

Superantigen Profile of *Staphylococcus Aureus* Isolates from Patients with Atopic Dermatitis in Sri Lanka

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Abstract

Background: *Staphylococcus aureus* colonizes most patients with atopic dermatitis. *Staphylococcus aureus* is able to produce staphylococcal enterotoxins, staphylococcal enterotoxin-like toxins and Toxic shock syndrome toxin – 1. The presence of superantigen encoding genes has been associated with atopic dermatitis in other cohorts.

Aim: To determine the association of different Staphylococcal superantigens with clinical disease severity in a tropical disease setting in patients with atopic dermatitis.

Methodology: Skin swabs collected from 100 patients with atopic dermatitis and 120 controls were cultured. Severity of atopic dermatitis was graded using the Nottingham Eczema Severity Score. Bacterial DNA was extracted and superantigen genes were detected by separate PCRs.

Results: 52/59 (88%) of *Staphylococcus aureus* isolates from patients and 5/16 (31%) from healthy individuals had genes encoding at least one type of superantigen. Each superantigen type was expressed significantly higher ($P < 0.05$) in patients than in healthy individuals. Possession of genes for staphylococcal enterotoxin-like toxin type M ($P = 0.038$) and staphylococcal enterotoxin-like toxin type O ($P = 0.016$) were associated with milder disease. Possession of staphylococcal enterotoxin type B gene was significantly higher ($p = 0.0186$) in *Staphylococcus aureus* isolated from patients who were aged 5 years or older compared to *Staphylococcus aureus* isolated from younger patients. Strains possessing genes that encode the classical superantigens such as staphylococcal enterotoxin type A, B, C, E and Toxic shock syndrome toxin – 1 were significantly associated with patients with moderate to severe disease when they possessed not more than two superantigen genes in total.

Conclusions: Superantigens associate with atopic dermatitis in a cohort of patients from Sri Lanka consistent with a role in disease pathogenesis. Differences in superantigen gene possession are unlikely to explain differences in disease prevalence between populations from tropical and temperate climates.

Keywords: Atopic dermatitis; Superantigens; Clinical diseases severity; *Staphylococcus aureus*

Abbreviations: AD: Atopic Dermatitis; SA: *Staphylococcus aureus*; SAGs: Superantigens; Th: Helper T cell; SEs: Staphylococcal Enterotoxins; SEIs: Staphylococcal Enterotoxin-like Toxins; TSST-1: Toxic Shock Syndrome Toxin – 1; egc: enterotoxin gene cluster

Introduction

Atopic dermatitis (AD) is a chronic relapsing, itchy, inflammatory condition of the skin. It is one of the commonest skin diseases affecting around 10% to 30% of children and 2-3% of adults [1-3]. Studies have shown that superantigen producing strains of *Staphylococcus aureus* (SA) could be isolated from over 70% of patients with AD [4-6]; Superantigens (SAGs) of SA have been shown to play an important role in inducing skin inflammation in AD. They are thought to aggravate the disease by multiple pathways including activation of T cells that are important in the disease pathogenesis of AD. SAGs have shown to induce expansion of allergen specific Th2 cells [7], and stimulate the production of proinflammatory cytokines by T cells [8]. They also increase presentation of allergens such as house dust mite by keratinocytes [7] and have also shown to reduce activity of regulatory T cells [9,10].

Staphylococcal superantigens include staphylococcal enterotoxins (SEs), staphylococcal enterotoxin-like toxins (SEIs) and Toxic shock syndrome toxin – 1 (TSST-1). SEs and SEIs are a broad family of pyrogenic toxin superantigens [11]. Those that induce vomiting are

designated as SEs and the related toxins that lack emetic activity or have not been tested for it are designated as SEIs [12]. These include the SEs: SEA-E, SEG-I, SER-T and the SEIs: SEIJ-Q, SEIU, SEIU2, and SelV13. SA strains producing SAGs encoded by the enterotoxin gene cluster (SEG, SEI, SEIM, SEIN, SEIO) are commonly isolated from patients with AD [4,14]. Although high percentages of SAG producing SA strains have been isolated from patients with AD, a significant association of any particular SAG with clinical disease severity has not been observed [4,5,14,15]. However, sensitization to staphylococcal superantigens has found to be associated with higher disease severity [16,17].

Although the association of SAGs and AD is well established, the association in tropical climates is not known. Differential superantigen

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expression between populations is clearly of interest in order to determine the potential relative roles in disease; an absence of SAg expression in isolates from patients living in tropical climates would potentially argue against their role in disease pathogenesis. Therefore we have investigated the SAg profile of SA isolates from patients with AD in Sri Lanka.

Materials and Methods

Participants

A total of 100 patients with AD (40 male, 60 female patients; mean age $6 \pm$ SD 9.6 years, range 0.1– 46.8) were recruited from a tertiary care hospital in Sri Lanka. The study was approved by the local ethics committee, and all participants gave informed written consent. AD was defined according to the U.K. Working Party's Diagnostic Criteria [18] and severity was graded according to the Nottingham Eczema Severity Score : preliminary refinement of the Rajka and Langeland grading' by a dermatologist [19]. The patient group included 61 (61%) patients with mild AD, 30 (30%) patients with moderate AD and 9 (9%) patients with severe AD. The control group comprised of 120 (60 male, 60 female patients; mean \pm SD age $14.9 \pm$ 12.3 years, range 0.7–47) age-matched individuals (healthy volunteers) who did not have any history of asthma, AD or allergic rhinitis.

Isolation of *Staphylococcus aureus*

Skin swabs were collected from eczematous lesions and nonlesional areas from patients with AD (swabs from two lesions and two nonlesional areas) and also from healthy individuals (from left and right antecubital fossae). Swabs were inoculated onto mannitol salt agar (Hi-Media Laboratories, Mumbai, India) and SA was identified after overnight incubation at 37°C.

Bacterial DNA extraction and PCR for superantigen toxin genes

Bacterial DNA extracted using the DNeasy blood and tissue kit (Qiagen®) according to the manufacturer's instructions. The presence of genes encoding TSST-1, SEA-E, SEG-I and SEI/Q were detected for all the isolates by separate PCRs for each superantigen using the primer sets described previously [20,21] (Table 1). PCR products were separated on 1% w/v agarose (Promega) in 1×TAE buffer and visualized under a UV transmitter (Fotodyne™).

Statistical analysis

Data analysis was carried out using GraphPad Prism 4. Fisher's exact test was used to calculate statistical significance. A "P" value < 0.05 was considered significant.

Results

SAg expression in AD patients and in healthy individuals

The total numbers of patients with SA isolated was 59/100 (59%) compared to the number of controls 16/120 (13.3%). Skin colonization was seen in 57 (57%) patients compared to 10 (8%) controls and nasal colonization of SA was seen in 45 (45%) patients and in 9 (8%) controls. Two patients had nasal colonization only. 52/59 (88%) of SA isolates from patients and 5/16 (31%) from healthy individuals had genes encoding at least 1 type of SAg gene. The frequencies of each of these SAg in SA colonized patients with varying severity are shown in Table 2. Each type of SAg gene was present in significantly higher numbers of patients when compared to healthy individuals with the

exception of SE/L (P=0.14) and SE/H (not found). The presence of genes encoding SE/M (P=0.038) and SE/O (P=0.016) were associated with milder disease but this was not significant after correction for multiple comparisons.

Colonization with SA strains possessing genes for SAg subtypes in patients with varying clinical disease severity

In addition, the involvement of the body surface area showed a trend towards being less in patients colonized with SE/O (p=0.24, OR, 0.4802, CI = 0.3122 to 1.258). SEO gene possessing SA strains were found in 9 (45%) of patients who scored less than 3 points in the body surface area affected, whereas SEO was seen in 11 (28%) of patients who scored >3 points. Although not statistically significant, patients colonized with SEA gene possessing SA strains showed higher risk of developing moderate to severe AD (P = 0.2432, OR = 2.19, CI = 0.7026 to 4.645). None of the SAg was significantly higher in AD with other atopic diseases such as asthma, allergic rhinitis and food allergies (P>0.05) when compared to AD patients who did not have other atopic diseases. Further SAg were not significantly associated with duration of AD lesions, body surface area affected or sex of patients.

Possession of SEB gene was significantly higher (P=0.019) from SA isolated of patients who were aged 5 years or older compared to SA isolated of younger patients. Although not statistically significant, genes encoding SEC and SEE were also found at a higher frequency

Superantigenic toxin type	Sequence (5' to 3') for Forward/Reverse primers	Ta/°C	product size
SEA	GCAGGGAAACAGCTTTAGGC GTTCTGTAGAGTATGAAACACG	53	521bp
SEB	GTATGATGATAATCATGTATCAGCAATA CGTAAGATAAACTTCAATCTTCACATC	53	625bp
SEC	GAGTCAACCAGACCCTATGCC CGCCTGGTGCAGGCATC	55	614bp
SED	GAGACTAGCCGCAATCTATCC GCTGCATTTAGTAATGCTGGCTG	54	650bp
SEE	GGTAGCGAGAAAAGCGAAG GCCTTGCCGTAAGATCTAGCTC	54	450bp
SEG	TGAATGCTCAACCCGATCCTAAAT CAAACCAAAAACCTTGATTGTTCTTTCA	54	580bp
SEH	TTCACATCATATGCGAAAGCAGAA CAGATTTTAAAGTTTTATTGTCTTCA	50	625bp
SEI	CGTATGCTCAAGGTGATATTGGTG AAAACTTACAGGCAGTCCATCTCC	55	580bp
SEJ	TTTAGGATCCCTACAGAACCAAGG GTTTCCATGGATAGCAAAAATGAAAC	50	750bp
SE/K	GTGTCTTAATAATGCCAGCGCTC TTTGGTAGCCCATCATCTCC	55	650bp
SE/L	CACCAGAATCACACCGCTTA TCCCCTTATCAAAAACCGCTAT	51	450bp
SE/M	TTTTGCTATTTCGAAAATCATATCGCA TCAACTTTGTCCTTATAAGATATTTCTAC	54	687bp
SE/N	TGAGATTGTTCTACATAGCTGCAA AATTAGATGAGCTAACTGTTCTATTATCAC	54	720bp
SE/O	TAGTGTAACAATGCATATGCAAATG ATTATGTAATAAATAAACATCAATATGATA	50	721bp
SE/P	GGAAGCTAAAGCAGAGACAC CCCCTTTTCATATGAAGTGCCACC	51	660bp
SE/Q	GCTTCAAGGAGTTAGTTCTGG CTCTCTGCTTGACCACTGCCGGTG	57	510bp
TSST-1	GAAATTTTTTCATCGTAAGCCCTTTGTTG TTCATCAATATTTATAGGTGGTTTTTCA	53	625bp

Table 1: Base sequences, annealing temperature selected for the primer set (Ta), and product size of amplified products and the control for the superantigen primer sets.

Superantigenic toxin type	No of isolates from		Odds ratio	95% Confidence interval	P value (fisher exact test)	P _c
	patients with mild AD (total n=28) n/(%)	patients with moderate to severe AD (total n=31) n/(%)				
SEA	05 (17.8)	10 (32)	2.19	0.7026 to 4.645	0.24	4.13
SEB	04 (14.3)	4 (12.9)	0.89	0.2490 to 3.277	1.00	17.00
SEC	07 (25.0)	8 (25.8)	1.04		1.00	17.00
SED	05 (17.9)	4 (12.9)	0.68	0.1634 to 2.842	0.72	12.29
SEE	04 (14.3)	5 (16.1)	1.15	0.3361 to 3.793	1.00	17.00
SEG	13 (46.4)	10 (32.3)	0.55	0.3638 to 1.327	0.29	5.04
SEH	00 (0)	00 (0)				0.00
SEI	13 (46.4)	13 (41.9)	0.83	0.5085 to 1.604	0.79	13.53
SEJ	02 (7.1)	3 (9.7)	1.39		1.00	17.00
SEK	03 (10.7)	4 (12.9)	1.23		1.00	17.00
SEL	01 (3.6)	5 (16.1)	5.40		0.20	3.36
SEM	17 (60.7)	10 (32.3)	0.31	0.2943 to 0.9593	0.04	0.65
SE/N	12 (42.9)	9 (29)	0.55	0.3373 to 1.360	0.30	4.95
SE/O	14 (50)	6 (19.4)	0.24	0.1724 to 0.8689	0.01	0.27
SE/P	05 (17.9)	4 (12.9)	0.68		0.72	12.29
SE/Q	03 (10.7)	4 (12.9)	1.39		1.00	17.00
TSST-1	06 (21.4)	6 (19.4)	0.88		1.00	17.00

Table 2: Association of superantigen production with severity of atopic dermatitis (AD).

in patients aged 5 years or older compared to patients who were younger. When SEB, SEC and SEE were grouped together, SA isolates from patients 5 years or older produced at least one type of these SE when compared to younger patients, which was statistically significant (P=0.0001).

Multiple SAg gene possession and disease severity

SA was isolated from 28 (46%) patients with mild AD and 25 (89%) isolates had genes encoding the SAgS. Out of 31 SA isolates from patients with moderate to severe AD 27 (87%) were positive for SAg genes. Strains possessing genes that encode the classical superantigens such as SEA, SEB, SEC, SEE and TSST were significantly associated with patients with moderate to severe disease when they possessed not more than 2 SAg genes in total. Such SA strains comprised 12 out of 27 (44%) of SAg gene positive strains isolated from patients with moderate to severe AD when compared to 3/25 (12%) of SAg gene positive strains from patients with mild AD (P= 0.014).

Discussion

Our data show that SAg gene positive SA strains are expressed at a high frequency in patients with AD. Each SAg type was found at a significantly higher frequency (P<0.05) in patients with AD than in healthy individuals colonized with SA, with the exception of SEL and SEH. Our results indicate that a very high percentage (88%) of SA strains isolated from patients with AD is positive for genes that encode SAgS, which supports their role in the pathogenesis of AD. This high percentage is compatible with the findings from previous studies from other cohorts where 70 –100% of strains isolated were positive for SAg genes [4-6,22].

Our previous studies have shown that SA skin colonisation rates (57%) were comparatively lower in AD patients in Sri Lanka when compared to SA colonisation rates of patients from European countries, United States [23] and some Asian countries [15]. In these countries the SA colonization rates were between 80% - 100% [24,25]. However, SA colonisation rates in healthy Sri Lankan individuals (13%) were comparable to the rates reported in healthy European individuals (6-30%) [26]. Although, SA colonization rates were lower in AD patients in Sri Lanka, the presence of genes encoding SAg in SA isolates were

similar to those from countries with a high prevalence. Therefore, differential superantigen gene possession cannot explain differences in disease prevalence between populations from tropical and temperate climates.

Out of the superantigens SEM, (46%), SEI (44%), SEG (39%), SE/N (36%) and SE/O (34%) were the most abundant. All these genes are located on an accessory genetic element known as the enterotoxin gene cluster (egc) [13]. These SAgS have also been shown to be the most frequent enterotoxins (37-48%) produced by SA colonising patients with AD [4,14]. egc-genes have found to be the most prevalent SAg genes in commensal and invasive SA isolates with reported frequencies between 52 and 66% [27-29]. However these studies have not found these egc coded SAgS to be significantly associated with clinical disease severity.

Although a significantly positive relationship was not observed between any individual SAg and disease severity, the presence of genes encoding SEM (P=0.0380) and SE/O (P=0.0158) showed a trend towards being associated with milder disease. SEM and SE/O are found to induce 21.3 and 7.1 subpopulations of Vβ T cells respectively [30]. These two subpopulations are not induced by the majority of other SAg types or induced comparatively weakly [30]. These two subpopulations of Vβ T cells may have a modified response when induced. We did not find any significant association with any of the SAgS with duration of AD lesions, body surface area affected or sex of patients. The presence of SAg genes was also not significantly higher in patients with AD with other atopic diseases such as asthma, allergic rhinitis and food allergies than those with AD alone. However, possession of genes encoding SEB alone and a SAg group including SEB, SEC and SEE were seen at a higher frequency in patients 5 years or older (P<0.05).

Several recent studies have not observed a significant association of any particular SAg with clinical disease severity of AD [4,5,14,15]. Yet these studies have also shown high percentages of SAg gene positive SA in patients with AD. Other studies have found allergic sensitization to staphylococcal superantigens to be associated with higher disease severity [16,17]. Therefore, it will be important to investigate sensitization and Vβ T cell expansion studies in SA colonized patients with AD in Sri Lanka.

Overall these data show in a population of individuals in Sri Lanka, that proportional superantigen gene possession associates with the presence of AD, and confirms the findings observed in cohorts from temperate climates. This would support a role of superantigen expressing SA in the pathogenesis of AD and suggests that differential superantigen gene possession cannot explain differences in disease prevalence between populations from tropical and temperate climates.

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