptic and anti-inflammatory agents. Patients whose skin was still colonized with *S. aureus* during antiseptic and anti-inflammatory treatment showed significantly higher disease activity. Gong *et al.*⁴² described the frequency of colonization with *S. aureus* in patients with AD following topical treatment with hydrocortisone butyrate ointment alone or in combination with topical mupirocin ointment. The study showed a decreased skin colonization of *S. aureus* from 60% to about 35% when the combination of both substances was used.⁴² Here, for the first time, we have investigated the secretion pattern of α -toxin from *S. aureus* strains, which were still detectable after topical antiseptic and anti-inflammatory treatment of AD. We found a high proportion (62.5%) of α -toxin-secreting *S. aureus* strains in this group. We speculate that α -toxin might hinder eradication of *S. aureus*. To confirm this hypothesis prospective investigations with colonization analysis before and after treatment are necessary. It is known that α -toxin may lead to cell damage in keratinocytes, so that it is also possible that sublytic concentrations of α -toxin promote defects in the skin barrier.^{35,43,44} Because α -toxin hitherto has been investigated only in conjunction with mouse models to infectious diseases no data exist concerning the influence of α -toxin in the epidermal barrier.

It is a fact that patients with AD show a higher risk to develop other atopic diseases like allergic asthma or allergic rhinoconjunctivitis. We demonstrate that highly atopic individuals with higher total IgE levels and specific IgE levels to birch pollen and timothy grass pollen were more often colonized with *S. aureus* and that these patients more frequently suffered from allergic bronchial asthma. Moreover, colonized patients had significantly higher serum levels of total and specific IgE to birch pollen and timothy grass pollen. Surprisingly, α -toxin was detectable more often in this group of patients. We did not find a difference in disease severity between patients colonized with α -toxin-positive and -negative *S. aureus* strains. As most patients colonized with *S. aureus* had a high disease severity irrespective of α -toxin production, possible differences between the two subgroups of toxin-producers and nonproducers might have been masked. However, for specific IgE to birch pollen we showed a significant difference between patients colonized with α -toxin-positive and -negative *S. aureus* strains. This may point to a susceptibility of patients with higher Th2 polarization towards α -toxin-producing *S. aureus*.¹⁶ Further studies are needed to elucidate whether there is a direct link between Th2 polarization and cellular stimulation with α -toxin in AD.

From these data, we conclude that α -toxin may be a critical factor which impairs the elimination of *S. aureus* and that α -toxin-secreting *S. aureus* strains are found more frequently in patients with severe AD in comparison with other studies.²⁷ Further investigations are needed to reveal cellular mechanisms that are related to the activity of α -toxin in the skin and the higher percentage of *S. aureus* found in patients with AD.

Acknowledgment

This study was supported by a grant of the Dr Karl Wilder-Stiftung.

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