

IgE-binding components of staphylococcal enterotoxins in patients with atopic dermatitis

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Background: The exacerbation of atopic dermatitis may be associated with infection of the skin with *Staphylococcus aureus* (*S. aureus*). *S. aureus* isolated from the skin of patients with atopic dermatitis secretes enterotoxin A, B, and toxic shock syndrome toxin 1. This is of interest because these patients may develop specific IgE antibodies against components from staphylococci.

Objective: The objective was to demonstrate IgE-sensitization to components of *Staphylococcus aureus* enterotoxins A and B (purified and partially purified), toxic shock syndrome toxin 1, and the bacterial cell component lipoteichoic acid, in patients with atopic dermatitis.

Methods: Blood samples from 34 patients with atopic dermatitis and 10 controls were tested by leukocyte histamine release to the enterotoxins and lipoteichoic acid. The toxins were separated by sodium dodecylsulfate polyacrylamide gel electrophoresis and analyzed by IgE-immunoblotting with sera from the same patients.

Results: The majority of patients (96%) with clinical signs of skin infection produced specific IgE-antibodies to all three toxins. Nearly half of the patients produced IgE to enterotoxin A and B. Only 63% of the patients with atopic dermatitis showed cellular response judged by the release of histamine from patient basophils when challenged in vitro with the toxins. This may indicate clinically unimportant sensitization in a number of patients. The immunoblotting revealed that the major allergens of the toxins were 24 and 28 kD proteins. Partially purified toxins showed a higher frequency of leukocyte histamine release responses than purified toxin. The only obvious difference was a difference in the content of pure toxin of the two preparations. Lipoteichoic acid showed nonspecific activity.

Conclusion: These findings suggest that staphylococcal enterotoxins may act as specific allergens and induce IgE-antibodies to enterotoxins that may exacerbate the skin inflammation in some patients with atopic dermatitis.

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INTRODUCTION

Atopic dermatitis is a chronic inflammatory skin disorder with a genetic predisposition to develop IgE-mediated responses. External factors may contribute to the pathogenesis of the

skin disorder, partly because of IgE sensitization and possibly also a T cell response. Clinical observations have led investigators to focus on the microflora of the skin as a potential pathogenic factor in atopic dermatitis.

Studies show that *S. aureus* has a 100 to 1000 times higher density in different types of skin lesions compared with normal skin areas of patients with atopic dermatitis.¹ In addition, a significantly higher frequency of *S. aureus* colonies are found in apparently normal skin of patients with atopic dermatitis compared with skin of healthy subjects.^{1,2} IgE sensitization to intact bacteria, cell wall and super-

natant proteins of sonicated cells of *S. aureus* have been demonstrated.^{3,4} The clinical importance of these findings is still uncertain but reduction of microbial load by treatment with antimicrobial reagents combined with steroids may be more effective than treatment with steroids alone.⁵

Recently, *S. aureus* isolated from the skin of patients with atopic dermatitis was shown to secrete enterotoxins A, B, and toxic shock syndrome toxin I.⁶ These toxins are best known as the causative agents of food poisoning but are implicated in other types of infections as well and may act as superantigens.⁷ This suggests several mechanisms by which they may contribute to the chronic inflammation observed in atopic dermatitis. First, toxins secreted on the skin surface could penetrate the skin on damaged skin sites and stimulate Langerhans' cells and/or macrophages to produce cytokines. Second, the toxins may stimulate the TCR $\alpha\beta$ receptor on T cells causing production of cytokines that modulate tissue inflammation. Third, it is reported that 57% of patients with chronic atopic dermatitis have circulating IgE against enterotoxins A, B, and/or toxic shock syndrome toxin 1.⁶ This suggests that toxins can induce IgE-mediated inflammation and contribute to the exacerbation of atopic dermatitis.

The aim of this study was to identify possible proteins acting as allergens of staphylococcal enterotoxins by analyzing the sera of patients with atopic dermatitis.

MATERIAL AND METHODS

Patients

This study was undertaken according to the Helsinki declaration on human

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investigations and was approved by the local ethics committee (protocol KF01-444/93 and KF01-435/93). Informed consent was obtained from all subjects. Thirty-four patients fulfilling the Hanifin criteria for atopic dermatitis⁸ and with clinical findings suggesting skin infection (honey-colored crusting, extensive serous weeping, folliculitis, and pyoderma) were recruited from the Departments of Dermatology, University Hospital of Copenhagen and Bispebjerg Hospital. The median age was 31 years (range 18 to 60 years).

Heparinized whole blood samples from all patients were tested for basophil histamine release to a panel of enterotoxins and lipoteichoic acid. Five atopic donors (with positive skin prick test results to inhalation allergens and with no clinical signs of skin infection or eczema) and five nonatopic donors served as negative controls. Furthermore, a serum pool consisting of serum from normal individuals, and a sample of human myeloma-IgE (>4000 U/mL) was included as negative controls. All sera were immunoblotted with the toxins and lipoteichoic acid.

Staphylococcal Enterotoxins

Staphylococcal enterotoxin A and B, partially purified enterotoxin A and partially purified enterotoxin B, and toxin shock syndrome toxin 1 were delivered lyophilized by Toxin Technology, Sarasota, Florida and lyophilized lipoteichoic acid by Sigma Chemicals. The toxins and lipoteichoic acid were reconstituted with 50% glycerol to obtain protein concentrations of 1 mg/mL and 5 mg/mL, respectively. Reagents were stored at 4 °C. Before the patient study was undertaken, toxins and lipoteichoic acid were examined for analytical interference or non-specific histamine release; none was observed. In addition, dose-response curves were established in several patients before including patients in the study.

Histamine Release Test

The leukocyte histamine release test was performed as previously described with glassmicrofiber-coated microtiter plates, that selectively bind histamine.^{9,10} Briefly, 25 μ L of whole blood was incubated with 25 μ L toxin or lipoteichoic acid. The stock solutions of enterotoxin A (10 μ g/mL), enterotoxin B (10 μ g/mL), toxic shock syndrome toxin 1 (10 μ g/mL), and lipoteichoic acid (1 mg/mL) were diluted in Pipes-AMC buffer (10 mM piperazine-N,N'-bis(2-ethanesulfonic acid), 140 mM sodium acetate, 5 mM potassium acetate, 30 mM Trizma-T-1503, 0.5 mM calcium chloride, and 1 mM magnesium chloride, pH 7.4). Ten times higher concentration was chosen for partially purified enterotoxin A and partially purified enterotoxin B (100 μ g/mL) as the absolute amount of pure toxin in this preparation was unknown at the beginning of the study. Aliquots were run in duplicate and tested at six tenfold dilutions to ensure a representative histamine release curve. In addition, all blood samples were tested with a dilution range of anti-IgE (Behringwerke). After one hour incubation at 37 °C the glassfiber-coated microtiter wells were rinsed and cell debris and proteins were enzymatically removed. Released histamine was coupled to o-phthalaldehyde and measured spectrophotofluorometrically with a Gilson fluorometer.^{9,10} Released histamine was expressed as nanogram histamine per milliliter and calculated on the basis of a histamine standard curve.^{9,10}

Sodium Dodecylsulfate-Polyacrylamide Gel Electrophoresis

The toxins and lipoteichoic acid were separated in a discontinuous buffer system by sodium dodecylsulfate-polyacrylamide gel electrophoresis according to the method of Laemmli¹¹ with a 16% separation gel and a 5% stacking gel for three hours at 70 mA per gel. The assays were performed in a vertical slab cell apparatus (Hoefer Instruments, San Francisco, CA). The toxins and lipoteichoic acid were boiled for five minutes with a reducing

sample buffer (containing 2- β -mercaptoethanol) and 100 μ g of each sample was loaded on 0.5-cm gel. The gels were stained with Coomassie Brilliant Blue. For immunoblotting, 10 μ g of each toxin and 100 μ g lipoteichoic acid were used per 0.5-cm gel (one person). The molecular weights were evaluated from a prestained protein standard (14.3 to 200 kD, Amersham International, Buckinghamshire, UK).

IgE-Immunoblotting and Autoradiography

Transfer of proteins to nitrocellulose (pore size 0.2 mm) was performed with a semidry electroblotter (JKA-Biotech, Copenhagen, Denmark) at 0.8 mA/cm² for 90 minutes in a discontinuous buffer system (anodic buffer 1: 0.3 M Tris with 20% methanol, pH 10.4 and anodic buffer 2: 25 mM Tris with 20% methanol, pH 10.4 and cathodic buffer: 25 mM Tris, 40 mM 6-Amino-*n*-hexanoic acid with 20% methanol, pH 9.4). For IgE-immunoblotting, the nitrocellulose paper was cut into 0.5-cm wide strips and incubated for ½ hour with TBS buffer (0.05 M Tris, 0.15 M NaCl, 0.005 M NaN₃, 0.5% Tween, pH 7.4) to which another 1% Tween was added for blocking. Sera were diluted 1:10 in TBS and incubated overnight at room temperature. After the strips had been washed 3 times in TBS, 1 mL ¹²⁵I rabbit anti-human IgE (Pharmacia) diluted to 200,000 CPM/mL TBS was added to each strip. After overnight incubation the strips were washed, air-dried, placed in filmcassettes and sensitive radiographic films (Hyperfilm-MP, CODE RPN.6, Amersham) were developed at day 1, day 7, and day 14, respectively.

Total IgE

The automated HYTEC enzyme immunoassay system using anti-IgE coated paper discs was used to measure total IgE in the sera.¹³ The assay is calibrated according to the World Health Organization serum standard (International Reference Preparation 75/502) and values are reported in

kU/L. The dynamic range of the assay is 0.5 to 2000 kU/L.

Data Evaluation

The proteins detected by IgE-immunoblotting were divided into three categories: weak/visible, moderate, and strong by a visual evaluation. The immunoblot was considered positive if at least one protein band was visible.

The histamine release data were evaluated by calculating the area under the curve (AUC) by numerical integration by the formula $\frac{1}{2}c_1 + c_2 + c_3 + c_4 + c_5 + \frac{1}{2}c_6$ where "c" is the histamine released (in ng/mL whole blood) at a given concentration (symbolized by numbers 1 to 6). An AUC of ≥ 40 ng histamine/mL blood constitutes a positive histamine release response.¹⁴

RESULTS

Patients

The patient population showed a median total IgE level greater than 2,000 IU/mL (range 64 to greater than 2000 IU/mL). Sera from 7 out of 34 patients were not used for immunoblotting because they were basophil non-responders (neither reacting to anti-IgE nor toxins). Thus, the evaluable study subjects included 27 patients (14 women and 13 men) and 10 control subjects (5 atopics and 5 nonatopics). None of the controls reacted to the toxins or lipoteichoic acid; however, anti-IgE induced histamine release in all study subjects.

Frequency Distribution of Test

Results

Seventeen patients (63%) showed leukocyte histamine release to either toxins or lipoteichoic acid, whereas all but one patient (96%) showed IgE-binding to the toxins or lipoteichoic acid in immunoblotting. Two nonatopic controls showed (weak) IgE-binding to lipoteichoic acid and one atopic control showed (weak) IgE-binding to toxic shock syndrome toxin 1 (data not shown). Furthermore, myeloma-IgE

(4000 U/mL) bound to enterotoxins A and B.

All three toxins were able to induce a histamine release response in the atopic dermatitis patients. The majority of patients reacted to enterotoxins A or B (Table 1). Generally, the partially purified toxins induced histamine release in more patients than the purified toxins. In contrast, IgE-immunoblotting did not show this difference.

Sodium Dodecylsulfate-Polyacrylamid Gel Electrophoresis Analysis

Separation of the toxins by sodium dodecylsulfate-polyacrylamid gel electrophoresis (Table 2) revealed a wider molecular weight range for the partially purified toxins (partially purified toxin A < 14.3 to >200 kD and partially purified toxin B < 14.3 to 62 kD) than for the purified toxins (toxin A <14.3 to 96 kD and toxin B <14.3 to 54 kD). The most intense staining was observed at the molecular weights corresponding to the unreduced toxins (toxic shock syndrome toxin 1 24 kD, toxin A and toxin B 28 kD). This suggests that only a minor part of the toxins is reduced to subunit size. Lipoteichoic acid was not stained by the Coomassie dye.

IgE-Immunoblotting Analysis

The most frequent IgE-binding components of the toxins and lipoteichoic acid are summarized in Table 3. It is observed, that components of the partially purified toxins greater than 100

kD (partially purified toxin A) and 32 kD (partially purified toxin B) do not bind IgE. When comparing the toxins a general pattern appears: when the patient sera reacted they typically showed binding to all toxins indicating multiple sensitization directed against several toxins. Only four patients were monosensitized. This is illustrated in Figure 1 showing the individual immunoblot and histamine release data from ten patients.

Components of Staphylococcal Enterotoxin A that Bind Enterotoxin A Specific IgE

Generally, the sera from patients with atopic dermatitis showed similar IgE-binding patterns with enterotoxin A and partially purified enterotoxin A (Fig 1a). The major IgE-binding component of toxin A was a 28-kD protein recognized by 13 sera (48%) and 10 patients (37%) reacted to this protein only. Less frequent components were 33-, 16- and 15-kD proteins. All but one of 15 patients (52%) reacted to the 28-kD protein of partially purified toxin A and six patients (27%) recognized this protein only. A few patients showed binding to 45-kD and 16-kD proteins.

Components of Staphylococcal Enterotoxin B that Bind Enterotoxin B Specific IgE

Figure 1b depicts almost similar IgE-binding patterns of enterotoxin B and partially purified enterotoxin B. The partially purified toxin B preparation contains a number of IgE-binding components not observed in the purified toxin B preparation. Seventeen patients (63%) showed binding to purified enterotoxin B, and all of them recognized the major protein (28 kD) and 13 patients (48%) reacted only to this protein. Less frequent IgE-binding proteins were the 54, 26, 22, 16, 15, and <14.3 kD proteins. Eighteen patients showed IgE-binding to partially purified toxin B and one recognized the 28-kD protein only. A few sera identified 62, 54, 50, 38, 35, 33, 32, and 18 kD proteins.

Table 1. Frequency Distribution of Patients Reacting with Histamine Release (HR) and Sera Binding Specific IgE to Purified Staphylococcal Enterotoxin A (SEA) or B (SEB), Partially Purified Toxins (pp), Toxic Shock Syndrome Toxin 1 (TSST), and Lipoteichoic Acid (LTA)

Toxin	HR Positives	Immunoblot Positives
SEA	3 (11%)	13 (48%)
ppSEA	8 (30%)	15 (56%)
SEB	3 (11%)	17 (63%)
ppSEB	9 (33%)	18 (67%)
TSST-1	4 (15%)	11 (41%)
LTA	11 (41%)	13 (48%)

Table 2. Molecular Weights (in kD) of Toxins Separated by Sodium Dodecylsulfate-Polyacrylamid Gel Electrophoresis*

SEA	ppSEA	SEB	ppSEB	TSST
	<200 (two bands)		62	50
	175	54	54	44
	165		50	43
	140		35 (two bands)	24
	130		33	21
96	96		32	<14
	80	30	30	
	66	28	28	
54	54	26 (two bands)	26 (two bands)	
	50		22	
	45		20	
33	33	17	17	
	30	16	16	
28	28		14	
<1	<14	<14	<14	
4		<14	<14	

* Sodium dodecylsulfate-polyacrylamid gel electrophoresis sequence for *Staphylococcus aureus* toxins. The purified *Staphylococcus aureus* toxin A (SEA), B (SEB), partially purified toxins (pp), and toxic shock syndrome toxin 1 (TSST).

Table 3. Frequency Distribution of Patient Sera Reacting to IgE-Binding Components of Toxins and Lipoteichoic Acid (LTA)*

Toxins	Molecular Weight in kD											
	96	54	32	30	28	26	24	22	19	16	15	14
SEA					11							4
ppSEA	4	4		9	15						4	4
SEB					17							
ppSEB			10		18	6		5	9	9	6	
TSST-1							11					
LTA							13					

* Components to which fewer than 4 sera reacted are not included in the table.

Components of Toxic Shock Syndrome Toxin 1 that Bind Specific IgE

Thirteen patients (48%) recognized IgE-binding proteins of toxic shock syndrome toxin 1 (Figure 1c). All patients showed binding to the major protein (24 kD) except one patient who reacted to a <14.3-kD protein. Less frequent components were 50, 42, 30, 20, and <14.3-kD proteins. It was observed that one negative atopic control reacted weakly to the 24-kD protein (data not shown).

Components of Lipoteichoic Acid that Bind Lipoteichoic Acid Specific IgE

Thirteen patient sera (48%) recognized lipoteichoic acid (24 kD) (Fig 1c).

Two nonatopic negative controls reacted weakly with lipoteichoic acid.

DISCUSSION

Exacerbation of atopic dermatitis may be caused by skin infection with *Staphylococcus aureus*. The clinical association, however, between skin infections and type I-mediated skin sensitivity to bacteria is still debatable.

This study demonstrates that patients with atopic dermatitis and clinical signs of skin infection develop specific IgE-antibodies to staphylococcal enterotoxins or bacterial cell components. About 20% of the patients had non-responding basophils and were excluded from further analysis. The reason for non-responding basophils is

unknown but may be related to increased spontaneous histamine release (histamine depleted basophils) or subcellular mechanisms.

Sixty-three percent of the patients show leukocyte histamine release to staphylococcal toxins and lipoteichoic acid. This is in agreement with another study that reported that 57% of patients with atopic dermatitis (selected similarly to this study) showed specific IgE against toxic shock syndrome toxin 1, staphylococcal enterotoxins A or B.⁶ Our histamine release data showed a remarkably lower frequency of patients positive to the purified preparation of enterotoxins A and B (11%) than to the partially purified preparation of enterotoxin A (30%) and B (33%). It is possible that additional elements in the partially purified toxins are responsible for this difference. The staining patterns of the immunoblots did not indicate this, since the staining patterns and the number of sera positive to purified and partially purified toxins were similar. By contacting the manufacturer, it was determined that one milligram of partially purified toxin contained one milligram of pure toxin. This results in a ten times higher toxin content of the partially purified toxins compared with the purified toxins. Thus, the toxin concentrations of enterotoxin A, B, and toxic shock syndrome toxin 1 in the histamine release test may have been too low. On the other hand, a synergistic effect on the histamine release response mediated by additional components in the partially purified toxins cannot be excluded.

The IgE-immunoblotting revealed more IgE-sensitized patients (53%) than the leukocyte histamine release test (24%). Partly, this difference can be explained by the use of lower concentrations of the purified toxins in the histamine release test. Half of the sera not responding to the partially purified toxins in the histamine release test, however, showed toxin-specific IgE antibodies in immunoblotting. Since human myeloma-IgE at a concentration of 4000 U/mL binds to the 28-kD component of enterotoxin A and en-

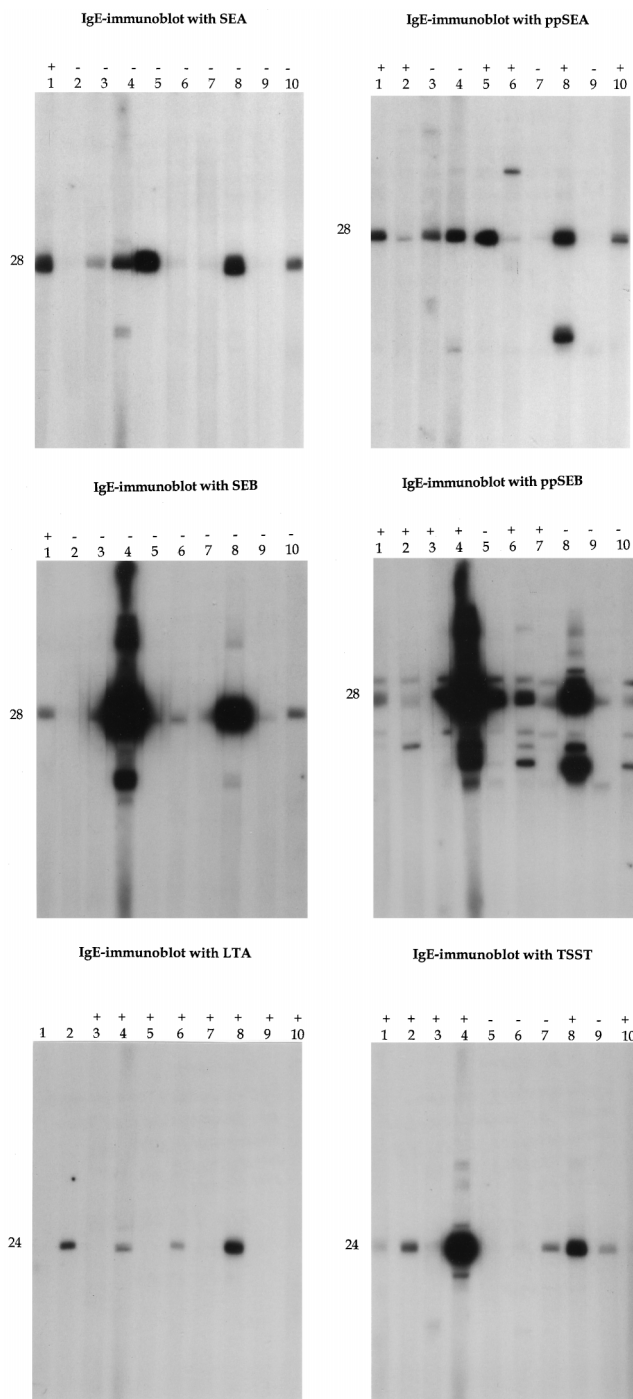


Figure 1a. Representative immunoblots and histamine release results of ten patients. The most frequently IgE-binding component (in kD) of purified enterotoxin (SEA) and partially purified enterotoxin A (ppSEA) is marked on the figure. The top row shows individual histamine release (HR) test results where (+) is a positive result and (-) is a negative result.

Figure 1b. Representative immunoblots and histamine release results of ten patients. The most frequently IgE-binding component (in kD) of purified enterotoxin B (SEB) and partially purified enterotoxin B (ppSEB) is marked on the figure. The top row shows individual histamine release (HR) test results where (+) is a positive result and (-) is a negative result.

Figure 1c. Representative immunoblots and histamine release results of ten patients. The most frequently IgE-binding component (in kD) of lipoteichoic acid (LTA) and toxic shock syndrome toxin 1 (TSST) is marked on the figure. The top row shows individual histamine release (HR) test results where (+) is a positive result and (-) is a negative result.

terotoxin B, it is possible that some patient sera with very high total IgE levels will show nonspecific binding to the toxins. On the other hand many patients with atopic dermatitis may have low titers of circulating antibodies directed against the toxins because of frequent staphylococcal colonization of damaged skin sites. This is rendered by the observation that the atopic control group did not show IgE-binding (except one) despite the fact that they had elevated IgE levels.

Another study demonstrated that *S. aureus* isolated from the skin of atopic dermatitis patients secreted enterotoxins (A, B, and toxic shock syndrome toxin 1).⁶ We employed the same techniques and demonstrated secretion of enterotoxin A and toxic shock syndrome toxin 1 in four isolates (data not reported). Together these findings suggest that the route of sensitization may be via the skin. The clinical importance of this IgE sensitization is yet unknown but perhaps the leukocyte histamine release test being a cellular test may distinguish cellular activity from clinically unimportant IgE circulating antibodies. Lipoteichoic acid was also included in the study to examine the importance of toxins compared with a material representing the whole bacteria. When evaluating the frequency distributions of test results (Table 1) it seems that lipoteichoic acid is just as important as the toxins; however, two controls showed IgE-binding to lipoteichoic acid. Possibly, persons having previously been infected with this organism have a low titer of IgE-antibodies. Why this is not observed with toxins may be due to the fact that normal individuals generally are not carriers of toxin-secreting staphylococci.

Sodium dodecylsulfate-polyacrylamid gel electrophoresis revealed that the purified toxins all contained IgE-binding components exceeding the molecular weight of the toxins themselves (main components). The 54-kD and 50-kD components may be dimers as this phenomenon has been observed upon lyophilization (according to the manufacturer). The 96-kD component

may be a trimer of the 33-kD component or both of these may be impurities (95% purity of toxins). Possibly, components smaller than toxins are subunits or impurities. The partially purified toxins are approximately 50% pure with components ranging from <14.3 to >200 kD. The additional components are other staphylococcal extracellular proteins including protease, nuclease, lipase, and hemolysins. The IgE-binding components of the partially purified toxins (that are not toxins) are probably mainly hemolysins since they have molecular weights corresponding closely to the molecular weight of the additional IgE-binding components found in this study.¹⁵

In conclusion, a cellular induced IgE-mediated response to toxins and lipoteichoic acid in patients with atopic dermatitis was demonstrated by leukocyte histamine release. Then the IgE-binding components of the toxins were identified by IgE-immunoblotting. Two major protein components of the toxins (24-kD and 28-kD proteins) seemed more important than several additional proteins identified in the partially purified toxins.

The study supports the clinical impression that, in addition to other mechanisms, staphylococcal infection may exacerbate inflammation in some patients with atopic dermatitis by IgE-mediated mechanisms due to sensitization to toxins secreted by staphylococci in the skin of patients with atopic dermatitis. In many patients, however, the sensitization may be clinically irrelevant.

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