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Evaluation of recombinant SEA-TSST fusion toxoid for protection against superantigen induced toxicity in mouse model

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ABSTRACT

Treatment of *Staphylococcus aureus* infections has become complicated owing to growing antibiotic resistance mechanisms and due to the multitude of virulence factors secreted by this organism. Failures with traditional monovalent vaccines or toxoids have brought a shift towards the use of multivalent formulas and neutralizing antibodies to combat and prevent range of staphylococcal infections. In this study, we evaluated the efficacy of a fusion protein (r-ET) comprising truncated regions of staphylococcal enterotoxin A (SEA) and toxic shock syndrome toxin (TSST-1) in generating neutralizing antibodies against superantigen induced toxicity in murine model. Serum antibodies showed specific reactivity to both SEA and TSST-1 native toxins. Hyperimmune serum from immunized animals protected cultured splenocytes from non-specific superantigen induced proliferation completely. Passive antibody administration prevented tissue damage from acute inflammation associated with superantigen challenge from *S. aureus* cell free culture supernatants. Approximately 80% and 50% of actively and passively immunized mice respectively were protected from lethal dose against *S. aureus* toxin challenge. This study revealed that r-ET protein is non-toxic and a strong immunogen which generated neutralizing antibodies and memory immune response against superantigen induced toxic effects in mice model.

1. Introduction

Staphylococcus aureus is an important pathogen responsible for various illnesses ranging from mild skin and soft tissue infections and food poisoning to serious staphylococcal pneumonia, endocarditis, sepsis and toxic shock syndrome (Lowy, 1998; Chambers and DeLeo, 2009; Spaulding et al., 2012). Treatment of *S. aureus* infections has become very complicated owing to the emergence of antibiotic resistant strains such as methicillin resistant *S. aureus* (MRSA) which are also resistant to multiple antibiotics (DeLeo and Chambers, 2009; Grundmann et al., 2006). Virulence of *S. aureus* is mediated by both surface associated and secretory proteins. Although vaccination is a common practice to control infectious diseases, yet there is no licensed vaccine available till date to *S. aureus* infections. This failure is largely due to the multiple arrays of virulence factors secreted by the organism, evasion of the host's immune system and leukolytic toxins which lyse the phagocytic cells (Roy Chowdhury et al., 2013; Otto, 2010). Staphylococcal enterotoxins (SEs) and toxic shock syndrome toxin (TSST) are the Pyrogenic Toxin Super Antigens (PTSAs) responsible for toxic shock syndrome in deep tissues and vaginal tissues respectively (Poli et al., 2002). SEs constitutes a family of more than 15 proteins (26–28 kDa) with a typical disulphide loop having significant homology at structural and sequence levels and contributes to emetic activity. SEA is also a potent stimulator of T-lymphocytes and is the leading cause of staphylococcal food poisoning. SEA is one of the most potent SEs with an exceedingly low half-maximum stimulating dose of 0.1 pg/ml and is atypical in having two binding sites, a high affinity β chain and a low affinity α chain of MHC-II molecules of antigen presenting cells (Narayan et al., 2009). TSST-1 (22 kDa) lacks disulphide loop and shows no homology with other staphylococcal antigens but shows similar biological activities (Müller-Alouf et al., 2001). It was earlier reported that immunization of rabbits with secreted toxins provided complete protection from lethal *S. aureus* challenge (Spaulding et al., 2012). However, they observed that at least 50% of rabbits were incompetent to raise antibodies when native TSST-1 toxin was administered. However, same animals produced anti-TSST antibodies when the superantigenicity was removed by mutations. This is due to the hyper responsiveness to superantigens leading to immune dysfunction by

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an unknown mechanism thus rendering them highly susceptible to toxic shock syndrome. This same phenomenon has also been observed with certain humans (Spaulding et al., 2012).

Several active and passive immunization strategies involving whole *S. aureus* cells of avirulent or mutant strains (Pellegrino et al., 2010), recombinant proteins (Uppalapati et al., 2014), mutated versions of toxin components (Spaulding et al., 2012; Gampfer et al., 2002a,b), native toxins (Spaulding et al., 2012) or intravenous immunoglobulin therapy (Darenberg et al., 2003, 2004) and neutralizing antibodies (Varshney et al., 2011; Sully et al., 2014) have been studied to combat against *S. aureus* infections. Vaccines and antibodies targeting surface components such as capsular polysaccharides have been met with little to moderate success due their limited role in *S. aureus* virulence and pathogenesis mechanisms (O'Riordan and Lee, 2004; Schaffer and Lee, 2009). Interfering with crucial virulence determinants is a promising approach in combating bacterial infections. Present strategies for *S. aureus* vaccine development are focused on antigens with proven role in pathogenesis (Otto, 2010). Many studies were established for the application of neutralizing antibodies against pathogens and toxins (Tilahun et al., 2010; Parma et al., 2011; Peddayelachagiri et al., 2014). Most studies involving passive immunotherapy approaches are aimed at neutralizing the *S. aureus* virulence determinants involving mainly toxins. Passive antibody therapy could be given to patients to confer immediate and specific protection against *S. aureus* infections for short periods such as during hospitalizations and surgery.

As multivalent vaccine approach is gaining more attention to prevent *S. aureus* infections, we constructed a fusion protein comprising truncated regions of SEA and TSST toxins. Polyclonal antibodies generated against the structural chimera were tested for their binding and neutralizing properties *in vitro*. Further, the fusion protein was evaluated for its toxoid potential against superantigen induced lethality in mouse model.

2. Materials and methods

2.1. Materials

Dehydrated media, antibiotics and supplements were procured from Himedia laboratories, India. Primers used in the study were synthesized at primer synthesizing facility at Sigma Aldrich, Bangalore, India. pET22b vector and hosts were procured from Novagen, USA. *S. aureus* ATCC-700699 strain was procured from Microbiologics, USA.

2.2. Growth conditions and crude toxin preparation

S. aureus ATCC-700699 strain was positive for both SEA and TSST-1 toxins and hence this strain was used throughout the study for cloning purpose and crude toxin preparation during animal challenge experiments. This strain also harbors gene for SEC3 variant. Though this strain showed amplification for alpha hemolysin gene somehow the toxin was not observed in the culture filtrates when tested by Western blot analysis with commercial anti-alpha hemolysin antibodies (Sigma). Crude toxin preparation was undertaken by inoculating *S. aureus* ATCC-700699 in BHI broth and allowed to grow for 18–20 h with vigorous shaking. Cell free culture filtrates were passed through disposable 0.22 μM filters and concentrated by methanol chloroform method according to the method of Wessel and Flügge (1984). Presence of both SEA and TSST-1 toxins in concentrated crude toxins was confirmed by WB before initiating the experiments.

2.3. Purification of r-SEA-TSST protein

Chimeric *sea-tsst* gene encompassing truncated immunodominant regions of SEA and TSST-1 toxins was constructed in our earlier work (Reddy et al., 2012) employing a novel gene fusion technique based on hetero-staggered PCR. This fusion gene was cloned into pRSET A vector and the expressed denatured protein when immunized elicited antibodies against both SEA and TSST-1 toxins. This gene construct was subcloned into pET22b vector for functional expression of r-SEA-TSST protein in native confirmation in periplasm employing the primers listed in Table 1. Recombinant pET22b-*sea-tsst* plasmid was transferred into BL21(DE3) host and analyzed for expression by SDS-PAGE and Western blot analysis with anti-histidine antibodies. Recombinant SEA-TSST protein was purified under native conditions from one litre induced culture from periplasmic extract using Ni-NTA agarose (Qiagen, Germany) according to manufacturer's instructions.

2.4. Multiple sequence alignment with related SA's

Portions of *sea* and *tsst* genes used in *sea-tsst* fusion product were checked for sequence homology with related enterotoxins of *S. aureus* using Clustal Omega tool. Percent identity was created using Clustal 2.1. Structure alignment of SEA and TSST portions used in r-ET with other superantigens was carried out with CN3D structure alignment tool.

2.5. Immunization and generation of hyperimmune sera

Female Balb/C mice were received from central animal housing facility at Defence Food Research Laboratory (DFRL), Mysore city, India. Mice were provided with protein rich food pellets and Aquaguard filtered water. All procedures involving animals were performed as per the standard operation procedures approved by Institutional Ethical Committee at DFRL, India. Blood was drawn two days prior to immunization and the pooled serum was used as control in all the experiments. Total of 32 six week old female Balb/C mice (four groups of eight mice each) were used in the present study. Two groups were immunized with 50 μg each of recombinant SEA-TSST fusion protein in emulsion with Freund's complete adjuvant by subcutaneous route. Three booster doses were given with same quantity of protein in emulsion with Freund's incomplete adjuvant at 15 days interval. Remaining groups of mice served as controls receiving only 500 μl of Freund's complete and three doses of incomplete adjuvants via intraperitonial route. Blood was drawn by retro orbital sinus bleeding, serum was collected and tested for antibody titers and antibody isotypes.

2.6. Antigenicity testing

Ability of r-SEA-TSST protein to induce antibodies against both SEA and TSST was observed by Western blot analysis. Culture supernatants of *S. aureus* strains positive for both SEA and TSST toxins were concentrated by methanol: chloroform precipitation and resolved on 12% SDS-PAGE analysis. Proteins were transferred on nitrocellulose paper by electroblotting and blocked with 5% non fat

Table 1
List of primers used for sub cloning the *sea-tsst* fusion gene in pET22b from pRSET A-*sea-tsst* plasmid (Reddy et al., 2012).

Primer	Sequence (5′–3′)	Product (bp)
SEA-F (*Nco* I)	CATG**CCATGG**[a]*AT*GGCTTTTTTACAGATCATTCG	774
TSST-R (*Xho* I)	CCG**CTCGAG**[a]ATGTGGATCCGTCATTCATT	

[a] Nucleotides in bold represents restriction enzyme recognition sequences.