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Inverse relationship between toxic shock syndrome toxin-1 antibodies and interferon-γ and interleukin-6 in peripheral blood mononuclear cells from patients with pediatric tonsillitis caused by *Staphylococcus aureus*

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ABSTRACT

Introduction: Pediatric tonsillitis is frequently caused by *Staphylococcus aureus*, which is the most common pathogen that causes serious pyogenic infections in humans and endangers human health. *S. aureus* produces numerous potent virulence factors that play a critical role in the pathogenesis of the infection caused by this bacterium, and one of the most important toxins produced by *S. aureus* is toxic shock syndrome toxin-1 (TSST-1). The aim of this study is to investigate the first time the levels of IFN-γ and interleukin IL-6 in TSST-1-stimulated PBMCs from pediatric tonsillitis patients and the correlation of these cytokine levels with TSST-1-specific IgG in serum.

Methods: TSST-1 gene of *S. aureus* was cloned and expressed in a prokaryotic expression system, and purified recombinant TSST-1 protein was used for measuring TSST-1-specific antibodies in the serum of patients with pediatric tonsillitis caused by *S. aureus*. Moreover, the levels of interferon (IFN)-γ and interleukin (IL)-6 in TSST-1-stimulated peripheral blood mononuclear cells (PBMCs) from pediatric tonsillitis patients were investigated.

Results: In patients with pediatric tonsillitis caused by *S. aureus*, significantly higher levels of serum TSST-1-specific IgG ($P < 0.05$) and IgG1 ($P < 0.05$) were detected than in healthy children. Moreover, PBMCs from the patients exhibited higher IFN-γ ($P < 0.05$) production in response to TSST-1 than did PBMCs from healthy children. In patients with pediatric tonsillitis caused by *S. aureus*, the positive rate of TSST-1-specific IgG was 70%, and the patients who tested negative for TSST-1-specific IgG exhibited significantly higher levels of IFN-γ ($P < 0.05$) and IL-6 ($P < 0.05$) than did the IgG-positive patients, in accord, the levels of TSST-1-specific IgG correlated inversely with the levels of IFN-γ and IL-6 in patients PBMCs stimulated with TSST-1.

Conclusions: TSST-1 induces humoral and cellular immunity in pediatric tonsillitis caused by *S. aureus*, which suggests that TSST-1 may play an important role in the pathogenesis of pediatric tonsillitis.

1. Introduction

Staphylococcus aureus is the most common pathogen that causes serious pyogenic infections in humans and endangers human health, and the emergence and spread of methicillin-resistant *S. aureus*, in particular, is considered to be a worldwide public health problem [1,2]. *S. aureus* produces a variety of potent

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virulence factors that play a critical role in the pathogenesis of the infection caused by this bacterium [3]. One of the most important toxins produced by *S. aureus* phage group I is toxic shock syndrome toxin-1 (TSST-1), which belongs to the pyrogenic superantigen family that retains its biological activities after treatment with heat and digestive enzymes [4,5]. TSST-1 stimulates T lymphocyte to proliferate and release large amounts of inflammatory factors that elicit strong immune responses, which then results in uncontrolled inflammation and multi-organ damage [6].

Tonsillitis is a common infection of the palatine tonsils that occurs mostly in children, and recurrent tonsillitis, in particular, is a clinical problem for which patients might ultimately undergo tonsillectomy [7]. Among bacterial etiological agents, *S. aureus* has been considered as one of the most important pathogens that cause pediatric tonsillitis [8]. Although major advances have been made in the understanding of the pathogenesis of *S. aureus* infection, few studies have been reported on TSST-1 pathogenesis in *S. aureus*-induced tonsillitis. In this study, we investigated for the first time the levels of IFN-γ and interleukin IL-6 in TSST-1-stimulated PBMCs from pediatric tonsillitis patients and the correlation of these cytokine levels with TSST-1-specific IgG in serum.

2. Materials and methods

2.1. Serum and PBMC collection

A total of 40 serum specimens and 40 PBMC specimens were isolated from patients with pediatric tonsillitis caused by *S. aureus*, which was identified by means of tonsillar tissue culture [9]; 40 serum and 40 PBMC specimens were also isolated from healthy children. The children's ages ranged from 11 days to 12 years old. All samples were collected at Guangzhou Women and Children's Medical Center between June 2013 and August 2014. The collected serum was stored at −80 °C, and the PBMCs were stored in liquid nitrogen. Informed consent was obtained from the guardians of all study participants. The protocol was approved by the Committee on the Ethics of research of Guangzhou Women and Children's Medical Center.

2.2. Cloning, expression, and purification of TSST-1

S. aureus was cultured at 37 °C in Luria Broth (LB) for 12 h and then collected through centrifugation at 12,000 rpm for 1 min. Next, the genomic DNA of *S. aureus* was extracted using MiniBEST Bacterial Genomic DNA Extraction Kit Ver.2.0(Takara, China).According to the full-length sequence of the TSST-1 gene in GenBank, we designed the following primers to amplify the gene: sense: 5′-cgcgaattcatgtttaagaaaaaaatgttag-3′; antisense: 5′-aacctcgagttaattatgtcctttcacttt-3′. The underlined bases are *Eco*RI and *Xho*I recognition sites, respectively. PCR was performed in a 50-μL final volume that included 1 μL of 0.5 μg bacterial genomic DNA, 4 μL of dNTPs (2.5 mM), 5 μL of 10× PCR Buffer (with 2.5 mM $MgCl_2$), 1 μL each of 10 μM sense and antisense primers, 0.5 μL of rTaq polymerase (5 U/μl), and 37.5 μL of ddH_2O. The PCR amplification steps were the following: an initial denaturation step at 94 °C for 5 min, followed by 34 cycles of 45 s at 94 °C, 45 s at 53 °C, and 2 min at 72 °C, and extension for 15 min at 72 °C after the last cycle. An Eppendorf MasterCycler Gradient Thermal Cycler was used, and the obtained PCR products were electrophoresed on 1% (w/v) agarose gels and visualized under a UV transilluminator after ethidium bromide staining.

The PCR products were purified using a PCR purification kit and double-digested with *Eco*RI and *Xho*I, and then subcloned into the prokaryotic expression vector pGEX-4T-1. *Escherichia coli* BL21 was transformed with the recombinant plasmid and dispersed onto LB agar plates containing 50 μg/mL ampicillin. The positive plasmid DNAs were extracted and confirmed by means of sequencing and digestion with restriction enzymes (*Eco*RI and *Xho*I). A single colony of BL21 containing the GST-TSST-1 plasmid was cultured overnight in LB medium containing 50 μg/mL ampicillin, and then the cultures were diluted 1:50 in 400 mL of fresh LB medium and grown at 37 °C until the culture's optical density (OD) at 600 nm reached 0.40. The expression of GST-TSST-1 was induced with 1.0 mM isopropyl β-D-thiogalactoside (IPTG) for 6 h at 30 °C. The culture was collected and centrifuged at 8000 rpm for 20 min. A 1-mL aliquot of the culture was pelleted by centrifuging it at 8000 rpm for 5 min, and the cell pellet was resuspended in 60 μL of 20 mM PBS and denatured in 5× denaturing buffer at 100 °C for 10 min. After centrifugation at 8000 rpm for 5 min, 10 μL of the supernatant was loaded onto a 10% gel for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Protein bands were visualized by staining with Coomassie Brilliant Blue G-250. The recombinant protein was separated by means of SDS-PAGE, transferred to polyvinylidene fluoride membranes, and immunoblotted with an anti-GST-tag mouse monoclonal antibody and peroxidase-conjugated goat anti-mouse IgG; the immunoreactive bands were visualized using a DAB Horseradish Peroxidase color development kit (Boster, China). Recombinant GST-TSST-1 protein was purified by using a GST MiniSpin Purification Kit according to manufacturer instructions (Thermo Scientific, USA). The column-bound fusion protein was cleaved using the protease thrombin in order to remove the GST tag, after which the obtained TSST-1 protein was dialyzed against PBS at 4 °C for 24 h [10]. Protein samples were subjected to 10% SDS-PAGE and visualized through Coomassie staining. The Bradford assay was used to determine the final concentration of purified TSST-1. Protein aliquots were stored at −80 °C.

2.3. Measurement of TSST-1-specific IgG by using ELISA

We coated 96-well polystyrene plates with 0.5 μg/mL TSST-1 protein in carbonate buffer solution (CBS; 0.05 M, pH 9.6) overnight at 4 °C, and then washed the wells thrice with PBS containing 0.05% Tween 20 and blocked with CBS containing 2% BSA for 2 h at 37 °C. Next, diluted serum samples from pediatric patients with *S. aureus* infection and healthy children were added to the plates and incubated for 1 h at 37 °C. As the blank control, the antibody dilution buffer was added to 10 wells coated with TSST-1 protein. After washing 3 times with PBS containing 0.05% Tween 20, peroxidase-conjugated goat anti-human IgG antibodies (0.16 μg/mL) were added and the plates were incubated for 1 h at 37 °C, and then the reaction was developed by using o-phenylenediamine dihydrochloride as the substrate (for 5 min at 37 °C). Lastly, the reaction was terminated by adding 2 M H_2SO_4 and the OD at 490 nm was measured using an ELISA reader [11]. The mean OD_{490} of three repeated wells was calculated, and the final OD value was obtained after subtracting the background OD. TSST-1-specific IgG1 and IgG2 were detected using peroxidase-conjugated goat anti-human IgG1 and IgG2 antibodies (SouthernBiotech, USA).

2.4. ELISA measurement of IFN-γ and IL-6 in PBMCs

PBMCs were isolated by means of density-gradient centrifugation by using Ficoll-Hypaque Solution 1.077 [12]. Next, PBMCs were plated at a concentration of 1×10^5 cells/well at 1 mL/well in 24-well plates and cultured in culture medium for 48 h at 37 °C with 5% CO_2 [13]. The culture medium consisted of complete Roswell Park Memorial Institute (RPMI) 1640 supplemented with 2 mM L-glutamine, 10% fetal bovine serum, and 100 μg/mL streptomycin and 100 U/mL penicillin. The PBMCs were divided into 3 groups,