

Presence of staphylococcal exfoliative toxin A in sera of patients with atopic dermatitis

S. Yagi*, N. Wakaki*, N. Ikeda*, Y. Takagi†, H. Uchida‡, Y. Kato* and M. Minamino*

*Club Cosmetics Co., Ltd., Research and Development Division, Nara, Japan, †Department of Dermatology, Jikei University School of Medicine, Tokyo, Japan and ‡Department of Pediatrics, International Medical Center of Japan, Tokyo, Japan

Summary

Background It has been reported that the toxins that *Staphylococcus aureus* produces are associated with the exacerbation of atopic dermatitis (AD). It has been shown in many studies that staphylococcal enterotoxin (SE) A and SEB contribute to AD by humoral immunity through IgE production as a superantigen. On the other hand, little attention has been paid to the relationship between AD and exfoliative toxin x (ETx).

Objective We investigated the toxins that are frequently detected from the skin of patients and how these toxins affect AD.

Methods *S. aureus*, isolated from the skin of 100 patients with mild to severe AD, were examined for the producibility of toxins by polymerase chain reaction. Serum samples were obtained from 21 patients with mild and moderate AD. The levels of SEB, ETA, total IgE, specific IgE, and specific IgG in sera were measured by ELISA.

Results SEB was most frequently detected from *S. aureus* on the skin of these patients as previously reported. And ETx, to which little attention has been paid so far, was frequently detected next to SEB. Furthermore, ETA was detected from the sera of almost all the AD patients. SEB was not detected at all. Although the level of ETA in the AD group was significantly higher than that of controls, ETA-specific IgE was not detected from their sera. High levels of ETA tended to be detected from infantile patients. Although there were no significant differences in the levels of ETA-IgG between AD and the controls, its prevalence was more than twice as high as the controls in AD.

Conclusion These results suggest that many AD patients were exposed to ETx. We conclude that ETx may contribute to exacerbation of AD, particularly in infants, by a mechanism that is not through specific IgE production, unlike SEB.

Keywords atopic dermatitis, exfoliative toxin A, serum, specific IgE, specific IgG, staphylococcal enterotoxin, *Staphylococcus aureus*

Submitted 1 July 2002; revised 16 September 2002; accepted 20 February 2003

Introduction

Atopic dermatitis (AD) is a chronic inflammatory skin disease with a tendency toward repetition of exacerbation and remission. The cause of AD is believed to be a complex interplay of genetic background, immune dysregulation, and epidermal barrier dysfunction [1]. The infection of microorganisms has also been well known as a factor of pathogenesis of AD. Colonization of *Staphylococcus aureus* on the skin of patients with AD has previously been reported [2–6]. There are many studies that explain the effect of *S. aureus* on AD. Most strains of *S. aureus* isolated from AD patients can produce superantigenic toxins such as staphylococcal enterotoxin (SE) A, SEB, and toxic shock syndrome toxin-1 (TSST-1). Various studies demonstrated that the incidence of toxin-producing *S. aureus* in AD is more frequent than that in

healthy subjects [7, 8]. These toxins can lead to very high levels of IgE in their sera by stimulating the immune response, and exacerbate AD [9–12]. Superantigens can bind to MHC class II molecules on epidermal Langerhans cells or macrophage, and cause the release of pro-inflammatory mediators such as IL-1 α and TNF- α [13, 14]. In addition, through cross-linking of MHC class II molecules on antigen-presenting cells and the β element of the T cell receptor, T cells can be stimulated to proliferate and secrete a range of inflammatory cytokines [15, 16]. In the skin, Langerhans cells or keratinocytes can function as accessory cells in the T cell response to superantigens [15]. AD is frequently characterized by IgE hyper-responsiveness to various environmental antigens, including anti-*S. aureus* IgE [17]. Furthermore, in some studies, toxin-specific IgE antibodies have been detected in patients with AD, and some of these studies show a correlation with disease severity [12, 18, 19]. However, some authors reported a negative view of this point in that the presence of serum IgE is not essential for the development of AD [20, 21]. Moreover, there is one previous study of IgG

Correspondence: Shoko Yagi, Club Cosmetics Co., Ltd., Research and Development Division, Institute of Cosmetic Sciences, 145-1, Ichibu-cho, Ikoma-shi, Nara 630-0222, Japan. E-mail: syagi@clubcosmetics.co.jp

antibodies to toxins in AD, although they have not found evidence to suggest that the inability to produce IgG antibody to toxins is a factor in the development or ongoing inflammation response in AD [22]. *S. aureus* undoubtedly exacerbates AD, but it is unknown whether this is through an IgE-mediated pathway, superantigenic affection, or some other mechanisms.

In this paper, we examine the relationship between AD and these toxins from a slightly different angle. First, the detection rates of toxins isolated from patients were examined, and it was confirmed that SEB and ETx were frequently detected. Therefore, to investigate how both toxins affect AD, we built up a hypothesis that the toxins produced at the skin surface penetrate into circulation and reach dermal capillaries before acting on skin cells on AD. Based on this hypothesis, we examined whether ETx and SEB are present in the sera of patients with AD or not. At the same time, the levels of total serum IgE, specific IgE (against SEB and ETA), and specific IgG against ETA were evaluated. Furthermore, we examined the effect of ETA with the 3D skin model (living skin equivalent (LSE)).

Methods

Patients

All patients and their parents provided informed consent for participation in this study.

The determination of toxin production by *S. aureus* involved 100 patients with mild to severe AD. They were 54 males and 46 females aged 0–23 years (mean, 6 years), included 55 infants (0–5 years), 28 children (6–11 years), and 17 adults (12–23 years). The severity of AD was scored on the basis of the modified Leicester system [23] that gave a disease activity score with a maximum of 90 points (47 patients with severe and 53 patients with mild and moderate). In this system, five clinical features (erythema, papule, excoriation, oozing, and lichenification) were graded at six body sites (head, trunk, elbow, hand, knee, and foot) on a scale of 0 (none) to 3 (severe). We defined 0–19 as mild, 20–49 as moderate, and 50–90 as severe. The features of these patients are summarized in Table 1.

Serum samples were obtained from 21 patients with mild and moderate AD and analysed for total serum IgE, specific IgE (against SEB and ETA), specific IgG against ETA, and toxin levels. Each patient's profiles are shown in Table 2. Control samples were obtained from 38 healthy subjects. We enquired into a past history of atopic disease and family history, and divided the controls into three groups according

Table 2. Profiles of 21 patients with AD

Patient no.	Age (years)	Sex (F/M)	Leicester's disease activity score
1	0	M	
2	0	M	3
3	0	M	5
4	1	F	2
5	2	M	20
6	2	F	8
7	2	F	10
8	2	M	6
9	3	M	15
10	3	M	22
11	3	M	4
12	4	M	3
13	4	F	15
14	4	M	19
15	5	F	19
16	6	F	6
17	8	F	22
18	11	M	5
19	14	F	22
20	22	F	9
21	23	M	13

to the results. Of the 38 controls, 14 were defined as non-atopic controls, 19 who had a family history of atopic respiratory disease were defined as controls with atopic predisposition, and five who had a past history of AD were excluded from the control groups and included in the AD patients group. The characteristics of these subjects are summarized in Table 3.

None of these patients was suffering from staphylococcal scalded skin syndrome (SSSS).

Bacterial strains

Bacterial strains were isolated from four skin areas (of the patients shown in Table 1) – nasal cavity, non-lesional, dry-lesional, and exudative-lesional skin areas using a sterile cotton tips. They were cultured on mannitol salt agar (Nissui, Tokyo, Japan) plates, which is a selective medium for *S. aureus*. After 72 h incubation at 37 °C, the grown colonies that presented yellow were regarded as *S. aureus*.

Detection of genes for staphylococcal toxins by polymerase chain reaction (PCR)

Identified *S. aureus* strains were obtained and suspended in 1 mL of phosphate-buffered saline (PBS). After washing,

Table 1. Patients who were included in the detection examination of a toxin production gene

	Severe	Mild and moderate	Total
Infants	22	33	55
Childhood	15	13	28
Adults	10	7	17
Total	47	53	100

Table 3. Patients and healthy subjects who had serum collected

	Controls		
	Without predisposition	With predisposition	AD group
AD patients			21
Healthy subjects	14	19	5
Total	14	19	26

Table 4. Base sequences for the toxin-specific oligonucleotide primers

Gene	Primer	Oligonucleotide sequence	Size
SEA	Sense	TTGGAACGGTTAAACGAA	120
	Anti-sense	GAACCTTCCCATCAAAAACA	
SEB	Sense	TCGCATCAAAGTGACAAACG	478
	Anti-sense	GCAGGTACTCTATAAGTGCC	
SEC	Sense	GACATAAAAGCTAGGAATTT	257
	Anti-sense	AAATCGGATTAACATTATCC	
SED	Sense	CTAGTTTGGTAATATCTCCT	317
	Anti-sense	TAATGCTATATCTTATAGGG	
TSST-1	Sense	ATGGCAGCATCAGCTTGATA	350
	Anti-sense	TTTCCAATAACCAACCCGTTT	
ETA	Sense	CTAGTGCATTTGTTATTCAA	119
	Anti-sense	TGCATTGACACCATAGTACT	
ETB	Sense	ACGGCTATATACATTCAATT	200
	Anti-sense	TCCATCGATAATATACCTAA	

S. aureus strains were resuspended in 0.5 mL of PBS with 100 µg/mL of lysostaphin (Sigma Chemical, St Louis, MO, USA) and incubated at 37 °C for 1 h to extract DNA.

The oligonucleotide primers used in this study are shown in Table 4. The PCR was performed in a 25 µL reaction mixture using Ready-To-Go PCR Beads (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA) and the primers for 30 cycles. The amplification cycle was as follows: denaturation at 95 °C for 2 min, annealing of primers at 55 °C for 2 min, and primer extension at 72 °C for 1 min with autoextension. Then, 5 µL of PCR products were applied to 2% agarose gel. Electrophoresis was performed at room temperature at 100 V for 80 min in Tris-borate EDTA (TBE) buffer. After electrophoresis, the gel was stained with ethidium bromide, and DNA was visualized with a UV illuminator.

ELISA for detection of ETA and SEB in sera, and protein A

Flat-bottomed polystyrene microtiter plate (Nunc, Roskilde, Denmark) wells were filled with 100 µL of a 5.0 µg/mL solution of rabbit anti-staphylococcal ETA IgG (Toxin Technology, Inc., Sarasota, FL, USA) in 0.01 mol/L Na₂CO₃ buffer (pH 9.1). The plates were covered and left overnight at 4 °C. The following day, the plates were washed three times with 0.05 % Tween 20 in PBS (T-PBS) and then filled with 200 µL of 0.1% bovine serum albumin (BSA) (Sigma Chemical) in T-PBS. After being incubated at 37 °C for 1 h, the plates were washed three times with T-PBS. Next, 100 µL of serum samples were placed in the wells. The plates were incubated at room temperature for 2 h and washed three times with T-PBS. Then, 100 µL of a 1:300 dilution of horseradish peroxidase (HRP)-conjugated sheep anti-ETA (Toxin Technology) was placed in the wells. The plates were incubated at room temperature for 30 min and washed three times with T-PBS. Finally, 100 µL of substrate solution (TMB peroxidase EIA Substrate Kit, BIO-Rad Lab. Hercules, CA, USA) was placed in the wells. After 20 min, 100 µL of 1 N H₂SO₄ solution was added in each well to stop the reaction, and results were read at 450 nm.

SEB was detected with rabbit SEB IgG (Toxin Technology) as primary antibodies. Secondary antibodies were HRP-

conjugated sheep anti-SEB IgG (Toxin Technology). ELISA was performed in the same manner as above.

For determination of the binding affinity for protein A of the IgG used in the above methods, a 0.01–10 000 ng/mL solution of protein A (Sigma Chemical) in T-PBS was placed in the wells instead of serum samples. ELISA was performed in the same manner as above.

Western blot

ETA was electrophoresed on 12.5% SDS-PAGE for 45 min at 200 V. The ETA on the SDS-PAGE gel was electrophoretically transferred onto a nitrocellulose membrane for 6 h at 30 mA using a Semi-Dry Transblot apparatus. The membrane was washed in T-PBS and blocked with 3% BSA in T-PBS for 15 min. Rabbit anti-staphylococcal ETA IgG (used in ELISA) diluted to 1:50 in 1% BSA in T-PBS was added to the membrane and incubated for 45 min. The membrane was washed twice in T-PBS for 10 min per time. Sheep anti-rabbit IgG F(ab)₂ conjugated to HRP (Wako, Osaka, Japan) and diluted to 1:100 was then added and incubated for 45 min, washed as described above, and developed with 0.2% 3,3'-diaminobenzidine in Tris-HCl.

Assay of total and specific IgE

The levels of total serum IgE and SEB specific IgE were measured with the UniCAP 100 (Pharmacia & Upjohn, Tokyo, Japan). The specific IgE is considered to be positive at the level over 0.35 IU/mL in this system.

The level of ETA-specific IgE was measured by ELISA according to the method of Leung et al. [9]. Flat-bottomed polystyrene microtitre plate (Nunc) wells were filled with 100 µL of 10 µg/mL solution of highly purified staphylococcal ETA (Toxin Technology) in 0.05 M Na₂CO₃ buffer (pH = 9.5). The plates were covered and shaken gently overnight at 37 °C. The following day, the plates were washed three times with T-PBS. Next, 100 µL of serum to be tested was placed in the pre-coated wells. After being incubated at room temperature for 2 h, the wells were washed three times with T-PBS. Then, 100 µL of a 1:1000 dilution of alkaline phosphatase-conjugated mouse anti-human IgE (Sigma Chemical) was placed in the wells. After being incubated at 37 °C for 1 h while being shaken gently, the plates were washed three times with T-PBS. Finally, 200 µL of *p*-nitrophenylphosphate solution (Sigma Chemical) was placed in each well and incubated at room temperature. After 1 h, 50 µL of 3 N NaOH solution was added in each well to stop the reaction. Results were read at 405 nm.

Assay of ETA-specific IgG

ETA-specific IgG was also measured by ELISA according to the method of Campbell and Kemp [22]. Flat-bottomed polystyrene microtitre plate wells were coated with 100 µL/well of ETA at 1 µg/mL in carbonate buffer, pH 9.6, and incubated for 16 h at 4 °C. Plates were washed with T-PBS, blocked with 1% gelatin/ T-PBS for 2 h and washed a further four times. Samples (100 µL/well) were added at 1:500 (1% gelatin/T-PBS) and incubated at 37 °C for 90 min. Biotinylated monoclonal anti-human IgG (Pharmingen, San Diego, CA, USA) 100 µL/well (0.5 µg/mL) was added, incubated at

room temperature for 45 min, washed, and avidin peroxidase (EY Laboratories, Inc., San Mateo, CA, USA) diluted at 1:400 (100 µL/well) was then added and incubated at room temperature for a further 30 min. After final washing, 100 µL/well of substrate solution was added and incubated at room temperature for 20 min, and the reaction was stopped by the addition of 100 µL/well of 2 M H₂SO₄. Results were read at 450 nm.

An arbitrary unit was defined as normal pool serum ($n = 14$) 1:500 = 100 laboratory units. Concentrations of samples (1:500) were then calculated from the standard curve.

Stimulation of LSE with ETA

TESTSKINTM (TOYOBOKO Co., Osaka, Japan) was cultured with medium in the presence of 30 µg/mL of ETA in an atmosphere containing 5% CO₂ at 37 °C for 24 h.

Histology

After the culture period, this TESTSKINTM was fixed in 10% formalin. A paraffin section was stained with hematoxylin and eosin, and observed with light microscopy (Olympus, Tokyo, Japan).

Statistical analysis

Intergroup differences in the level of ETA were statistically evaluated by the Mann–Whitney non-parametric *U*-test, and those in the level of total serum IgE and ETA-IgG were statistically analysed by the unpaired *t*-test.

Results

Producibility of toxins by *S. aureus* from patients with AD

We studied the production of toxins (SEA, SEB, SEC, SED, TSST-1, ETA, and ETB) by *S. aureus* isolated from AD patients. Of the 100 patients tested, 81 (81.0%) were found to have *S. aureus* with a gene for at least one toxin. Figure 1a shows the detection rate of *S. aureus* that produces toxins from each skin area. Of the 81 patients, 57 patients (70.4%) had *S. aureus* that produces toxins in the nasal cavity, 33 patients (40.7%) in the non-lesional area, 50 patients (61.7%) in the dry-lesional area, and 61 patients (75.3%) in the exudative-lesional area. Figure 1b shows the detection rate of *S. aureus* that produces each toxin. Of the 81 patients, 22 patients (27.2 %) had *S. aureus* that produces SEA, 44 patients (54.3%) had SEB, 22 patients (27.2%) had SEC, four patients (4.9%) had SED, 13 patients (16.0%) had TSST-1, 25 patients (30.9%) had ETA, and 28 patients (34.6%) had ETB. Thus, the gene of SEB was most frequently detected as reported so far; Etx, which did not attract attention until now was also detected frequently. The production rate of each toxin was quite similar at all skin areas, and there is no point that should be mentioned especially (data not shown). Furthermore, Figure 1c shows the results when the patients are classified according to their AD severity. The detection rate was similar in mild and severe AD. We detected *S. aureus*, which has a gene for one or more toxins, in 37 (78.7%) of 47 severe AD patients, and 44 (83.0%) of 53 mild and moderate AD patients. In 37 severe patients, 12 (32.4%) were SEA producers, 23 (62.2%) were SEB producers, nine (24.3%) were SEC producers, two (5.4%) were SED producers, four (10.8%) were TSST-1 producers, 11

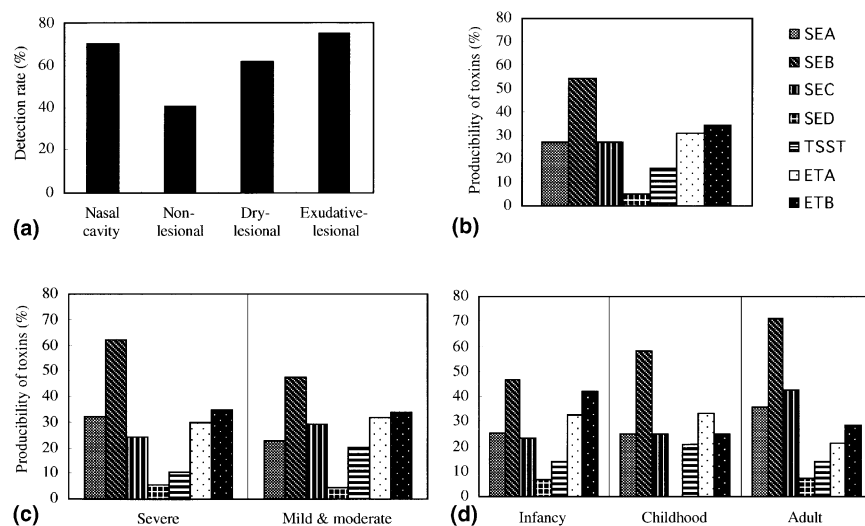


Fig. 1. Producibility of toxins in AD. (a) Producibility according to areas. *S. aureus* was isolated from the four skins areas as described previously. The rate of gene possession for toxin production was examined. High productivity was checked from a nasal cavity and wet-lesional skin. (b) Detection rate of each toxin. The rate of possession of the gene of each toxin was examined. SEB was most frequently detected followed by ETA and ETB. (c) Producibility at each severity. One hundred patients were divided into two groups (mild and moderate AD, and severe AD) as described previously. We detected *S. aureus* with a gene for one or more toxins from 81 of 100 patients: 37 of 47 severe AD, and 44 of 53 mild and moderate AD. SEB was most frequently detected in both groups (severe AD: 62.2%; mild and moderate AD: 47.7%). Next, ETx was most frequently detected from both groups (severe AD: ETA 29.7%, ETB 35.1%; mild and moderate AD: ETA 31.8%, ETB 34.1%). (d) Producibility at each age. One hundred patients were divided into three groups (infancy, childhood, adulthood) as described previously. We detected *S. aureus* with a gene for one or more toxins from 81 of 100 patients: 43 of 55 infants, 24 of 38 children, and 14 of 17 adult patients. SEB was most frequently detected in all groups (infancy: 46.5%, childhood: 58.3%; adulthood: 71.4%). Next, ETx was most frequently detected in infancy and childhood patients (infancy: ETA 32.6%, ETB 41.9%; childhood: ETA 33.3%, ETB 25.0%).

Table 5. Prevalence of exposure to ETA

	Controls (%)		AD group (%)
	Without predisposition	With predisposition	
Probability of ETA positive	57.1 (8/14)	63.2 (12/19)	88.5 (23/26)
Probability of ETA-IgG positive	14.3 (2/14)	15.8 (3/19)	34.6 (9/26)
Prevalence of exposure to ETA	57.1 (8/14)	68.4 (13/19)	96.2 (25/26)

(29.7%) were ETA producers, and 13 (35.1%) were ETB producers. The number of each toxin producer in 44 mild and moderate AD was 10 (22.7%), 21 (47.7%), 13 (29.6%), 2 (4.5%), 9 (20.5%), 14 (31.8%), and 15 (34.1%), respectively. As this result shows, when we compared the two cases, SEB was most frequently detected and ETx was the next most frequently detected. Figure 1d shows the rate of producers of each toxin when the patients are classified according to their age (infancy, childhood, and adulthood). We detected *S. aureus* that has a gene for one or more toxins in 43 (78.2%) of 55 infants, 24 (85.7%) of 30 children, and 14 (82.4%) of 17 adults. The number of each toxin producers in 43 infants were 11 (25.6%), 20 (46.5%), 10 (23.3%), 3 (7.0%), 6 (14.0%), 14 (32.6%) and 18 (41.9%), respectively. The number of each toxin producer in 24 children was 6 (25.0%), 14 (58.3%), 6 (25.0%), 0 (0%), 5 (20.8%), 8 (33.3%) and 6 (25.0%), respectively. The producers in 14 adults were 5 (35.7%), 10 (71.4%), 6 (42.9%), 1 (7.1%), 2 (14.3%), 3 (21.4%), and 4 (28.6%), respectively. The rate of SEB producers was the highest in all groups. ETx was also detected frequently. The incidence of ETx in infancy was especially high.

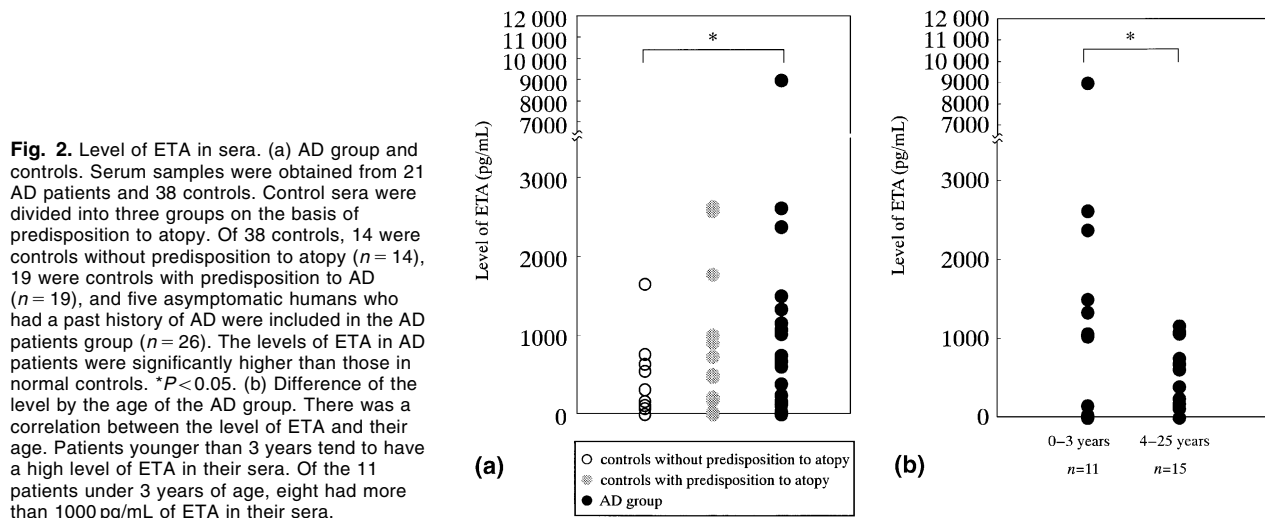
Presence of toxins and specific IgE in sera

Toxins in the sera of patients SEB was not detected in sera of the AD group and controls. On the other hand, ETA was detected from the sera of almost all the patients and some

controls. Detectable ETA in sera was found in 57.1% (8 of 14), 63.2% (12 of 19), and 88.5% (23 of 26) of controls without predisposition, controls with predisposition, and the AD group, respectively (Table 5). The level of ETA (Fig. 2a) in the AD group (mean 1192 pg/mL, range 0–9020 pg/mL) was significantly higher than that in non-atopic controls (mean 305 pg/mL, range 0–1654 pg/mL). In almost all the non-atopic controls, the level of ETA was less than 1000 pg/mL. On the contrary, a much higher level of ETA was measured in the sera of the AD group. Over 1000 pg/mL of ETA was detected in only 1 (7.1%) of 14 non-atopic controls, 4 (21.1%) of 19 controls with predisposition to atopy, and 11 (42.3%) of 26 AD group. A high level of ETA was measured in only infantile patients (Fig. 2b). In order to judge whether the existence of ETA in sera is a transient or continuous phenomenon, some serum samples, which were obtained 3 months later, were evaluated again. In the result, ETA of about the same concentration was detected (data not shown).

To date, there is no paper that reports having detected ETA from the sera of AD patients. We tried to confirm that the rabbit anti-ETA IgG used in ELISA binds with ETA specifically. As a result of performing Western blot analysis, binding of the antibody and ETA was visualized as a single band (Fig. 3). The detection sensitivity was 100 ng. Evaluation of interaction of the antibody and protein A was performed by ELISA. There is a fear that the ELISA system for the detection of ETA gives a false-positive result due to interference by staphylococcal protein A, which is produced by *S. aureus* and has the ability to bind the Fc fragment of rabbit IgG. However, the ELISA assay using rabbit anti-ETA-IgG as the capture antibodies was unable to detect binding of pure protein A up to 10 µg/mL (Table 6). From these findings, it has been confirmed that the antibody used in ELISA had detected ETA specifically.

Of the 21 patients, 18 had ETA in their sera. On the other hand, the gene for ET was detected only from three of 21. It does not necessarily have a high-concentration of ET in serum just because it has a gene for ET. In addition, it does not necessarily have a gene for ET just because it has a high level of ET in serum. Thus, it is estimated that the ET production gene and serum ET do not have any correlation in these 21 patients.



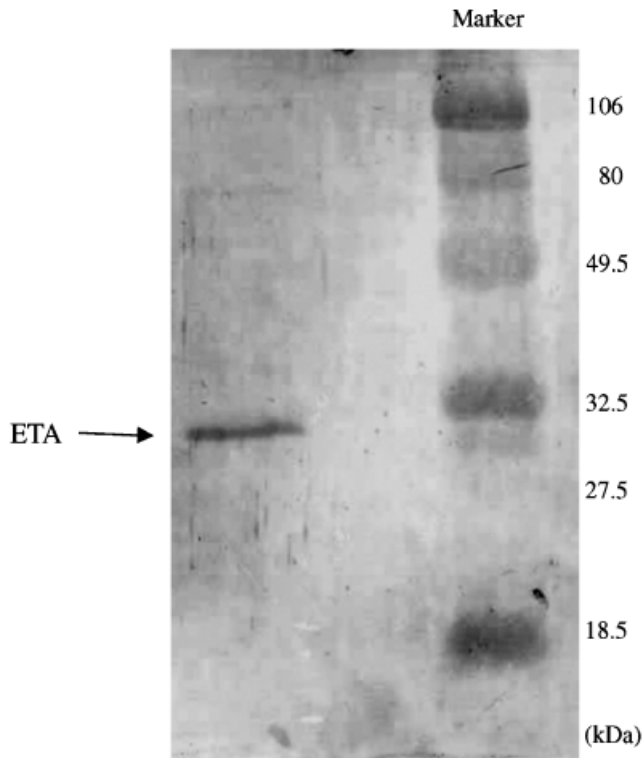


Fig. 3. Evaluation of the antibody by Western blot analysis. ETA was separated by 12.5% SDS-PAGE and transferred to nitrocellulose paper. ETA was detected with rabbit anti-staphylococcal ETA IgG (used for the detection of ETA in sera). Binding of this antibody and ETA was observed.

In this study, informed consent was not obtained from patients with severe AD. However, it was shown clearly that the prevalence of a gene for ET in mild and moderate AD was high as in severe AD. Therefore, we presumed that the relationship between AD and ET could be surmised only by evaluation of mild and moderate AD and that evaluation of severe AD was not indispensable.

Total serum IgE and SEB/ETA-specific IgE The total serum IgE in patients (mean 572.0 IU/mL, range 2.1 – 3012 IU/mL)

Table 6. Detection of Protein A by the ELISA system for detection of ETA

Concentration of protein A (ng/mL)	Response of protein A to the ELISA for detection of ETA
0.01	Undetectable
0.1	Undetectable
1.0	Undetectable
10	Undetectable
100	Undetectable
1000	Undetectable
10000	Undetectable

was significantly higher than that in non-atopic controls (mean 139.7 IU/mL, range 12.1 – 720.0 IU/mL) (Fig. 4a). A high level of total serum IgE was detected from adult patients rather than from infants (Fig. 4b).

The positive ratio of SEB specific IgE in the AD group was 19.2% (five of 26) and in controls it was 6.1% (two of 33). ETA-specific IgE was not detected from sera of the AD group (Table 7).

ETA-specific IgG Detectable ETA - IgG was found in 14.3% (two of 14), 15.8% (three of 19), and 34.6% (nine of 26) of controls without predisposition, controls with predisposition, and the AD group, respectively (Table 5). There was no significant difference in the concentration of ETA-IgG between the AD group and controls (Fig. 5a).

As for the AD group, subjects older than 4 years had a higher proportion of ETA-IgG (46.7%) than subjects younger than 3 years old (18.2%). A significantly higher concentration of ETA-IgG was detected from adult patients rather than from infants ($P < 0.05$) (Fig. 5b).

Both or either prevalence of ETA and/or ETA-IgG (prevalence of exposure to ETA) were 57.1% (8 of 14), 68.4% (13 of 19), and 96.2% (25 of 26) of controls without predisposition, controls with predisposition, and the AD group, respectively (Table 5).

Effect of ETA in TESTSKINTM

When TESTSKINTM was incubated with a medium that contained 30 µg/mL of ETA for 24 h, spontaneous superficial

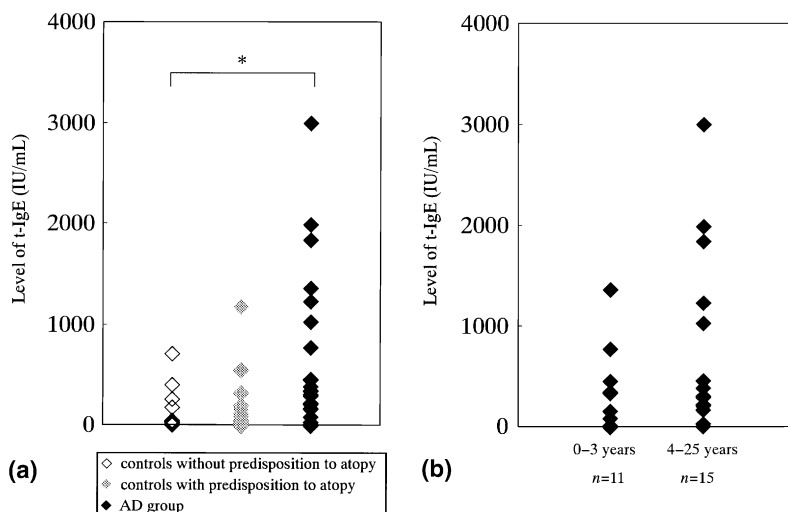


Fig. 4. Level of total serum IgE. (a) AD group and controls. Serum samples were obtained and divided as described previously. The levels of serum total-IgE in the AD group were significantly higher than those in non-atopic controls. * $P < 0.05$. (b) Difference of the level by the age of the AD group. A high level of total serum IgE was found in patients older than 4 years.

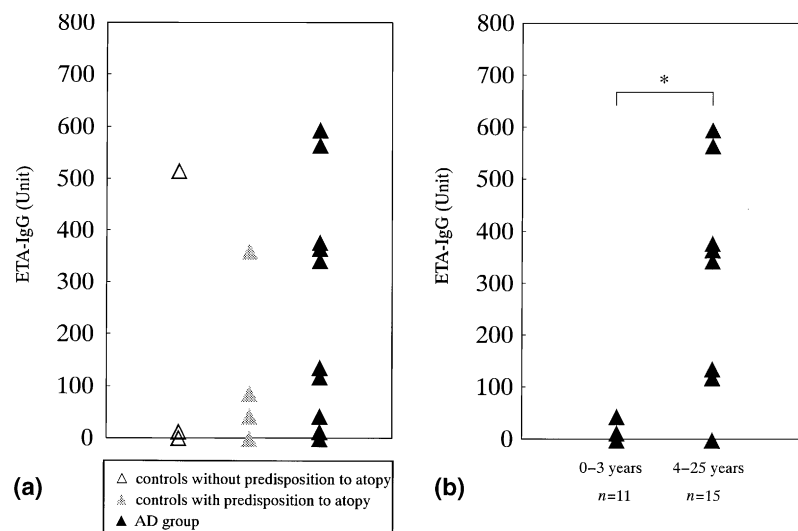


Fig. 5. Level of ETA-IgG. (a) AD group and controls. The level of ETA-IgG in each group did not show any significant difference. (b) Difference of the level by the age of the AD group. Patients older than 4 years had a significantly higher level of ETA-IgG in sera. * $P < 0.05$.

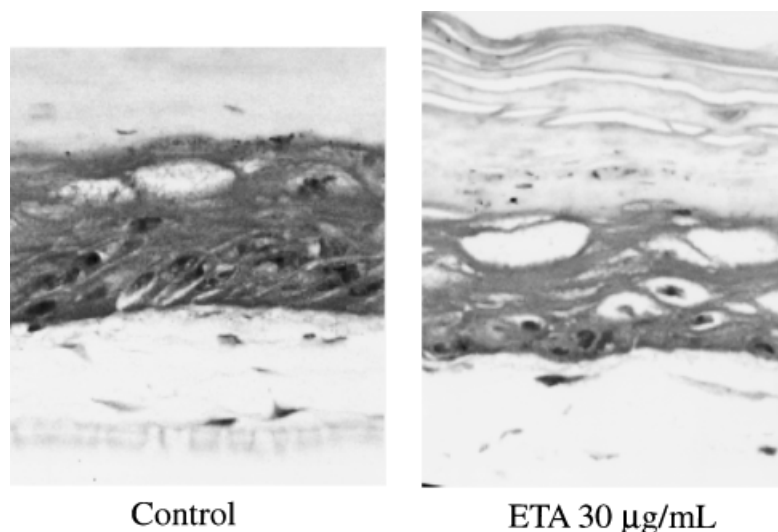


Fig. 6. Condition of epidermal cleavage induced by ETA. When TESTSKINTM was cultured with a concentration of ETA of 30 µg/mL for 24 h, spontaneous intra-epithelial cleavages were noted around the granular layer in TESTSKINTM.

Table 7. Positive ratio of toxin specific IgE

	SEB specific IgE (%)	ETA specific IgE (%)
AD group	19.2 (5/26)	0 (0/26)
Controls	6.1 (2/33)	0 (0/33)

cleavage was noted around the granular layer in TESTSKINTM (Fig. 6).

Discussion

SEB was most frequently detected on the skin of AD patients in this study, as reported so far [12]. We should notice that not only SEB but also ETx was frequently detected in AD

patients. A number of researchers have reported that *S. aureus* that produces various toxins is frequently detected in patients with AD [2–6], and SEB has been suggested to be the mediator to induce the AD lesion as a superantigen [11, 24–26]. However, there are few reports that mention the relationship between AD and ETx [27]. Furthermore, the significant association of ETx with AD has not always been reported [28, 29]. In this study, *S. aureus* that produces ETx was frequently detected on the skin of patients with AD, particularly in infants. Therefore, we called attention to the action of ETx on AD. ETx is known to be a causative agent of SSSS. It seems that ETB is more closely related to SSSS than ETA [29–32]. SSSS is an exfoliative dermatitis characterized by generalized tender erythematous rash with fever, and primarily affects infants and young children. The pathological process of the disease has been partly clarified. ETx is produced by *S. aureus*, which infects and colonizes in

the nasal cavity before being absorbed into the systemic circulation. ETx diffuses through dermal capillaries and may contribute to the pathogenesis by reaching the zona granulosa of the epidermis [33–35]. To date, some researchers have already analysed staphylococcal toxins of AD patients. They investigated the secretion of toxin from *S. aureus* on the skin, and the presence of IgE antitoxin in sera [4, 9, 11, 12]. However, the presence of the toxin itself in the sera of AD had not been investigated in these studies. We built up a hypothesis that ETx acts on the epidermis from the bloodstream even in patients with AD as previously mentioned, and determined the presence of toxins (SEB and ETA) in sera. As a result, we found that the majority of AD patients have ETA in sera. According to clinical diagnosis, these AD patients were not suffering from SSSS, as described previously. In order to judge whether the existence of ETA in sera is a transient or continuous phenomenon, serum samples that were obtained 3 months later were evaluated. This resulted, in the detection of ETA of about the same concentration. It seems that the existence of ETA in sera is a continuous phenomenon. On the contrary, SEB was not detected in their sera. Since patients with AD had a high level of ETA in their sera, we were concerned with the possibility of false-positive results as a result of non-specific binding of protein A to the Fc fragment of capture antibodies (rabbit anti-ETA IgG). The interaction between protein A produced by many *S. aureus* and the Fc fragment of rabbit IgG has been investigated [36, 37]. With whole-IgG double antibody ELISA, a false-positive result due to protein A may occur. However, this is unlikely because the ELISA was unable to detect the binding of a high level of protein A at levels up to 10 µg/mL in this study. We also checked by Western blot that false-positive reactivity to protein A did not occur. It is safe to say that these ELISA systems do not detect a binding of protein A. Furthermore, we proved that this antibody bound to ETA by Western blot analysis. Our results show that almost all the AD patients have ETA in their sera and do not have SEB. A high level of ETA was measured in only infantile patients, and not in adult patients. This agrees with the report that clearance of ET from the sera of adult mice is more rapid than that of neonatal mice [33]. In addition, we detected specific-IgE antibody to SEB in the sera of patients with AD. SEB may contribute to chronic inflammation and exacerbation of AD as a superantigen through the IgE-mediated immune response. It has been reported that an increase in the level of SEB specific IgE correlates well with severity of AD [12, 18, 19]. In spite of the fact that many patients have ETA in their sera, ETA-specific IgE was not detected in their sera. It has been reported that the relationship between susceptibility and immune response to ETA in some mammals could be divided into three groups: (1) with resistant skin and high productive ability of antibody to ETA; (2) with resistant skin and low productive ability of antibody to ETA; (3) with sensitive skin and various titres of antibody to ETA. It is believed that humans and mice have susceptibility and very low immunogenicity to ETA [38, 39]. In another study, it has also been reported that ETA specific IgE was not detected in the sera of patients with AD [9].

In this study, we also evaluated ETA-IgG in the sera of patients with AD. At present, there is only one previous

report about IgG against toxin of *S. aureus* in AD patients [22]. Campbell and Kemp examined SEA-IgG, SEB-IgG, and TSST-1 IgG in AD. Although they expected that IgG to superantigens acts protectively against AD, in fact a higher level of SEB-IgG was observed in AD than controls. Thus, they concluded that increased responses to SEB relate to increased colonization and hence exposure to toxin-producing *S. aureus* in AD, and inflammation of AD is not caused by the lack of ability to produce IgG to toxin. There have been no previous studies of ETA-IgG antibodies in AD. In this study, we evaluated ETA and ETA-IgG in the sera of AD at the same time. As a result, the probability that both or either of the factors were detected was 57.1%, 68.4%, and 96.2% in controls without predisposition, controls with predisposition, and the AD group, respectively, and it turned out that almost the entire AD group was exposed to ETA. Although there were no significant differences in the concentration of ETA-IgG among the three groups, its prevalence was more than twice the controls in the AD group. Furthermore, ETA-IgG had a tendency to be detected from subjects with a comparatively low level of ETA in sera. It follows from this that ETA-IgG may contribute to the clearance of ETA from sera as a neutralizing antibody. Moreover, in AD, the prevalence of ETA-IgG increased and inability to produce IgG to ETA is not observed; nevertheless, a high level of ETA was detected in sera. It is likely that both the increases in ETA and ETA-IgG result from too much ETA that has penetrated into circulation. Besides, it can be explained that the high level of ETA in the sera of infants is due to the immaturity of the immune system of IgG production.

The effect of *S. aureus*-IgG in AD was suggested by Gabrielsen and Brandtzaeg [40] as early as in 1985. They measured four immunoglobulin classes (IgG, IgA, IgM, and IgE) against *S. aureus* in AD, and showed that the IgG and IgE classes were significantly higher in patients than in controls. They discussed that the persistent production of specific IgG may be of pathogenetic importance as antibodies of this class can cause a hypersensitivity reaction of both type 2 and type 3. They also discussed that an IgE-mediated reaction to *S. aureus* may initiate the lesion and dominating itching symptom; subsequently, a type 3 reaction mediated by IgG may be more important for chronicity and relapse of the disease.

Our data showed that a high level of ETA exists in the sera of AD, and suggested that the ETA in sera may trigger the immune response through IgG without IgE-mediated reaction. From these findings, it seems that ETA has little effect of activating humoral immunity through B cell activation and IgE production as SEB in AD. We have not found evidence to suggest that ETA-IgG is a factor in the development or ongoing inflammation of AD. Nevertheless, we proved the existence of a high level of ETA in the sera of patients with AD as well as SSSS. ETA has recently been demonstrated to have potent T cell proliferation [41]. We did not investigate the T cell reactivity against ETA of these patients. However, in another study of ours, we investigated the effect of ETA in lymphocytes from healthy volunteers *in vitro*. As a result, the lymphocytes proliferated in response to ETA, and ETA stimulation induced the lymphocytes to increase their IFN-γ and IL-2 production. In addition, it is suggested that signals

from lymphocytes stimulated with ETA can induce the production of pro-inflammatory cytokines by fibroblasts and keratinocytes by conducting a study with lymphocyte-conditioned medium (unpublished data). In terms of these views, it is estimated that ETA is concerned as a protein antigen (not as superantigen) with the immune response of humoral immunity through the production of IgG and cellular immunity, where T cells can be stimulated and proliferate to secrete a range of inflammatory cytokines. Although ETA has not been noted as an exacerbation factor on AD, it may trigger allergic reaction through different mechanisms from SEB.

It is still uncertain whether the fact that ETA exists in the sera of patients with AD results from or leads to exacerbation of AD. However, in this study, we showed that ETA induced exfoliation in TESTSKINTM by the action not from surface but inside the skin. Therefore, it seems highly possible that the pathogenetic system of SSSS holds true for that of AD. In other words, in AD patients, ETA absorbed into circulation may diffuse through dermal capillaries, cause an immune reaction, and injure the barrier function of the skin surface, as ETB acts on SSSS. On these grounds, we have come to the conclusion that patients with AD have ETA in their sera, and the ETA may play important roles in the pathogenesis or exacerbation of AD, particularly in infants.

Acknowledgements

We acknowledge the generous assistance of the late Dr Tae Takeda (National Children's Medical Research Center, Tokyo, Japan). We also wish to thank Dr Yuko Hayakawa (Department of Dermatology, Jikei University School of Medicine) for cooperation.

References

- Cooper KD. Atopic dermatitis: recent trends in pathogenesis and therapy. *J Invest Dermatol* 1994; 102:128–37.
- Svejgaard E. The role of microorganisms in atopic dermatitis. *Sem Dermatol* 1990; 9:255–61.
- Ring J, Abeck D, Neuber K. Atopic eczema: role of microorganisms on the skin surface. *Allergy* 1992; 47:265–9.
- Hoeger PH, Lentz W, Boutonier A, Fournier JM. Staphylococcal skin colonization in children with atopic dermatitis: prevalence, persistence, and transmission of toxigenic and nontoxigenic strains. *J Infect Dis* 1992; 165:1064–8.
- Williams JV, Vowels B, Honig P, Leyden JJ. *Staphylococcus aureus* isolation from the lesions, the hands, and anterior nares of patients with atopic dermatitis. *J Emerg Med* 1999; 17:207–11.
- Nishijima S, Namura S, Higashida T, Kawai S. *Staphylococcus aureus* in the anterior nares and subungual spaces of the hands in atopic dermatitis. *J Intern Med Res* 1997; 25:155–8.
- Zollner TM, Wichelhaus TA, Hartung A et al. Colonization with superantigen-producing *Staphylococcus aureus* is associated with increased severity of atopic dermatitis. *Clin Exp Allergy* 2000; 30:994–1000.
- Bunikowski R, Mielke ME, Skarabis H et al. Evidence for a disease-promoting effect of *Staphylococcus aureus*-derived exotoxins in atopic dermatitis. *J Allergy Clin Immunol* 2000; 105:814–9.
- Leung DY, Harbeck R, Bina P et al. Presence of IgE antibodies to staphylococcal exotoxins on the skin of patients with atopic dermatitis. Evidence for a new group of allergens. *J Clin Invest* 1993; 92:1374–80.
- Akiyama H, Toi Y, Kanzaki H, Tada J, Arata J. Prevalence of producers of enterotoxins and toxic shock syndrome toxin-1 among *Staphylococcus aureus* strains isolated from atopic dermatitis lesions. *Arch Dermatol Res* 1996; 288:418–20.
- Tada J, Toi Y, Akiyama H, Arata J, Kato H. Presence of specific IgE antibodies to staphylococcal enterotoxins in patients with atopic dermatitis. *Eur J Dermatol* 1996; 6:552–4.
- Nomura I, Tanaka K, Tomita H et al. Evaluation of the staphylococcal exotoxins and their specific IgE in childhood atopic dermatitis. *J Allergy Clin Immunol* 1999; 104:441–6.
- Leung DYM. The immunologic basis of atopic dermatitis. *Clin Rev Allergy* 1993; 11:447–68.
- Marrack P, Kappler J. The staphylococcal enterotoxins and their relatives. *Science* 1990; 248:705–11.
- Tokura Y, Yagi J, O'Malley M et al. Superantigenic staphylococcal exotoxins induce T-cell proliferation in the presence of Langerhans cells or class II-bearing keratinocytes and stimulate keratinocytes to produce T-cell-activating cytokines. *J Invest Dermatol* 1994; 102:31–8.
- Strange P, Skov L, Baadsgaard O. Interferon gamma-treated keratinocytes activate T-cells in the presence of superantigens: involvement of major histocompatibility complex class II molecules. *J Invest Dermatol* 1994; 102:150–4.
- Bos JD, Wierenga EA, Smitt JHS, Heijden FI, Kapsenberg ML. Immune dysregulation in atopic eczema. *Arch Dermatol* 1992; 128:1509–12.
- Breuer K, Wittmann M, Bosche B, Kapp A, Werfel T. Severe atopic dermatitis is associated with sensitization to staphylococcal enterotoxin B (SEB). *Allergy* 2000; 55:551–5.
- Lin YT, Yang YH, Hwang YW et al. Comparison of serum specific IgE antibodies to staphylococcal enterotoxins between atopic children with and without atopic dermatitis. *Allergy* 2000; 55: 641–6.
- Halbert AR, Weston WL, Morelli JG. Atopic dermatitis: is it an allergic disease? *J Am Acad Dermatol* 1995; 33:1008–18.
- Peterson RDA, Page ARP, Good RA. Wheal and erythema allergy in patients with agammaglobulinaemia. *J Allergy* 1962; 33:406–11.
- Campbell DE, Kemp AS. Production of antibodies to staphylococcal superantigens in atopic dermatitis. *Arch Dis Child* 1998; 79:400–4.
- Sowden JM, Berth JJ, Ross JS et al. Double-blind, controlled crossover study of cyclosporin in adults with severe refractory atopic dermatitis. *Lancet* 1991; 338:137–40.
- Strange P, Skov L, Lisby S, Nielsen PL, Baadsgaard O. Staphylococcal enterotoxin B applied on intact normal and intact atopic skin induces dermatitis. *Arch Dermatol* 1996; 132:27–33.
- Bunikowski R, Mielke M, Skarabis H et al. Prevalence and role of serum IgE to the *Staphylococcus aureus*-derived superantigens SEA and SEB in children with atopic dermatitis. *J Allergy Clin Immunol* 1999; 103:119–24.
- Morishita Y, Tada J, Sato A et al. Possible influences of *Staphylococcus aureus* on atopic dermatitis – the colonizing features and the effects of staphylococcal enterotoxins. *Clin Exp Allergy* 1999; 29:1110–7.
- Capoluongo E, Giglio AA, Lavieri MM et al. Genotypic and phenotypic characterization of *Staphylococcus aureus* strains isolated in subjects with atopic dermatitis. Higher prevalence of exfoliative B toxin production in lesional strains and correlation between the markers of disease intensity and colonization density. *J Dermatol Sci* 2001; 26:145–55.
- Capoluongo E, Giglio A, Belardi M et al. Association between lesional *S. aureus* strains from patients with impetigo and exfoliative toxin production. No association with Smal PFGE patterns. *New Microbiol* 2000; 23:21–7.

- 29 Kanzaki H, Ueda M, Morishita Y, Akiyama H, Arata J, Kanzaki S. Producibility of exfoliative toxin and staphylococcus coagulase types of *Staphylococcus aureus* strains isolated from skin infections and atopic dermatitis. *Dermatology* 1997; 195:6–9.
- 30 Opal SM, Johnson-Winegar AD, Cross AS. Staphylococcal scalded skin syndrome in two immunocompetent adults caused by exfoliative B-producing *Staphylococcus aureus*. *J Clin Microbiol* 1988; 26:1283–6.
- 31 Murono K, Fujita K, Yoshikawa H. Detection of staphylococcal exfoliative toxin by slide latex agglutination. *J Clin Microbiol* 1988; 26:271–4.
- 32 Sarai Y, Nakahara H, Ishikawa T, Kondo I, Futaki S, Hirayama K. A bacteriological study on children with staphylococcal toxic epidermal necrolysis in Japan. *Dermatologica* 1977; 154:161–7.
- 33 Plano LR, Adkins B, Woischnik M, Ewing R, Collins CM. Toxin levels in serum correlate with the development of staphylococcal scalded skin syndrome in a murin model. *Infect Immun* 2001; 69:5193–7.
- 34 Merlsh ME, Glasgow A. The staphylococcal scalded-skin syndrome. *N Engl J Med* 1970; 282:1114–9.
- 35 Ladhani S, Joannou CL, Lochrie DP, Evans RW, Poston SM. Clinical, microbial, and biochemical aspects of the exfoliative toxins causing staphylococcal scalded-skin syndrome. *Clin Microbiol Rev* 1999; 12:224–42.
- 36 Ladhani S, Robbie S, Garratt RC, Chapple DS, Joannou CL, Evans RW. Development and evaluation of detection system for staphylococcal exfoliative toxin A responsible for scalded-skin syndrome. *J Clin Microbiol* 2001; 39:2050–4.
- 37 Tarkhanova IA, Rudenskii AI, Sycheva IM, Kulberg AI. Inter-relationship between the structure of IgG and its Fc-fragment and their ability to react with protein A of *Staphylococcus aureus*. *Biull Eksp Biol Med* 1980; 89:318–20.
- 38 Machida K, Sakurai S, Kondo I, Ikawa S. Relationship between susceptibility and immune response to staphylococcal exfoliative toxin A in mammalian species. *Microbiol Immunol* 1988; 32:1079–84.
- 39 Machida K. Immunological investigations on pathogenesis of staphylococcal scalded skin syndrome. *Rinsho Byori* 1995; 43:547–56.
- 40 Gabrielsen T, Brandtzaeg P. Enzyme-linked immunosorbent assay (ELISA) for isotype-specific quantitation of antibodies to *Staphylococcus aureus* in patients with atopic dermatitis. *Acta Derm Venereol (Stockh)* 1985; 114:61–6.
- 41 Monday SR, Vath GM, Ferens WA et al. Unique superantigen activity of staphylococcal exfoliative toxins. *J Immunol* 1999; 162:4550–9.