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Gram-positive bacterial superantigen outside-in signaling causes toxic shock syndrome

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Staphylococcus aureus and Streptococcus pyogenes (group A streptococci) are Gram-positive pathogens capable of producing a variety of bacterial exotoxins known as superantigens. Superantigens interact with antigen-presenting cells (APCs) and T cells to induce T cell proliferation and massive cytokine production, which leads to fever, rash, capillary leak and subsequent hypotension, the major symptoms of toxic shock syndrome. Both S. aureus and group A streptococci colonize mucosal surfaces, including the anterior nares and vagina for S. aureus, and the oropharynx and less commonly the vagina for group A streptococci. However, due to their abilities to secrete a variety of virulence factors, the organisms can also cause illnesses from the mucosa. This review provides an updated discussion of the biochemical and structural features of one group of secreted virulence factors, the staphylococcal and group A streptococcal superantigens, and their abilities to cause toxic shock syndrome from a mucosal surface. The main focus of this review, however, is the abilities of superantigens to induce cytokines and chemokines from epithelial cells, which has been linked to a dodecapeptide region that is relatively conserved among all superantigens and is distinct from the binding sites required for interactions with APCs and T cells. This phenomenon, termed outside-in signaling, acts to recruit adaptive immune cells to the submucosa, where the superantigens can then interact with those cells to initiate the final cytokine cascades that lead to toxic shock syndrome.

Introduction

In 2007 based on 2005 data, the Centers for Disease Control and Prevention (CDC), together with their collaborators, reported that *Staphylococcus aureus* is the leading cause of serious and fatal infectious diseases in the USA [1]. This results from the organism making many virulence factors and being a common commensal organism on human skin and mucosal surfaces; as many as <u>40%</u> of humans may be colonized on mucosal surfaces, such as anterior nares and vagina [2]. It is well known that persons with damaged skin, such as in atopic dermatitis, are nearly always colonized on skin surfaces with the organisms [3]. *S. aureus*

Abbreviations

APC, antigen-presenting cell; CA-MRSA, community-associated MRSA; HA-MRSA, hospital-associated MRSA; HLA, human leucocyte antigen; IFN-γ, interferon-γ; IL, interleukin; LPS, lipopolysaccharide; MCP-1, monocyte chemotactic protein 1; MHC II, major histocompatibility complex class II; MIP-3α, macrophage inflammatory protein 3α; MRSA, methicillin-resistant *S. aureus*; MSSA, methicillin-sensitive *S. aureus*; OB, oligosaccharide/oligonucleotide binding; PANDAS, pediatric auto-immune neuropsychiatric disorders associated with streptococcal infections; SAg, superantigen; SE, staphylococcal enterotoxin; SLO, streptolysin O; SMEZ, streptococcal mitogenic exotoxin; SSA, streptococcal superantigen; TCR, T cell receptor; TNF, tumor necrosis factor; TSS, toxic shock syndrome; TSST-1, toxic shock syndrome toxin 1.

strains are not restricted to human colonization and infection, as many animal species are also infected by the organisms. However, despite some strain crossover, <u>human strains tend to colonize humans and ani-</u> mal strains tend to <u>colonize animals</u>.

Streptococcus pyogenes (group A streptococcus) also has a significant disease association with humans. Unlike S. aureus, group A streptococci are considered primary pathogens of humans only in that infected persons typically become ill upon their first exposure to the organism, most often with either pharyngitis or impetigo, but more recently also with severe invasive illnesses, and humans transmit the organism to each other [4]. Group A streptococci and S. aureus, although sharing many properties, differ in two important aspects of disease causation, in that group A streptococci are more invasive in causing serious human illnesses, such as toxic shock syndrome (TSS), than S. aureus, and group A streptococci strains have the potential to cause delayed sequelae such as rheumatic fever, acute glomerulonephritis and guttate psoriasis, whereas S. aureus strains do not [4].

Staphylococcus aureus and group A streptococci are both Gram-positive cocci, although S. aureus strains are facultative aerobes whereas group A streptococci are aerotolerant anaerobes. Both organisms depend on production of a myriad of cell-surface and secreted virulence factors for host colonization and disease production. The cell-surface virulence factors include a variety of proteins referred to as microbial surface components recognizing adhesive matrix molecules [5]. These molecules, including fibronectin-binding proteins and collagen-binding proteins among many others, facilitate attachment to host cells, or interfere with host immune responses through the antiphagocytic action of proteins such as protein A (S. aureus) or protein G (group A streptococci). Although important in disease production, these proteins will not be discussed further in this review.

Both organisms also produce large numbers of secreted virulence factors, including families of superantigens (SAgs) and cytolysins. These two major classes of exoproteins interfere with immune system function systemically (SAgs) and locally (cytolysins). This review will focus on the role of SAgs in human illnesses, their structure and function, and will discuss cytolysins in their role of facilitating SAg penetration of epithelial barriers and microorganism invasion systemically.

The superantigen family

Nearly all *S. aureus* strains have the capacity to produce one or more SAg proteins, including toxic shock

syndrome toxin 1 (TSST-1), staphylococcal enterotoxins (SEs) serotypes A, B, C (including multiple SEC subtypes), D, E and I, and SE-like SAgs, including SE-like G, H, J-X [6-9]. There is no SE or SE-like F protein, as this designation was retired when the protein was renamed TSST-1. SEs and SE-like SAgs differ in that SEs are emetogenic when given orally to monkeys and eaten by humans, whereas SE-like proteins either lack emetogenic activity or have not been tested [8]. All of the staphylococcal SAgs, except SE-like X. are encoded on DNA genetic elements that are considered variable traits in that their SAg genes are present in some S. aureus strains but other SAg genes are present in other strains. Staphylococcal SAgs are encoded by genes located on bacteriophages (e.g. SEA), plasmids (e.g. SED) or pathogenicity islands (most SAgs), and in the case of SE-like X, the core chromosome [9].

Group A streptococci also produce numerous SAgs, including streptococcal pyrogenic exotoxin (SPE, scarlet fever toxin) serotypes A, C, G–M, streptococcal superantigen (SSA), and streptococcal mitogenic exotoxin Z (SMEZ with numerous subtypes) [4]. With the exception of SPE G and SMEZ, which are encoded by genes within the core chromosome, all remaining streptococcal superantigens are encoded by genes located on bacteriophages, many of which are defective and essentially trap the SAg gene in the chromosome [4].

Although originally described in S. aureus and group A streptococci, SAgs are increasingly being isolated from coagulase-negative staphylococci and other groups of β -hemolytic streptococci, particularly groups C and G [10-14]. The increasing presence of SAgs in coagulase-negative staphylococci is particularly disconcerting, since this gives these normal flora organisms greater potential to cause serious human illnesses. The SAgs in coagulase-negative staphylococci and other groups of β -hemolytic streptococci thus far are related serologically and biochemically to those present in either S. aureus or group A streptococci, but there is ample reason to believe that previously undescribed SAgs will also be found in such strains, expanding our horizons on the numbers and types of SAgs and their associated infections.

Biochemistry of superantigens

SAgs are simple, non-glycosylated proteins that are secreted from bacterial strains through cleavable signal peptides [4]. The proteins have relatively low molecular weights (19 000–30 000) and appear tightly folded. They are generally resistant to heat (e.g. TSST-1 can be boiled for up to 1 h without loss of biological

activity), proteolysis (this allows SEs to resist gut proteolysis and cause staphylococcal food poisoning), weak acids (SEs are resistant to 10% bleach for several minutes) and desiccation (TSST-1 remains completely biologically active when dried onto Petri dishes for more than 1 year); the proteins are easily <u>inactivated</u> by bases, such as 1 M NaOH [4,9].

Structural features of superantigens

SAgs contain two major structural domains: an amino-terminal oligosaccharide/oligonucleotide binding (OB) fold and a carboxy-terminal β -grasp domain. An epithelial cell binding domain, referred to as the dodecapeptide region, resides predominantly on the central α -helix in the β -grasp domain. The region for T cell receptor (TCR) binding resides in a groove between both major folds, a low-affinity major histocompatibility complex class II (MHC II) site is located in the OB fold, and a higher-affinity MHC II site is located in the β -grasp domain. The staphylococcal and streptococcal SAgs can be divided into five groups based on their amino acid sequences and shared structural features (Table 1) [9].

Group I is characterized by TSST-1, which has an amino acid sequence that is unique compared with other SAgs. Also included in this group are the variant TSST-1, referred to as TSST-ovine, and a recently identified SAg named SE-like X. The dodecapeptide region of TSST-1 (119-FDKKQLAISTLD-130) is also unique among SAgs in that it is contained solely on the central α -helix and a nearby loop, but not involving β -strands. These group I SAgs have low-affinity MHC II sites and TCR interaction sites on the back of the SAgs as seen in the standard view (Fig. 1); these SAgs lack emetogenic activity.

Group II SAgs are characterized by SEB, SEC and SPE A and the presence of a cystine loop structure in which the cystines are separated by 10–19 amino acids [15]. This cystine loop is required for emetogenic activity but its presence does not automatically confer emetogenic ability because SPE A contains the cystine

loop but is not emetogenic [15,16]. It appears that the structural conformation induced by the loop is more important than the loop itself for activity. Like group I SAgs, group II SAgs have only the low-affinity MHC II binding site, lacking the higher-affinity MHC II site, and interact with the α -chain of MHC II through their OB-fold domains. It is important to note that a zinc binding site has been found in SPE A, but its role in MHC class II binding remains unclear [17–19]. Group II SAgs interact with the TCR on the top front of the SAgs.

Group III SAgs, which include SEA, SED and SEJ, are similar to group II SAgs due to the presence of the cystine loop structure, but in the case of group III SAgs the loop is always nine amino acids long. Group III SAgs differ from groups I and II SAgs because group III proteins have both low- and higher-affinity binding sites for MHC II molecules. These SAgs can bind either the α -chains of MHC II molecules through their OB folds (low affinity) or the β -chains through their β -grasp domains (higher affinity). These SAgs are emetogenic as well as superantigenic. Interestingly, one member of this group, SEH, was shown to bind the TCR α -chain, as opposed to the TCR β -chain that is used by the other SAgs [20–22].

Group IV SAgs are all produced by group A streptococci. This group includes SPE C, SPE J and SMEZ, the SAgs do not contain a cystine loop structure, and the SAgs have both low- and higher-affinity binding sites for MHC II. These SAgs are non-emetogenic.

Most of the recently characterized SAgs, such as SE-like K and SE-like Q, are contained within group V. These SAgs have both low- and higher-affinity binding sites for MHC II, and do not have cystine loop structures, similar to group IV SAgs; however, group V superantigens also have similar 15 amino acid inserts that are not found in the other SAgs. The inserts are loop extensions that reside between the third α -helix and the eighth β -strand, referred to as the α 3- β 8 loop. These loops have been shown to be required for T cell activation for both SPE I and SE-like K, and are thought to be required for activity of

Table 1. Staphylococcal and streptococcal SAg groups defined by amino acid sequence and three-dimensional structure similarities.

Group	Example SAgs	MHC II binding	Structural features
 V V	TSST-1, TSST-ovine, SE-like X SEB, SPE A, SEC, SSA, SEG SEA, SEE, SEJ, SED, SEH SPE C, SPE J, SPE G, SMEZ-2 SEI, SE-like K, SE-like L, SE-like P, SE-like Q, SPE H	Low-affinity (α -chain) site Low-affinity (α -chain) site Low- and higher-affinity (α - and β -chain) sites Low- and higher-affinity (α - and β -chain) sites Low- and higher-affinity (α - and β -chain) sites	Unique amino acid sequence, no cystine loop Variable length cystine loop Nine amino acid length cystine loop No cystine loop No cystine loop and a 15 amino acid insert



Fig. 1. Crystal structures of representative superantigens from each group. The dodecapeptide region is highlighted in yellow. TSST-1 and SPE A residues that, when mutated to alanine, result in different levels of IL-8 induction from human vaginal epithelial cells are depicted as follows: no IL-8 release in red, low IL-8 release in orange, and high IL-8 release in green. Key residues known to be involved in VB-TCR binding are depicted in violet, while key residues involved in MHC II higher-affinity and low-affinity binding are shown in gray and teal. respectively (not all residues required for binding are shown). Group 1: TSST-1 (PDB 4TSS [150]); Vβ-TCR, H135/Q136 [151,152]; MHC II, G31/S32 [153,154]; epithelial, F119–D130 [75]. Group II: SPE A (PDB 1UUP [155]); Vβ-TCR, L24, N54/Y55, C90, C98 [156]; MHC II, L42-Y48 [157]; epithelial, T135-D146. The cystine loop is shown in brown: Y88-A97 (note that for SPE A the cystine loop is apparently positioned incorrectly to allow SPE A to be emetogenic) [19]. Group III: SEA (PDB 1ESF [158]); Vβ-TCR, S206/N207 [158,159]; MHC II, H187, H225, D227 (high) [158] and F47 (low) [158]; proposed epithelial, T145–D156; cystine loop, A97–A105 [160]. Group IV: SPE C (PDB 1AN8 [137]); Vβ-TCR, Y15, R181 [161]; MHC II, H201, D203 (high) [130,136]; proposed epithelial, L122–D133. Group V: SE-like K (PDB 3EA6, Shi K, Huseby M, Schlievert PM, Ohlendorf DH and Earhart CA, unpublished results); VB-TCR, H142, Y158 [24]; MHC II binding residues have not been defined but proposed important residues based on SEI, H207, D209, D211 (high) and N98 (low) [24,162]; proposed epithelial, T115-D126. The α3-β8 loop of SE-like K is also shown in pink: G141-G160 [23,24]. Images generated using PYMOL (DeLano Scientific LLC, South San Francisco, CA, USA).

all SAgs that fall into this group [23,24]. Additionally, it appears that the $\alpha 3-\beta 8$ loop dictates the V β -TCR specificity of the SAgs in this group [24]. The SAgs in

this group that have been tested lack emetogenic activity. For this reason, they are referred to as SE-like.

Disease associations of superantigens

Staphylococcal and streptococcal SAgs unquestionably cause TSS, and probably contribute significantly to other illnesses [4,6,7,9,25]. Staphylococcal TSS is an illness defined by the following criteria: fever, hypotension, erythematous rash, peeling of the skin upon recovery, and any three of a multi-organ component, often seen initially as vomiting and diarrhea (easily confused with flu-like symptoms) [4,26–28]. If one defining criterion is absent, the illness is referred to as probable TSS [29]. If more than one defining symptom is missing and other causes are ruled out, the illness can be considered toxin-mediated disease [30]. Staphylococcal TSS is separated into two major categories. menstrual and non-menstrual illness. Menstrual TSS, as its name implies, occurs during or within 2 days of women's menstrual periods, and the illness is most often associated with tampon use [26,27]; the tampon association primarily results from tampon-introduced oxygen being required for TSST-1 production [31–33]. The human vagina in the absence of tampons is normally anaerobic. TSST-1 is the cause of nearly all menstrual cases of TSS, probably because of its greater capacity than other SAgs to penetrate mucosal surfaces (discussed later in this review) [9,25,34]. One paper has suggested other SAgs may occasionally be associated with menstrual TSS [35]. Non-menstrual TSS takes on many forms and can be associated with nearly any type of S. aureus infection [29]. The most common non-menstrual TSS is illness following upper respiratory viral infection, described initially in 1987 by Mac-Donald et al. [36]. It is suggested that the multiple proteases produced by S. aureus strains activate the hemagglutinin of influenza virus, facilitating the ability of the virus to initiate infection, which in turn creates a damaged epithelial site allowing secondary S. aureus infection. We estimate that $> 10\ 000$ people in the USA succumb to this infection yearly. Non-menstrual TSS is primarily caused by TSST-1 (50%) and two SEs, SEB and SEC (50% together), which are produced in higher concentrations than other SAgs (up to 10⁶-fold more) [34]. Other SAgs are occasional causes of non-menstrual TSS, presumably by strains that have upregulated production of their SAgs. The overall incidence of TSS and probable TSS combined, including both menstrual and non-menstrual categories, is 1-3per 100 000 population with a case : fatality rate of 5-10%. Occasionally staphylococcal TSS cases are now being seen in association with necrotizing fasciitis

and myositis; this form of the illness appears to be newly emergent [37,38].

Staphylococcal TSS is managed clinically with use of antibiotics and fluid, electrolytes and vasopressors as needed to maintain blood pressure, sometimes activated protein C in an attempt to manage sepsisinduced microvascular thrombosis, and intravenous immunoglobulin to neutralize SAgs in severe cases [39,40]. It is important to know if TSS cases are associated with methicillin-resistant S. aureus (MRSA) or methicillin-sensitive S. aureus (MSSA), as antibiotic selection for treatment of cases is different. MRSA are managed with antibiotics such as vancomycin with clindamycin, whereas MSSA may be managed with B-lactam antibiotics. Today, MRSA appear in two forms, hospital-associated (HA-MRSA) and community-associated (CA-MRSA) [1,41]. HA-MRSA typically are <u>multi-antibiotic</u> resistant, but the majority of these strains do not make large amounts of SAgs [42]. In contrast, CA-MRSA strains, which include pulsedfield gel electrophoresis types USA200, USA300 and USA400 (according to CDC designations), typically are resistant to β -lactam antibiotics and sometimes clindamycin, and these strains produce high levels of SAgs [42]. USA200 CA-MRSA strains produce TSST-1, USA300 CA-MRSA produce SE-like X, and USA400 CA-MRSA produce either SEB or SEC (it is important to note that the corresponding MSSA strains also produce these same high-level SAgs). Thus, nearly all CA-MRSA, and their MSSA counterparts, have the ability to cause TSS-like illnesses.

Staphylococcal SAgs are also associated with and cause a myriad of other illnesses (Table 2) [4]. Note that each of these illnesses could be the basis of an entire review, and thus only key references are provided. Readers are encouraged to perform searches for additional references. It is particularly noteworthy that nearly every year since the initial description of staphylococcal TSS in 1980, new illnesses have been associated with staphylococcal SAgs. Four examples are presented. In 2005, Kravitz et al. [39] described for the first time staphylococcal purpura fulminans, which is a particularly severe form of TSS, characterized by TSS symptoms and rapidly progressing disseminated intravascular coagulation. When initially described, the illness was 100% fatal, but now that the treatment strategies have been modified, including administration of intravenous immunoglobulin to neutralize SAgs and activated protein C to alter progression of sepsisinduced microvascular thrombosis, the case : fatality rate has fallen to approximately 25%. In 2009, Assimacopoulos et al. [43] described extreme pyrexia syndrome associated with staphylococcal SAg production,

Table 2. Superantigen-associated illnesses.

Human illness	SAg association
Staphylococcal menstrual TSS Staphylococcal non-menstrual TSS 1. Soft tissue infection 2. Purpura fulminans	TSST-1 TSST-1, SEB, SEC, occasionally others
 A. Recalcitrant erythematous desquamating syndrome of AIDS 	
 5. Anaphylactic 6. Kawasaki-like 7. Scleroderma-like 8. Rheumatoid arthritis like Staphylococcal extreme pyrexia 	TSST-1, any SE or SE-like SAg
syndrome Staphylococcal food poisoning Atopic dermatitis Psaeudomembranous enterocolitis Severe nasal polyposis Perineal erythema Sudden infant death syndrome Streptococcal TSS	Any SE TSST-1, any SE or SE-like SAg TSST-1, any SE or SE-like SAg TSST-1, any SE or SE-like SAg TSST-1, any SE or SE-like SAg Any SAg Any SAg
PANDAS Acute rheumatic fever Guttate psoriasis	SPE C, L and M SPE C, L and M Any SPE, SMEZ or SSA

notably a truncated version of TSST-1, primarily by

CA-MRSA USA300 strains. This illness remains 100%

fatal and is characterized by acute onset of fever in excess of 108 °F (42 °C). Kotler *et al.* [44] recently re-

identified the staphylococcal SAg association with

pseudomembranous enterocolitis, an illness that once

was associated with staphylococcal SAgs but then later

nearly always associated with Clostridium difficile

infection, and now re-identified in part associated with

staphylococcal SAgs. One final recent illness associated

with staphylococcal SAgs is atopic dermatitis [3].

Nearly all persons with this very common intensely

itchy skin condition have cutaneous infections due to

SAg-producing S. aureus, particularly TSST-1 and SEs

A-C. It has been shown in both humans and animal

Superantigen outside-in signaling

models that SAgs induce skin reactions (rashes) that can be clinically diagnosed as atopic dermatitis [45,46]; one of the authors (P.M.S.) was diagnosed with atopic dermatitis after accidentally splashing SEA on his face. <u>Streptococcal scarlet fever</u> has been known for more than a century, and in the early 1900s this illness included a particularly malignant form that today is recognized as <u>streptococcal TSS</u> [47]. From about 1950 until the 1980s, and for an unknown reason, streptococcal scarlet fever became a mild illness referred to as scarlatina. Scarlet fever is caused by group A strepto-

cocci that produce SPEs (scarlet fever toxins). In 1987,

Cone et al. [48] described two cases of streptococcal TSS associated with SPEs. Later, in a larger and more comprehensive study that remains the definitive study today, Stevens and colleagues [49] described streptococcal TSS in the Rocky Mountain Western USA and its high association with SPE A. This illness has the same general features as staphylococcal TSS but there are some important differences. Streptococcal TSS nearly always occurs in association with bloodstream invasion by the causative streptococci and often occurs in the presence of necrotizing fasciitis and myositis, whereas <u>S. aureus TSS</u> usually occurs with the organisms remaining localized on mucosal or other body surfaces [4]. Streptococcal TSS is not tampon associated since the streptococci are fermentative, and thus their growth and SAg production are independent of oxygen. Finally, streptococcal TSS patients often, but not always, lack the rash that defines staphylococcal TSS patients. The SAg rash appears to result from SAg-amplified hypersensitivity, whether type I or type IV, and streptococcal TSS patients would be expected to lack multiple exposures to the causative SAg-producing organisms [46,50]. In contrast, women having vaginal colonization with TSST-1 positive strains may have monthly outgrowths of organisms during their menstrual periods, giving them significant SAg exposure and risk of development of hypersensitivity. For as yet unknown reasons, it is easier to develop hypersensitivity to SAgs than it is to develop protective IgG antibody responses. S. aureus vaginally in women grow from 10⁴ mL⁻¹ of vaginal secretions at times other than menstruation to as high as 10¹⁰ mL⁻¹ of secretions during menstruation [51].

Group A streptococcal TSS is primarily associated with M protein types 1, 3 and a distant third 18 [49,51–53]. Nearly all M1 and M3 strains produce SPE A, and thus this SAg's high association with TSS [54,55]. M18 strains nearly always produce SPE C, and this explains its association with TSS [56]; some of these strains also produce SPE A. Initially, nearly all group A streptococci associated with TSS produced one or both of these two SAgs. However, since the initial association of streptococcal TSS with SPE A and C, numerous other SAgs have been identified and associated with TSS. In a recent key paper, Kotb and her colleagues [57] showed that severe invasive diseases caused by group A streptococci, including streptococcal TSS, are linked to the responsiveness of the host to SAg interaction with MHC II on antigen-presenting cells (APCs). Exaggerated APC responses, with massive cytokine production, lead to TSS with or without necrotizing fasciitis and myositis, whereas attenuated APC responses to SAgs lead to pharyngitis. The exaggerated response of TSS patients to SAgs, whether of staphylococcal or streptococcal origin, may explain the unique and characteristic lack of development of protective antibody responses upon recovery in TSS patients [58]. There are TSS patients who have had more than six recurrences of TSS without development of protective antibodies.

Streptococcal TSS patients are generally managed clinically similarly to management of staphylococcal TSS patients with a few exceptions [40]. Standard treatment will include the use of antibiotics, often including clindamycin since this antibiotic may have the added benefit of inhibiting SAg production at subbacterial-growth-inhibiting concentrations [59]. Blood pressure is stabilized by use of fluids, electrolytes and vasopressors. Intravenous immunoglobulin has been shown to increase the survival rates in streptococcal TSS patients significantly, and activated protein C may be useful in management of sepsis-induced microvascular thrombosis seen in some streptococcal TSS patients [60,61]. Since streptococcal TSS often includes necrotizing fasciitis/myositis, surgical debridement is necessary as part of the management; necrotizing fasciitis and myositis may occur in staphylococcal TSS, but it is not common in that illness. Necrotizing fasciitis and myositis, combined with bloodstream invasion, make streptococcal TSS much more severe than staphylococcal TSS. Streptococcal TSS may be associated with up to a 50% or higher case : fatality rate, while streptococcal TSS with necrotizing myositis is closer to 90% fatal. The incidence of streptococcal TSS appears to be approximately the same as for staphylococcal TSS – between <u>1 and 3 per 100 000 per year</u>.

Streptococcal SAgs have also been associated with other illnesses. For example, researchers have shown that streptococcal SAgs are linked to development of guttate psoriasis [62], a form of psoriasis that recurrently follows group A streptococcal infections in susceptible individuals. Studies have shown the association of M18 group A streptococci, and consequently its associated SAgs, with rheumatic fever [63,64]. Numerous investigators are presently examining the association of streptococcal SAgs with pediatric autoimmune neuropsychiatric disorders associated with streptococcal infections (PANDAS) [65].

Pathogenesis of superantigens: outside-in signaling from mucosa leads to TSS

The ability of SAgs to stimulate massive cytokine production by T cells and APCs, particularly macrophages, with resultant TSS has been thoroughly documented and reviewed, and will be briefly discussed later. However, their abilities to induce cytokine production from epithelial cells lining the mucosal surfaces that *S. aureus* and group A streptococci colonize play a critical role in their abilities to induce TSS from mucosal surfaces. We refer to this epithelial cell interaction as the 'outside-in' signaling mechanism, where SAgs (a) initially stimulate pro-inflammatory cytokine and chemokine production from epithelial cells to promote SAg penetration through the mucosa, and (b) recruit adaptive immune cells (T cells and macrophages) to the submucosa which are critical for cytokine production and development of TSS.

Although S. aureus can be found vaginally in 20-30% of all women, only approximately 5% of those strains are capable of producing TSST-1, the main cause of menstrual TSS [66,67]. It is known that S. aureus most often secrete TSST-1 in tampons in association with the vaginal mucosa to cause TSS without the organisms themselves penetrating the mucosa [27]; therefore the ability of TSST-1 to interact directly with the vaginal epithelium is critical to its ability to initiate disease from the mucosal surface. Human vaginal tissue is dominated by non-keratinized stratified squamous epithelium with intercellular lipids including ceramides, glucosyl ceramides and cholesterol located in the surface layers [68-72]. Although dendritic APCs are present in the vaginal epithelium, these cells are vastly outnumbered by epithelial cells. As uniformly tight junctions are not present between vaginal epithelial cells, the intercellular lipids constitute a dominant permeability barrier. Previous work from our laboratory has shown that other exotoxins secreted by S. aureus, most importantly the cytolysin α -toxin, are both cytotoxic to epithelial cells and induce inflammation from vaginal epithelial cells at sub-cytocidal concentrations, which together disrupt the stratified epithelial barrier and allow TSST-1 to penetrate more freely through the epithelium [73,74]. Once TSST-1 reaches the lower, more metabolically active layers of the epithelium, the SAg binds to an unidentified receptor on epithelial cells, possibly CD40. The binding of TSST-1 to these epithelial cells leads to production of at least three major pro-inflammatory chemokines and cytokines, interleukin (IL) 8, macrophage inflammatory protein 3a (MIP-3a) and tumor necrosis factor (TNF) α , from the cells [73–75]. These chemokines and cytokines function to recruit adaptive immune cells to the submucosa where TSST-1 can then initiate the final cascade of events that leads to TSS.

A similar model has been proposed for streptococcal TSS since group A streptococci also colonize stratified squamous epithelia, including the throat and, to a

lesser extent, the vagina [76]. Historically, group A streptococci are known to cause both puerperal fever. which is a TSS-like illness that results from vaginal colonization by the bacterium, and scarlet fever, often originating in concurrence with pharyngitis. In the past 30 years vulvovaginitis caused by group A streptococci in prepubescent (and some adult) females has become more common [77–84]. In the group A streptococcal model of TSS initiation, the cytolysin streptolysin O (SLO) may damage the epithelium directly without eliciting strong inflammatory responses from the cells [73]. We have shown that the ability of SLO to damage mucosal surfaces directly in the absence of significant inflammation may allow both the causative bacteria and the SPEs to gain access to the submucosa. and subsequently the bloodstream. This is important for two reasons: (a) as is the case with TSST-1, SPEs induce strong pro-inflammatory chemokine and cytokine responses (IL-6, IL-8 and MIP-3 α) from stratified squamous epithelial cells which may also function to recruit adaptive immune cells to the submucosa, and (b) in most cases of streptococcal TSS, group A streptococci are found in the bloodstream, contrary to S. aureus during menstrual TSS where the causative staphylococci typically remain on the mucosal surfaces. Supporting this hypothesis, streptococcal SLO augments bacterial penetration of intact ex vivo porcine vaginal tissue, which is an excellent model of human vaginal tissue, whereas the staphylococcal α -toxin does not [73]. These two models of SAg penetration of mucosal surfaces are illustrated in Fig. 2. It is important to note that streptococcal TSS also occurs in association with breaks in the skin.

Superantigen and epithelial cell interactions

The ability of SAgs to interact with and elicit immune responses from epithelial cells has been documented for various cell lines. Kushnaryov et al. [85,86] first demonstrated that TSST-1 binded specifically to human epithelial cells, with 10^4 receptor sites per cell predicted for the toxin. Moreover, the toxin was visualized in clathrin-coated pits, indicating that it may be internalized via receptor-mediated endocytosis. Our laboratory has demonstrated that immortalized human vaginal epithelial cells have approximately the same numbers of receptor sites for TSST-1 [74]. We have also shown that TSST-1, SEB and SPE A induced proinflammatory chemokine and cytokine responses from two different lines of human vaginal epithelial cells (obtained from the University of Iowa and ATCC CRL-2616) [73-75]. More specifically, TSST-1, SEB



Fig. 2. Outside-in signaling from SAgs at mucosal surfaces leads to TSS: proposed models. In the case of *Staphylococcus aureus*, bacteria remain localized on the vaginal epithelium but secrete cytolysins, such as α -toxin, which are pro-inflammatory and act to disrupt the mucosal barrier. SAgs such as TSST-1 can then more easily penetrate the mucosa to reach the lower levels of the epithelium which are more reactive to the SAg. It is here that we believe SAgs act to stimulate the production of IL-8 and MIP-3 α , which in turn function to recruit adaptive immune cells to the submucosa. The SAgs can then interact with T cells and APCs to stimulate a cytokine cascade that leads to TSS. A similar situation is proposed for group A streptococci; however, its main cytolysin, SLO, causes more cell damage in the absence of a strong pro-inflammatory response. This allows SAgs, such as SPE A, and bacteria to penetrate the mucosal barrier, as is more often the case in streptococcal TSS. SPE A also stimulates IL-8 and MIP-3 α production from epithelial cells to recruit adaptive immune cells to the submucosa to initiate the cytokine cascade.

and SPE A all induced MIP-3 α and IL-8, whereas only SPE A induced IL-6; TSST-1 also induced low levels of TNF- α production [73,74]. In addition, TSST-1 has been shown to elicit TNF- α and IL-8 responses from primary human bronchial epithelial cells [87]. SEB induced increased IL-5, IL-6, IL-8 and granulocyte macrophage colony-stimulating factor responses from human nasal epithelial cells [88–90]. SEB also elicited increased IL-1 β and IL-8 from corneal epithelial cells [91].

Mucosal superantigen exposure leads to systemic inflammation and disease

Many studies have shown that mucosal exposure to SAgs leads to systemic inflammation and immune activation. Herz *et al.* [92] initially demonstrated that low doses of SEB administered intranasally to BALB/C or

C57BL/6 mice induced IL-4 production in bronchial alveolar lavage samples, as well as immune cell recruitment to the airway mucosa. Using a more relevant 'humanized' human leucocyte antigen (HLA) transgenic mouse model, Rajagopalan et al. [93,94] showed that intranasal SEB exposure induced the influx of neutrophils into the lungs and systemic immune activation, indicated by increased levels of IL-12, IL-6, TNF- α , interferon- γ (IFN- γ) and monocyte chemotactic protein (MCP-1) in the serum. Similar results showed increased serum IL-2 and IL-6 levels in rhesus macaques exposed to aerosolized SEB [95]. Intrabronchial exposure of rabbits to SEB or SEC led to hemorrhagic lesions in the lungs and lethality, indicating a strong systemic response to the SAgs [96]. Even conjunctival exposure of mice to SEB led to T cell activation and expansion in the cervical lymph nodes and spleen [97]. The streptococcal SAg SPE A also induced

airway inflammation and immune cell proliferation in the spleen when administered intranasally to HLA transgenic mice [93].

Previous work performed in our laboratory examined how SAgs cause disease from various mucosal surfaces using a rabbit endotoxin-enhancement model of TSS [16]. This model tests the ability of SAgs to induce cytokine production that synergizes with the cytokine release caused by lipopolysaccharide (LPS), thereby rapidly increasing the progression to shock and death [98,99]. TSST-1, SEC1 and SPE A were all capable of lethality when administered vaginally (Brosnahan and Schlievert, manuscript in preparation for SPE A; [16,75]). Vaginal exposure of HLA transgenic mice to SEB in the absence of LPS enhancement led to systemic immune activation, as evidenced by increased IFN- γ , IL-6 and MCP-1 serum levels and immune cell infiltration into the lungs and liver [100].

BALB/C mice fed bolus doses of 50 µg SEB demonstrated increased TCR variable region β -chain 8⁺ (TCRV β 8⁺) CD4⁺ and CD8⁺ T cell numbers in Peyer's patches, which are part of the gut-associated lymphoid tissue, after 12 h [101]. Increased IL-2, IFN- γ and TNF- α mRNA in Peyer's patches and mesenteric lymph nodes were also seen in as little as 1.5 h after administration, indicating localized lymphoid tissue activation after intestinal mucosal exposure to the SE [101].

A conserved superantigen dodecapeptide is involved in epithelial signaling

A dodecapeptide (12 amino acids) region that is relatively conserved among staphylococcal and streptococcal SAgs is important for interactions with epithelial cells. This region was initially identified by Wang et al. [102] in 1993 as an important region of streptococcal M5 protein, but was shown to be present in most SAgs. Although the region is separate from known MHC II and TCR binding sites on the SAgs, peptides containing all or part of this region in SEB and SEC have been shown also to be potentially important for T cell proliferation and cytokine induction from peripheral blood mononuclear cells [103,104]. Additionally, a peptide antagonist (YNKKKATVQELD) generated against the dodecapeptide region of SEB was shown to have broad-spectrum anti-inflammatory activity against multiple superantigens, including SEB, SEA, SPE A and TSST-1 [105]. The same peptide antagonist was capable of increasing survival of D-galactosamine-sensitized mice when administered prior to challenge with SEB or SPE A. However, it was more protective when administered after challenge with TSST-1. Subsequently, the mice given the peptide

antagonist prior to challenge with SEB, SPE A or TSST-1 developed immunity to the challenging toxin that allowed them to resist additional challenges (even to other superantigens) [105–108]. Using HLA transgenic 'humanized' mice (HLA-DQ8 or HLA-DR3), however, Rajagopalan *et al.* [109] demonstrated that these dodecapeptide antagonists did not prevent T cell proliferation or protect the mice from developing TSS, nor were the antagonists able to inhibit SEB binding to human HLA class II molecules.

Although the dodecapeptide region was disproven to be relevant for protection in models of TSS resulting from systemic SAg administration, Shupp et al. [110] showed that this same region was involved in transcytosis of SEs across intestinal epithelial monolavers. Both TSST-1 and SEB had been previously shown to transcytose human intestinal epithelial monolayers of Caco-2 cells, whereas SEA appeared only to diffuse passively across the barrier [111]. This result was supported in an in vivo model which demonstrated that SEB could be found at higher concentrations in the bloodstream than SEA after oral consumption by mice. However, Shupp et al. [110] demonstrated different results using the highly polarized T-84 human intestinal cell line, which indicated that both SEB and SEA transcytosed tight monolayers at similar levels, with TSST-1 crossing at a slightly higher level. Increased penetration of TSST-1 was also noted by our group, compared with SEC1 and SPE A, when given orally to rabbits [16]. Using a peptide generated against the conserved dodecapeptide region of SEB (152-KKKVTAQELD-161), Shupp et al. [110] demonstrated that transcytosis of SEA, SEB, SEE and TSST-1 through T-84 cells could be inhibited. Additionally, antisera generated against the peptide were also inhibitory.

After Shupp et al.'s demonstration that the dodecapeptide region was involved in intestinal epithelial interactions, our laboratory demonstrated that the Arad et al. [105] dodecapeptide antagonist generated against SEB (YNKKKATVQELD) did in fact inhibit TSST-1 induced IL-8 and MIP-3a signaling from human vaginal epithelial cells, indicating a role for the dodecapeptide in other epithelial interactions [74]. In order to determine which residues within the dodecapeptide region were important for SAg-induced epithelial cytokine responses, single-site alanine mutations were generated along the dodecapeptide of both TSST-1 and SPE A, and the ability of each mutant to elicit IL-8 responses from human vaginal epithelial cells was determined. Whereas residues at the carboxyterminal end of the TSST-1 dodecapeptide region (S127, T128 and D130) were important for stimulation of IL-8 production by human vaginal epithelial cells, residues near the amino-terminal end of SPE A (T135, N136, K137 and M139) proved to be important (Brosnahan and Schlievert, manuscript in preparation; [75]). In each region, two mutants tested (D130A for TSST-1 and K137A for SPE A) were lethal when administered intravenously to rabbits in an endotoxinenhancement model of TSS, but were unable to induce TSS after vaginal exposure (Brosnahan and Schlievert, manuscript in preparation; [75]). Therefore, interfering with residues within the dodecapeptide region that are required to elicit pro-inflammatory responses from vaginal epithelial cells blocks the ability of SAgs to induce TSS from the vaginal mucosa.

One carboxy-terminal residue, SPE A residue E144, was important for SPE-A induced epithelial inflammation, and although this mutant maintained superantigenicity in vitro with human peripheral blood mononuclear cells, the mutant was unable to induce TSS after intravenous administration to rabbits. Interestingly, this mutant displayed some lethality when administered vaginally to rabbits, killing one of three rabbits tested (Brosnahan and Schlievert, manuscript in preparation). Similarly, the K121A mutant of TSST-1, which also induced only low IL-8 levels from human vaginal epithelial cells and maintained superantigenicity in vitro, was unable to induce TSS after intravenous exposure, but killed three of three rabbits after vaginal exposure (Brosnahan and Schlievert, manuscript in preparation). Thus, although the mutants stimulated only low IL-8 levels from the human vaginal epithelial cells, this induction was enough to initiate the steps required to cause TSS from mucosal surfaces. These results indicate that signaling through epithelial cells in the vaginal mucosa by SAgs is crucial for initiating TSS from mucosal surfaces.

The conserved dodecapeptide sequence was visualized using WEBLOGO 3.0 (http://weblogo.threeplusone.com/), which demonstrates the probability of particular residues occurring at each site based on the dodecapeptide sequence of 10 different staphylococcal and streptococcal SAgs (Fig. 3). Highly conserved residues can be seen as larger letters. Residues 3 and 4 (typically both lysines) and 11 and 12 (typically leucine and aspartic acid, respectively) are thought to be strictly conserved among all SAgs, helping to preserve the overall structure [112]. The dodecapeptide region of various SAgs has been highlighted in Fig. 1. All regions are located on β-strands that move into conserved central α -helices, with the exception of TSST-1 in which the dodecapeptide is located solely in the α -helix. The highly conserved QELD carboxy-terminal sequence of the dodecapeptide is located in the central α -helix, perhaps providing an explanation for the



Fig. 3. Dodecapeptide amino acid sequence conservation. Amino acid residues within the dodecapeptide region of 10 staphylococcal and streptococcal SAgs (TSST-1, SEA, SEB, SEC3, SE-like K, SE-like Q, SPE A, SPE C, SMEZ and SSA) were used to show the conservation of specific residues among various SAgs. Commonly used residues appear larger than their less common counterparts. Image generated using the free WEBLOGO 3.0 program located at http://weblogo.threeplusone.com/. Hydrophobicity color scheme: blue is hydrophobic, green is hydrophilic, black is neutral.

conservation of this sequence among most SAgs (note that SPE C, SMEZ-2, SEK and SEQ have the sequence QEID instead of QELD).

Superantigen interactions with MHC II and TCR lead to TSS

The interaction of SAgs with T cells and APCs, such as macrophages, and the subsequent T cell activation and massive cytokine production from both cell types cause the symptoms that characterize TSS. SAgs stimulate T cells in the absence of proteolytic processing and presentation by MHC II proteins. Instead, the entire SAg proteins act as bridges between MHC II molecules expressed by APCs and the TCR of T cells. Normally, an antigenic peptide would be expected to stimulate 1 in 10 000 T cells based on specific reactivity to the peptide being presented; however, SAgs have the ability to stimulate up to 50% of the body's T cell population [113]. This relatively non-specific stimulation also leads to production of cytokines involved in TSS, including TNF- α and TNF- β that cause capillary leak and consequent hypotension. IL-1B that causes fever, and IL-2 and IFN- γ that cause rash [50,114–118].

The development of TSS, in particular streptococcal TSS, has been linked to the degree of cytokine responsiveness to the associated SAgs. A study by Norrby-Teglund *et al.* [119] demonstrated that higher levels of cytokines are produced in those individuals with invasive streptococcal infections compared with those with non-invasive infections. Subsequently, researchers showed a link between allelic variation of the HLA class II haplotypes (the human genes that encode MHC II molecules) and responsiveness to streptococcal SAgs, which influenced whether or not a patient would

develop streptococcal TSS [57,120,121]. Patients with the DRB1*15/DQB1*06 (DR15/DQ6) HLA haplotype had reduced T cell proliferation in response to streptococcal SAgs compared with those with other haplotypes. The DR15/DQ6 haplotype was also associated with protection from developing more severe streptococcal infections compared with other haplotypes [57]. These findings were confirmed using HLA transgenic mice. Mice expressing the protective HLA-DQB1*06 (DQ6) showed lower cytokine responses to infection with M1T1 group A streptococci, which allowed those mice to survive longer after intravenous infection [122]. Recently, the DR15/DQ6 haplotype was shown to skew the ratio of anti-inflammatory to pro-inflammatory cytokines that are produced in response to SAgs [123]. The presence of the DR15/DQ6 alleles resulted in increased levels of the anti-inflammatory cytokine IL-10, which is thought to provide the protective basis of this haplotype in SAg-mediated illnesses.

Because SAgs are not processed and presented in the context of MHC II as antigenic peptides, their ability to act as a bridge between MHC II and the VB-TCR can be classified into two different arrangements: (a) the SAg can act as a wedge on the side of the typical MHC II-Vβ-TCR interaction, interacting with the α -chain of MHC class II and the V β -TCR chain, or (b) the SAg interacts with the V β -TCR but is then situated between the TCR and the SAg-bound β -chain of MHC II, appearing to be three beads on a string [124,125]. Examples for each of these interactions can be seen in Fig. 4. In some cases, contact occurs between the SAg and the processed antigenic peptide being presented by MHC II, which affects the binding arrangement for TSST-1 in particular as it prevents MHC II from contacting the TCR [126,127]. This arrangement can also enhance the presentation of SAg to TCR; for example, one peptide tested by Wen et al. [128] enhanced the presentation of TSST-1 up to

Fig. 4. Superantigen interactions with TCR and MHC II. Ternary structure models of SPE A and SPE C interacting with TCR and MHC II molecules. (A) Cartoon and (B) surface views of SPE A ternary complex, based on the following binary complexes: SPE A/TCR-β (PDB 1L0Y [143]), TCR-αβ (PDB 1J8H [163]) and SEB/MHC II (PDB 1SEB [164]). (C) Cartoon and (D) surface views of SPE C ternary complex, based on the following binary complexes: SPE C/TCR-β (PDB 1KTK [143]), TCR-αβ (PDB 1J8H [163]) and SPE C-MHC II (PDB 1HQR [130]). Notice how SPE A binds the molecules on the side of the complex, acting as a wedge between them, while SPE C binds in between the two molecules, appearing to be three beads on a string. SAgs are shown in yellow, with residues critical for MHC II or TCR binding shown in red and the epithelial binding region highlighted in green. The epithelial binding site remains distinct from the MHC II and TCR binding sites and is not thought to be involved in this interaction. TCR *a*-chains are shown in dark blue, TCR β-chains are shown in light blue. MHC II α-chains are shown in orange, and MHC II β-chains are shown in pink. A zinc ion is shown as a green ball in the SPE C ternary complex, while peptides in the MHC II groove are shown in black in all complexes. Images generated using PYMOL (DeLano Scientific LLC, South San Francisco, CA, USA).



5000-fold, whereas none of the peptides tested affected the presentation of SEB. This differential contribution of the peptide to the presentation of TSST-1 and SEB may be linked to the fact that, although their MHC II binding sites overlap, SEB does not interact with the peptide in the MHC II groove [126].

Interactions with the α -chain of MHC II are relatively low-affinity interactions, whereas interactions with the β -chain of MHC II are of higher affinity and require Zn²⁺ as a co-factor [129–132]. Interactions with the low-affinity site on the α-chain of MHC II involve the amino-terminal OB domain of the SAgs, whereas interactions with the higher-affinity site on the β-chain of MHC II involve the carboxy-terminal domains. Some SAgs interact through both low- and higher-affinity sites [124,133,134], whereas others interact only through the low-affinity [126] or higheraffinity [135–137] sites. The higher-affinity interaction of some SAgs with the β -chain of MHC II results in a 10- to 100-fold increase in activity [138,139], but it is important to note that those SAgs are usually made in much smaller quantities by S. aureus and group A streptococci than those that only interact with the lowaffinity α -chain site.

SAgs were originally characterized by their ability to stimulate specific groups of T cells based on their V β -TCR chains [140]. The V β -TCR subsets that are stimulated are based on the amino acid sequence within the TCR binding site of each SAg. Evidence has surfaced recently, however, that SEH binds the $V\alpha$ chain of the TCR instead, and does not seem to bind the VB-TCR chain at all [20-22]. An important structural arrangement to note is that most SAgs bind the Vβ domains of TCRs through the top front groove of the SAg [141-143], but TSST-1 binds the Vβ-TCR using the top back portion of the molecule [144]. Thus, there may be considerably more variability in TCR binding than originally thought. TSST-1 also is the most specific SAg in that the toxin only binds and stimulates T cells bearing the V β 2-TCR subtype [113]. For a more in- depth review of superantigen-TCR binding, see Li et al. [125].

T cell stimulation due to SAgs may involve the Lckdependent pathway that is used during normal T cellantigenic peptide–MHC II interactions; however, new evidence has shown that SAgs can also stimulate an Lck-independent signaling pathway in T cells [145]. In this case, the SAg SEE activated the pertussis-toxininsensitive Gα11 protein, which led to Ca²⁺ influx via the actions of PLC-β, and subsequent activation of PKC, ERK-1 and ERK-2. The end result was that NF-AT and NF-κB were translocated to the nucleus, and IL-2 was made by the T cells. Interestingly, SAgs also induce a T regulatory phenotype from $CD4^+CD25^$ stimulated T cells [146]. These T_{regs} express the classic CD25 and FOXP3 markers and appear able to inhibit proliferation of $CD4^+CD25^-$ T cells, indicating that they function as normal T regulatory cells. Thus, the SAgs may act to skew the immune response by inducing massive T cell proliferation and cytokine release, followed by a T regulatory response that can dampen any further immune response against bacterial infection.

For the future

Examples are given of important directions of future SAg research. This list is by no means exhaustive.

Researchers continue to find new SAg-associated illnesses each year; our laboratory is currently in the process of describing two new SAg illnesses. Moreover, although we are quite familiar with the role of T cells and macrophages and their cytokines in TSS, there remain numerous symptoms of TSS that are of unknown origin. For example, there is significant muscle damage [26,27] and neurological changes that occur in TSS, but their origin is unknown [147]. Streptococcal SAgs in particular may be linked to Syndenham's chorea, occurring in cases of rheumatic fever, and to PANDAS. Additionally, characterization is merited for why some SAgs are produced in high concentrations whereas others are produced in minute amounts. It is also important to know the biological significance to human hosts of such differences.

The versatility of SAg interaction with human cells continues to expand with increasing study. At the present time, there are at least five known host cell receptor sites located on some SAgs. These include low- and higher-affinity MHC II sites, a V β -TCR site, a cystine loop structure required for emetogenic activity, and an epithelial cell binding domain. Our current studies suggest that CD40 molecules on many host cells represent additional targets of SAg interaction. The potential biological consequences of SAg binding CD40 are enormous.

We know much about the interaction of SAgs with MHC II and V β -TCRs, but we are only beginning to understand the significance of epithelial cell interactions and the role of outside-in signaling in human illnesses, not just for SAgs and TSS but for many other illnesses as well (e.g. HIV infection [148]). Collectively these early studies suggest that common mechanisms for microbes in general to cause infections across mucosal surfaces, counter-intuitively, depend on low-grade stimulation of the immune system to disrupt the mucosal barrier. We anticipate that future studies will lead to development of important, novel therapeutic

strategies to manage infections across these important barriers.

Lastly, coagulase-negative staphylococci and nongroup A streptococci have been shown recently to produce SAgs. The full extent of SAg production by these strains remains to be clarified. However, the importance of these findings cannot be over-stated. Potentially, the most effective way for pathogens to cause human disease, and that creating the most difficulty in demonstration of causation, is for the pathogen either to mimic normal flora or simply to be normal flora, but with addition of one or two important virulence factors. It is clear from studies of historical S. aureus strains, from as far back as the 1940s, that the SAg TSST-1 was present and certainly contributing to human illnesses [149]. However, the SAg was not identified until 1980 and was not shown definitively to cause TSS until 1984. It is likely to be even more difficult to perform similar studies with new SAgs, if present with coagulase-negative staphylococci or α -hemolytic oral streptococci which colonize all humans.

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