Vaccine 30 (2012) 5099–5109

Contents lists available at SciVerse ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Immunity to *Staphylococcus aureus* secreted proteins protects rabbits from serious illnesses

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ARTICLE INFO

Article history:
Received 22 January 2012
Received in revised form 23 May 2012
Accepted 25 May 2012
Available online 9 June 2012

Keywords:
Staphylococcus aureus
Superantigen
Cytolysin
Vaccine
Rabbit Model

ABSTRACT

Staphylococcus aureus causes significant illnesses throughout the world, including toxic shock syndrome (TSS), pneumonia, and infective endocarditis. Major contributors to *S. aureus* illnesses are secreted virulence factors it produces, including superantigens and cytolysins. This study investigates the use of superantigens and cytolysins as staphylococcal vaccine candidates. Importantly, 20% of humans and 50% of rabbits in our TSS model cannot generate antibody responses to native superantigens. We generated three TSST-1 mutants; G31S/S32P, H135A, and Q136A. All rabbits administered these TSST-1 toxoids generated strong antibody responses (titers > 10,000) that neutralized native TSST-1 in TSS models, both in vitro and in vivo. These TSST-1 mutants lacked detectable residual toxicity. Additionally, the TSST-1 mutants exhibited intrinsic adjuvant activity, increasing antibody responses to a second staphylococcal antigen (β-toxin). This effect may be due to TSST-1 mutants binding to the immune co-stimulatory molecule CD40. The superantigens TSST-1 and SEC and the cytolysin α-toxin are known to contribute to staphylococcal pneumonia. Immunization of rabbits against these secreted toxins provided complete protection from highly lethal challenge with a USA200 *S. aureus* strain producing all three exotoxins; USA200 strains are common causes of staphylococcal infections. The same three exotoxins plus the cytolysins β-toxin and γ-toxin contribute to infective endocarditis and sepsis caused by USA200 strains. Immunization against these five exotoxins protected rabbits from infective endocarditis and lethal sepsis. These data suggest that immunization against toxoid proteins of *S. aureus* exotoxins protects from serious illnesses, and concurrently superantigen toxoid mutants provide endogenous adjuvant activity.

1. Introduction

Staphylococcus aureus is a major pathogen worldwide, responsible for significant illnesses, many of which are life threatening such as toxic shock syndrome (TSS), infective endocarditis, sepsis, and pneumonia [1,2]. *S. aureus* has the ability to cause a wide variety of infections by production of numerous virulence factors, both cell-surface and secreted exoproteins [1,2]. Treatment of *S. aureus* infections can be challenging and expensive, especially with the high occurrence of antibiotic resistant infections, such as caused by methicillin-resistant *S. aureus* (MRSA) [3].

Infective endocarditis is a life threatening infection of the heart endothelium caused by many organisms [4,5]. In the past decade, *S. aureus* has emerged as a primary cause of infective endocarditis throughout the world, largely in elderly patients and intravenous drug users [4–8]. The illness is characterized by formation of large "cauliflower-like" vegetations on the endothelium of the heart. These vegetations are composed of host factors (tissue factor, fibronectin, and fibrinogen) and host cells, as well as microbial colonies. Infective endocarditis is difficult to treat, and there are many risks associated with the illness, including cardiac failure, embolisms, renal dysfunction, and mycotic aneurysms [4,5]. Treatment of *S. aureus* infective endocarditis typically requires

Abbreviations: CFUs, colony-forming units; LPS, lipopolysaccharide; MHC, major histocompatibility complex; MRSA, methicillin-resistant *S. aureus*; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SE, staphylococcal enterotoxin; TSS, toxic shock syndrome; TSST-1, TSS toxin-1; Vβ-TCR, variable part of the β-chain of the T cell receptor; HVECs, human vaginal epithelial cells; KSFM, keratinocyte serum-free medium.

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http://dx.doi.org/10.1016/j.vaccine.2012.05.067

extensive antibiotic regimens, often lasting ≥6 weeks, and many times surgery is required [4,5,7,8].

Although cell-surface virulence factors are critical for *S. aureus* attachment and vegetation initiation, recent research has also implicated secreted virulence factors as major contributors to infective endocarditis progression with *S. aureus*. Pragman et al. showed that the superantigen TSS toxin-1 (TSST-1) is highly important for infective endocarditis vegetation formation caused by strains that produce the superantigen [9]. In a rabbit model, the researchers showed that strains producing native TSST-1 had significantly larger vegetation sizes and increases of nearly 7 logs colony-forming units (CFUs)/vegetations compared to isogenic strains lacking TSST-1. We have further noted that strains lacking superantigens do not induce infective endocarditis in rabbits [9]. A research group recently published a study examining the genotype of strains isolated from infective endocarditis patients with persistent bacteremia and observed that the majority of them are pulsed-field gel electrophoresis type USA200 and carried the *tstH* gene that encodes TSST-1 [10]; there is a one:one correlation between the presence of *tstH* and TSST-1 protein production. Additionally, it has been published that 90% of infective endocarditis cases are associated with USA200 strains and production of TSST-1 [11]. These studies collectively suggest that TSST-1 is highly important for *S. aureus* in its ability to cause infective endocarditis. Recent studies from Mattis et al. showed that another superantigen, staphylococcal enterotoxin (SE) C, is highly important for infective endocarditis caused by strains that produce that superantigen (Mattis DM, Spaulding AR, Chuang-Smith ON, Sundberg EJ, Schlievert PM, Kranz DM. Enterotoxin C contributes to USA400 methicillin-resistant *S. aureus* infective endocarditis in rabbits. Infect Immun, submitted for publication). When these investigators treated rabbits with a specific SEC inhibitor after challenge with a strain known to cause infective endocarditis at a high level in the rabbit model, the microbes were significantly reduced in ability to cause disease. Studies have also shown that secreted cytolysins contribute to infective endocarditis. Huseby et al. recently published that the cytolysin β-toxin facilitates infective endocarditis progression [12]. Cheung et al. showed that a *S. aureus* mutant that no longer produced α-toxin, β-toxin, γ-toxin, and δ-toxin was drastically reduced in its ability to cause infective endocarditis [13], although because these studies used a regulatory mutant for their studies, numerous other factors may also have contributed to reduced ability to cause illness.

In the rabbit model of infective endocarditis, we also gain important information on the role of exoproteins in lethal sepsis, since *S. aureus* is administered intravenously in high concentrations. Our prior studies strongly suggest that superantigens are important in lethal sepsis [14].

We and others have shown that superantigens and cytolysins are critical determinants of staphylococcal pneumonia [15–18]. Rabbits actively immunized against TSST-1 and SEC, and animals passively protected from SEB are protected from highly lethal intrapulmonary *S. aureus* challenge [17]. In addition, mice immunized against α-toxin are protected from lethal pneumonia [16].

These data led us to consider the possibility of a vaccine against serious *S. aureus* infections using the major secreted virulence factors (cytolysins and superantigens) as immunizing agents. Here we report our studies related to TSST-1, SEC, α-toxin, β-toxin, and γ-toxin, alone and in combination for protection against staphylococcal pneumonia, infective endocarditis, and sepsis. Our studies show that vaccines containing these important secreted virulence factors lead to immunity that protects against illness and increases survival. Additionally, TSST-1 mutant toxoids have endogenous adjuvant activities, dependent on interaction with the immune co-stimulatory molecule CD40 that amplifies immune responses to other antigens.

2. Materials and methods

2.1. Bacterial strains and growth

S. aureus strain RN4220 containing plasmids encoding TSST-1, TSST-1 mutants, or SEC were used as sources of TSST-1, TSST-1 mutants, or SEC, respectively [19–21]. Strain RN4220 does not produce detectable endogenous superantigens. RN4220 was also used as the source of β-toxin [22]. *S. aureus* strain MNPE was the source of native α-toxin [23]. *Escherichia coli* clones were the sources of mutant α-toxin (H35L), as provided by Dr. Juliane Bubeck-Wardenburg, University of Chicago, and γ-toxin as expressed from a pET vector [16]. *S. aureus* strain MNPE was used in microbial challenge studies; this organism caused a fatal case of post-influenza TSS in Minnesota [24]. This organism is USA200; these organisms cause the majority of TSS cases [25]. MNPE has the following secreted virulence factor phenotype of importance for our studies: TSST-1^{high+}, SEC^{high+}, α-toxin^{high+}, β-toxin^{high+}, and γ-toxin^{+} [23]. For use in pneumonia and infective endocarditis/sepsis studies, the organism was grown overnight in 25 ml of Todd-Hewitt (Difco Laboratories, Detroit, MI) broth at 37 °C with shaking at 200 rpm under standard air conditions [26]. The organism was washed one time with phosphate-buffered saline (PBS; 0.005 M sodium phosphate, pH 7.2; 0.15 M NaCl) through centrifugation at 14,000 × *g*, 5 min, and then resuspended in Todd Hewitt medium at 2×10^9/0.2 ml volume for high-dose injection in pneumonia studies [17], and in PBS at 1×10^8/ml, with 2 ml being injected intravenously for infective endocarditis/sepsis studies [27].

2.2. Secreted virulence factor purification

All reagents used in preparation of superantigens were maintained pyrogen-free. For production of TSST-1, TSST-1 toxoids, SEC, native α-toxin, and native β-toxin, the organisms were grown overnight in dialyzed beef-heart media [28]. TSST-1, TSST-1 toxoids, SEC and β-toxin were precipitated from culture fluids with 4 volumes of absolute ethanol for two days (80% final concentration), resolubilized in distilled water, and then purified by thin-layer isoelectric focusing. Isoelectric focusing pH gradients were pH 3.5–10 for initial separation, followed by gradients of pH 6–8 for TSST-1, TSST-1 toxoids, and α-toxin and 7–9 for SEC and β-toxin [28]. Native α-toxin was produced comparably from *S. aureus* MNPE, except the toxin was precipitated from culture fluids with 80% final saturation of ammonium sulfate, followed by solubilization in distilled water and three days dialysis, and then followed by isoelectric focusing. The biologically inactive mutant of α-toxin (H35L) and an enriched preparation of γ-toxin were produced from *E. coli* clones in pET vectors and purified on nickel columns [16]. TSST-1, TSST-1 mutants, and SEC were homogeneous when tested by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and reversed-phase high-performance liquid chromatography [28]. Additionally, these proteins were negative for contaminating lipopolysaccharide (LPS), peptidoglycan, cytolysins, lipase, and proteases. Native α-toxin was further purified by reversed-phase high-performance liquid chromatography and was homogeneous [23]. The α-toxin mutant H35L and γ-toxin, as produced in *E. coli* contained minor *E. coli* contaminates that did not affect experimentation. Purified toxins were quantified using the BioRad protein assay [17].

2.3. Production of TSST-1 mutants

Three site-specific mutants of TSST-1 were prepared through use of the Quikchange method (Stratagene, La Jolla, CA). The initial plasmid was native *tstH*, on a shuttle plasmid pCE104, cloned into *E. coli* [20]. After performing mutagenesis, the resultant plasmids