Specific Serum Ig Recognizing Staphylococcal Wall Teichoic Acid Induces Complement-Mediated Opsonophagocytosis against *Staphylococcus aureus*

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Specific Serum Ig Recognizing Staphylococcal Wall Teichoic Acid Induces Complement-Mediated Opsonophagocytosis against *Staphylococcus aureus*

Dong-Jun Jung,* Jang-Hyun An,* Kenji Kurokawa,* Yoon-Chuel Jung,† Min-Jung Kim,* Youko Aoyagi,‡ Misao Matsushita,§ Shinji Takahashi,‡ Hee-Seung Lee,§ Kazue Takahashi,¶ and Bok Luel Lee*†

Wall teichoic acid (WTA) of *Staphylococcus aureus* is a major cell envelope-associated glycopolymer that is a key molecule in promoting colonization during *S. aureus* infection. The complement system plays a key role in the opsonization and clearance of pathogens. We recently reported that *S. aureus* WTA functions as a ligand of human serum mannose-binding lectin (MBL), a recognition molecule of the lectin complement pathway. Intriguingly, serum MBL in adults does not bind to WTA because of an inhibitory effect of serum anti–WTA-IgG. In this study, serum anti–WTA-IgG was purified to homogeneity using a purified *S. aureus* WTA-coupled affinity column to examine the biological function of human anti–WTA-IgG. The purified anti–WTA-IgG contained the IgG2 subclass as a major component and specifically induced C4 and C3 deposition on the *S. aureus* surface in the anti–WTA-IgG–depleted serum, but not in C1q-deficient serum. Furthermore, the anti–WTA-IgG–dependent C3 deposition induced phagocytosis of *S. aureus* cells by human polymorphonuclear leukocytes. These results demonstrate that serum anti–WTA-IgG is a real trigger for the induction of classical complement-dependent opsonophagocytosis against *S. aureus*. Our results also support the fact that a lack of the lectin complement pathway in MBL-deficient adults is compensated by Ag-specific, Ab-mediated adaptive immunity. The *Journal of Immunology*, 2012, 189: 000–000.

The complement system plays important roles in host defense responses, such as opsonization of pathogenic microbes, production of peptide mediators for phagocyte recruitment, and generation of membrane-attack complexes for bacteria killing and lysis (1, 2). The effector functions of the complement system can be activated through three pathways: the classical, the alternative, and the lectin pathway. The classical pathway is activated by Ab–Ag complexes, and the alternative can be initiated when spontaneously activated complement components bind to the surfaces of pathogens (3). Finally, the lectin pathway is initiated by the binding of serum lectins, mannose-binding lectin (MBL) and ficolins, to the carbohydrates on bacteria, fungi, and viruses (4). Because complement-mediated opsonization and polymorphonuclear leukocyte (PMN)-mediated phagocytosis are crucial to innate immunity and play important roles in the clearance of pathogens and apoptotic cells, deficiencies of complement components are correlated with inflammatory and immunological diseases (5).

*Staphylococcus aureus* is a major commensal Gram-positive pathogen causing wound infections, bacteremia, and sepsis, leading to morbidity and mortality in the clinic (6). *S. aureus*, like most Gram-positive bacteria, incorporates peptidoglycans (PGNs) or membrane-associated carbohydrate-based glycopolymers, such as wall teichoic acid (WTA) and lipoteichoic acid (LTA), into their cell envelopes (7). *S. aureus* WTA polymers are composed of an N-acetylmannosamine-(β-1,3)-N-acetylgalactosamine disaccharide with two glycerol phosphates attached to the C4 hydroxyl of the N-acetylmannosamine residue, followed by 11–40 ribitol-phosphate repeating units. The hydroxyls on the ribitol phosphate repeats are tailored with cationic α-alanine esters and N-acetylgalactosamine (8). WTA is covalently linked to PGN at the C6 position of N-acetylmuramic acid (Supplemental Fig. 1). LTA polymers are usually formed by glycerol-phosphate repeating units and are connected to glycolipids. Although WTA is dispensable for viability, it is suggested to be involved in the adherence of *S. aureus* to nasal epithelial cells (9). However, LTA seems to play a crucial role in cell division and membrane integrity, which might explain why it is indispensable for viability at 37˚C, but not 30˚C (10, 11). In addition, *S. aureus* cannot survive with both WTA and LTA deleted (11), indicating that teichoic acids play crucial roles in *S. aureus* fitness and cell-wall maintenance.

Recently, we reported that the purified human MBL/MBL-associated serine proteases (MASP) complex specifically binds to wild-type *S. aureus*, but not to the WTA-deficient *S. aureus ΔtagO* mutant, indicating that MBL recognizes WTA in vitro (12). However, intriguingly, the serum MBL/MASP complex in adults does not bind to WTA because of an inhibitory effect of anti–WTA-IgG. In contrast, the MBL/MASP complex in infants who...
not have yet fully developed their adaptive immunity can bind to *S. aureus* WTA and then induce complement C4 deposition (12). Furthermore, we also demonstrated that the adult serum that does not contain serum MBL [MBL(−) serum] has the ability to deposit C4 on wild-type *S. aureus*, as does the adult serum that contains sufficient amounts of serum MBL [MBL(+) serum], but the same serum cannot induce C4 deposition on *S. aureus* ΔtagO mutant cells at a low serum concentration. Based on these observations, we proposed that WTA-recognition serum Abs might activate the anti–WTA-IgG–mediated complement pathway in the MBL(−) adult serum. We also proposed that MBL(+) adults who lack a lectin complement pathway produce specific serum Abs against *S. aureus* WTA to compensate for the deficiency of lectin complement pathway-mediated innate immunity (12). However, there is no direct evidence supporting these hypotheses.

To address these questions, in this study, we purified *S. aureus* WTA, which was free of LTA and PGN, and human serum anti–WTA-IgG from commercially available human i.v. Igs (IVIGs) using WTA-coupled affinity column that was prepared with our WTA-IgG from commercially available human i.v. Igs (IVIGs). WTA, which was free of LTA and PGN, and human serum anti–WTA-IgG from commercially available human i.v. Igs (IVIGs) sera were used. Our results demonstrate that anti–WTA-IgG is directly involved in the recognition of *S. aureus* WTA and induces the activation of the complement system leading to opsonophagocytosis of *S. aureus*.

**Materials and Methods**

**Protein, sera, and bacteria**

Complement component proteins and Abs were obtained from Complement Tech (Tyler, TX). Clq-deficient sera were obtained from Calbiochem/EMD Biosciences (San Diego, CA). Human IVIG was obtained from SK Chemicals (Seoul, Korea). MBL-sufficient (+) and -deficient (−) sera were obtained from healthy volunteers who provided informed consent. Bacterial strains and functions of deleted genes in mutant strains are summarized in Table I. *S. aureus* RN4220 is used as a parental strain. Strains M0702 and M0107 are WTA-deficient ΔtagO mutant and IgG-binding protein A-deficient Δspa mutant of RN4220, respectively. The tagO gene encodes the first-step enzyme TagO for WTA biosynthesis, and the spa gene encodes protein A that binds to the Fc portion of IgG. Strain T258 is a WTA- and protein A-deficient ΔtagO, Δspa double mutant (12). S. aureus RN4220 is an LTA- and protein A-deficient ΔltaS, Δspa double mutant. In the strain T775, the ltaS gene, which encodes polyglycerophosphate synthase for LTA polymerization, was disrupted in spa deleted M0107 cells (11). To make this strain, first, we amplified a DNA fragment of ~1.1 kb of the internal ORF of the ltaS gene by PCR using primers pMltaS-5P-HindIII (5′-CATGGTCTAGGAACGTCTTCATGAGCA-3′) and pMltaS-5P-BamHI (5′-CATCAACGTGGTGAGATCCCTGTGTGT-3′). Second, the resultant fragment was inserted into the HindIII and BamHI sites of pMutin-T3, creating pMltaS. The pMltaS plasmid was introduced into M0107 cells harboring pM102-ltaS (11), and erythromycin-resistant transformants generated by homologous recombination were selected. From these cells, targeted integration of pMltaS at the chromosomal ltaS gene was confirmed by PCR, resulting in T775 strain as pMltaS::pM102-ltaS. Finally, T772 strain was transformed with pM101 (13), which is incompatible with pM102-ltaS because they possess the same replicon, but has a different marker from pM102 (namely, kanamycin resistance). A kanamycin-resistant transformant, T775 (ΔltaS::pM101, Δspa::pM102-ltaS), was obtained at 30 °C. The loss of pM102-ltaS in T775 was confirmed by chloramphenicol sensitivity and the high temperature-sensitive growth phenotype of T775 as a ΔltaS mutant (11). Strain T363 is Lgt-deficient Δgt mutant. The lgt gene encodes Lgt, the first-step enzyme for lipoprotein lipid modification; thereby Δgt mutant loses lipid modification of all the bacterial lipoproteins and their TLR2-stimulating activity (14). Construction of strain T363 (Δgt::pHloe) will be described elsewhere (M. Nakayama, K. Kurokawa, K. Nakamura, B.L. Lee, K. Sekimizu, H. Kabagawa, K. Hiramatsu, H. Yagita, K. Okumura, T. Takai, D.M. Underhill, A. Aderem, and K. Ogasawara, submitted for publication). T363 strain is an Lgt- and WTA-deficient Δgt ΔltaS double mutant, which was constructed from T363 via phage 80α transduction. All of the bacterial strains were cultured with Luria-Bertani medium supplemented with antibiotics wherever required.

**Purification of *S. aureus* WTA and LTA**

*S. aureus* WTA was purified as described previously (15). Because the phosphodiester linkage between WTA and N-acetylmycolic acid of monomeric-PGN is easily hydrolyzed by acid (16) (see Supplemental Fig. 1), the obtained WTA-coupled PGN were treated with 5% (w/v) trichloroacetic acid. To further purify WTA by homogeneity, we used an ion-exchange column as described previously (12). LTA was purified from *S. aureus* strain T373 that was depleted from WTA and lipoproteins to avoid contamination of LTA by WTA and lipoproteins. The purification was performed as described previously (17). The binding specificity of WTA toward anti–WTA-IgG was examined by ELISA using the purified WTA- or LTA-coated plates.

**Measurements of anti–WTA-IgG binding and C4, C3 deposition on *S. aureus* cells by flow cytometry**

C4 and C3 deposition was measured as described previously (12). First, 2.0 × 10^7 *S. aureus* cells were fixed with ethanol and then incubated with different 2% human sera, purified anti–WTA-IgG, or IVIG in 500 μl incubation buffer (10 mM Tris [pH 7.4], 140 mM NaCl, 0.05% Tween 20, 1% human serum albumin [HSA], 10 mM CaCl_2, 10 mM MgCl_2) at 30°C for 1 h. After washing with washing buffer (10 mM Tris-HCl [pH 7.4], 140 mM NaCl, 0.05% Tween 20), recovered bacterial cells were suspended in 100 μl incubation buffer. To detect bound C4, we used mouse anti-human C3 mAb (diluted 1:500; Bioporto) and goat F(ab') 2 anti-mouse IgG mAb conjugated with FITC (diluted 1:200; Beckman Coulter). To detect bound C3b, we used mouse anti-human C3 IgG mAb conjugated with FITC (1:200 dilution; Beckman Coulter). To detect bound anti–WTA-IgG on bacteria, we used mouse anti-human IgM mAb (1:5000 dilution; Sigma Aldrich) and goat F(ab')2 anti-mouse IgG mAb conjugated with FITC (1:200 dilution; Beckman Coulter). Then *S. aureus* cells were sonicated for 15 s to make single cells before flow cytometry analyses (Model FC500; Beckman Coulter). To measure C3 deposition in the alternative pathway alone, we used Mg^2+-EGTA buffer (5 mM MgCl_2, without CaCl_2, and 10 mM EGTA in incubation buffer).

**Determination of serum levels of anti–WTA-IgG and MBL**

Levels of human serum anti–WTA-IgG and MBL were determined by ELISA as described previously (12). Recombinant human MBL was expressed in a CHO cell line and chromatographically purified as described previously (18). In brief, WTA (5 μg) or mannan (5 μg)-coated microplates were incubated with 0.1 μl human serum in 50 μl buffer E (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20) containing 1% BSA for 2 h at 4°C, and bound IgGs were detected with mouse anti-human IgG mAb conjugated with HRP (Human Ads). To quantify MBL amounts in serum, we used mouse anti-human MBL mAb (diluted 1:1000; Doeble). The concentration was estimated by comparison with those of controls. The resulting plates were developed with the substrate, 3,3′,5,5′-tetramethylbenzidine (Zymed Laboratories) in the dark and stopped with 2 N H_2SO_4. The absorbance at 450 nm was recorded using a microplate reader (Thermo Scientific). Data are representative of at least three independent experiments.

**Preparation of WTA-coupled Sepharose resin**

To make WTA-coupled Sepharose resin, we used the method of Click-Chemistry (19). The detailed preparation methods and synthetic methods of WTA-conjugated Sepharose resin are described in Supplemental Fig. 1B.

**Affinity purification of anti–WTA-IgG from human IVIG using a WTA-coupled Sepharose column**

Anti–WTA-IgG was affinity purified as described previously (12) with minor modifications. In brief, 2 ml IVIG (50 mg protein/ml) diluted in 10 ml buffer D (20 mM phosphate buffer, pH 7.4) was applied to a WTA-coupled Sepharose column (1 × 5 cm) equilibrated with buffer D. After washing this column with buffer D, bound proteins were first eluted with buffer D containing 1 M NaCl. Then bound IgG was eluted with 0.1 M glycine buffer (pH 2.5) and immediately neutralized with 1 N KOH to pH 7.5. The purity and identity of the purified IgG were confirmed by SDS-PAGE under reducing conditions and by N-terminal sequencing. The specific IgG subclass type and concentration in the purified anti–WTA-IgG were determined using a commercially available kit (Human IgG Subclass Profile; Invitrogen) according to the manufacturer’s instructions.
Depletion of \textit{S. aureus}-recognizing Ig and serum MBL from intact human serum

The serum depleted of both serum MBL and \textit{S. aureus}-recognizing IgG were prepared using \textit{S. aureus} \Delta\textit{spa} mutant cells as described previously (20). In brief, \textit{S. aureus} \Delta\textit{spa} mutant cells, which were collected from 200 ml cultured medium, were treated with 1% formaldehyde to fix bacteria and then kept on ice for 30 min with vortexing every 10 min. After fixation, 100 mM ethanolamine (pH 8.0) containing 150 mM NaCl was treated to inactivate residual aldehyde groups. Fixed bacteria were washed twice with PBS and divided into three parts. Human serum (5 ml) was incubated with one part of the fixed bacteria on ice for 30 min with mixing every 10 min. Subsequently, the bacteria were removed by centrifugation at 8600 \times g for 10 min at 4°C; then the supernatant was taken. The same absorption process was repeated three times successively and bacteria were removed at each step by centrifugation. Finally, the obtained serum depleted of both MBL and \textit{S. aureus}-recognizing IgG was filtered with a 0.2-\mu m filter and kept at \(-80^\circ\text{C}\) until use.

PMN preparation

PMNs were prepared as described previously (20) with some modifications. In brief, 4 ml whole human blood containing 40 \mu l heparin (1000 U/ml) was poured onto 4 ml Polymorph prep solution (Nycomed Pharm As, Torshov, Norway). After centrifugation at 450 \times g for 30 min at 20°C, both the plasma and mononuclear phases were carefully discarded. The PMN phase (1.5 ml) was collected into another tube containing 1.5 ml 50% RPMI medium 1640. Then PMN was pelleted by centrifugation at 250 \times g for 5 min at 20°C, washed three times with 4 ml RPMI medium 1640, and resuspended in 1 ml RPMI medium 1640 containing 0.4% HSA and 10 mM HEPES. PMNs were counted by a Burker-Turk cell counting chamber and adjusted to \(4.3 \times 10^8\) cells/ml by adding RPMI medium 1640 containing HSA and HEPES. PMN viability was confirmed by dye exclusion test and was >95%.

Opsonophagocytosis assay

This experiment was performed as previously described (20) with some modifications. In brief, \textit{S. aureus} M0107 or T258 mutant strains grown to a postexponential growth phase culture in Luria-Bertani medium were washed, killed with 70% ethanol, labeled with 0.1 mM FITC (Sigma) in 0.1 M Na\textsubscript{2}CO\textsubscript{3} buffer (pH 8.5) for 30 min at room temperature, and resuspended in HBSS. FITC-labeled bacteria (equivalent to 1.5 \times 10^8 CFU) were opsonized with 10% prepared sera with or without anti–WTA-IgG or IVIG (0.6 \mu g) in 20 \mu l HBSS containing 2 mM Ca\textsubscript{Cl\textsubscript{2}}, 1 mM Mg\textsubscript{Cl\textsubscript{2}}, 10 mM HEPES, 150 mM NaCl, and 0.4% HSA for 30 min at 37°C with shaking. Then the PMN suspension (1.5 \times 10^5 cells, 35 \mu l) prepared earlier was added to 5 \mu l of the opsonized bacteria (corresponding to 3.7 \times 10^5 CFU: multiplicity of infection \(\approx 25\)) and incubated at 37°C for 60 min with shaking. Finally, phagocytosed FITC-labeled \textit{S. aureus} cells in PMNs were counted under fluorescent phase-contrast microscopy. More than 100 PMNs were counted. Extracellular FITC-labeled \textit{S. aureus} were quenched by 0.2% trypan blue.

Data processing and statistical analysis

Results from quantitative analyses are expressed as the mean \(\pm\) SD of the data from at least three independent experiments, unless otherwise stated in the text. Other data are representative of at least three independent experiments that yielded similar results. Statistical analyses were performed using Student t test, and \(p\) values <0.05 were considered significant and are indicated in the figures.

Results

Purification and biochemical characterization of human serum anti–WTA-IgG

It is essential to purify WTA-recognizing IgG from human serum to determine the biological functions of serum anti–WTA-IgG. For this purpose, \textit{S. aureus} WTA was first purified to homogeneity, and specificity of anti–WTA-IgG was evaluated by using WTA-deficient \textit{S. aureus} monomeric PGN-attached WTA was used to identify WTA-recognizing IgG. In this study, we removed monomeric-PGN from WTA by treatment with trichloroacetic acid to exclude the possibility of copurification of PGN- and WTA-recognizing IgGs (Supplemental Fig. 1A). Then WTA-conjugated Sepharose resin was prepared using Click-Chemistry (Supplemental Fig. 1B). Next, to purify serum anti–WTA-IgG, we used commercially available IVIG as a human IgG source. After loading IVIG onto a WTA-coupled column, bound anti–WTA-IgG was eluted with 20 mM phosphate buffer (pH 7.5) containing 1 M NaCl and then with 0.1 M glycine buffer (pH 2.5). The elution pattern of the WTA-Sepharose column is shown in Fig. 1A. The purity of the anti–WTA-IgGs was confirmed by SDS-PAGE under reducing conditions (Fig. 1B). The N-terminal sequences of bands A and B in Fig. 1B were determined as H chain IgG and L chain IgG, respectively (Fig. 1C). In ELISAs, purified anti–WTA-IgG specifically recognized WTA only (Fig. 1D), but not LTA, suggesting that purified anti–WTA-IgG has high binding specificity to its ligand molecule. An average of 2.5 \mu g anti–WTA-IgG was obtained by 0.1

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<th>Table I. Bacterial strains used in this study</th>
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<td>Stains</td>
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<tr>
<td>--------------</td>
</tr>
<tr>
<td>RN4220</td>
</tr>
<tr>
<td>M0702</td>
</tr>
<tr>
<td>M0107</td>
</tr>
<tr>
<td>T258</td>
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<tr>
<td>T775</td>
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<tr>
<td>T363</td>
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<td>T737</td>
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Protein encoded by the deleted gene and its mutant phenotype.

\textit{lg}r, First-step enzyme for lipoprotein lipid modification: loss of lipid modification of all the bacterial lipoproteins and of their TLR2 ligand activity; \textit{baa}, the enzyme for LTA polymerization: loss of LTA; \textit{spa}, protein A for Ig-binding protein: loss of protein A; \textit{tagO}, first-step enzyme for WTA biosynthesis: loss of WTA.

![FIGURE 1. The purification and binding specificity of anti–WTA-IgG.](http://www.jimmunol.org/Downloadedfrom)
M glycan elution from 50 mg IVIG proteins (0.005% purification yield). Because specific human serum IgG recognizing S. aureus WTA was obtained, the subclass of the purified anti–WTA-IgG was determined. As shown in Table II, anti–WTA-IgG eluted by 0.1 M glycan elution mainly consisted of the IgG2 subclass (73%) and was largely enriched from IVIG, which contains the IgG1 subclass as a major component. However, anti–WTA-IgG eluted by 1 M NaCl elution consisted of 55% IgG2 and 33% IgG1 subclasses. In our preliminary experiments, because anti–WTA-IgG eluted by 0.1 M glycan elution induced a stronger PMN-mediated opsonophagocytosis response against S. aureus compared with anti–WTA-IgG eluted by 1 M NaCl elution, anti–WTA-IgG eluted by 0.1 M glycan elution was used in the further experiments.

Binding specificity of purified anti–WTA-IgG against S. aureus cells

To further confirm whether this purified anti–WTA-IgG also has binding specificity to WTA expressed on the surface of S. aureus mutant strains, we used three S. aureus mutant strains. First, the IgG-binding protein A-deficient Δspa mutant (11) was used to exclude the possibility of nonspecific binding between S. aureus protein A and purified anti–WTA-IgG. Second, both the WTA- and IgG-binding protein A-deficient ΔtagO, Δspa double mutant (12) and the LTA- and IgG-binding protein A-deficient Δltas, Δspa double mutant were used. As shown in Fig. 2, anti–WTA-IgG recognized both the Δspa mutant and the Δltas, Δspa double mutant (Fig. 2, panels 2-2, 2-6), but not the ΔtagO, Δspa double mutant (Fig. 2, panel 2-4). This result confirms that purified anti–WTA-IgG also has binding specificity to WTA expressed on the S. aureus cell surface. Under the same conditions, all three mutant cells bound to IVIG (Fig. 2, panels 2-1, 2-3, 2-5), indicating that IVIG may contain all of the different IgGs recognizing other surface molecules of S. aureus. Taken together, these results demonstrate that purified serum anti–WTA-IgG specifically recognizes purified S. aureus WTA and exposed WTA on the bacterial surface.

Anti–WTA-IgG specifically induces C3 deposition on the surface of S. aureus

To investigate the biological functions of anti–WTA-IgG during complement activation, we prepared the serum depleted of both S. aureus-recognizing IgG and MBL by absorption with the Δspa mutant cells. After preparation, we estimated the residual amounts of serum MBL and anti–WTA-IgG in the Δspa mutant-treated serum by ELISA and found that MBL and anti–WTA-IgG were not detectable in the serum (Table III). To further characterize the Δspa mutant-treated serum, the proteins bound to Δspa mutant cells were eluted with 0.1 M glycine buffer (pH 2.5) and then analyzed by SDS-PAGE to determine what kinds of serum proteins were bound on the Δspa mutant cells (Fig. 3A, lanes 3–5). When the N-terminal sequences of three commonly bound bands on Δspa mutant cells were determined, bands A, B, and C of elution fractions (first to third) were confirmed as HSA, IgG H chain, and IgG L chain, respectively. We supposed that HSA of band A may be a contaminant from the tremendous amount of HSA in the intact serum, but specific human serum IgGs were mainly enriched on the surface of S. aureus Δspa mutant cells. To further analyze the proteins bound to Δspa mutant cells, we further eluted with 0.3 M mannose to obtain MBL bound on the Δspa mutant cells; then eluates were analyzed by Western blot using anti-MBL Ab (Fig. 3B). As expected, a human MBL band was detected from the first and second eluate fractions of Δspa mutant cells (Fig. 3B, lanes 2, 3). These results suggest that serum MBL readily binds to S. aureus WTA in the condition of small amounts of serum anti–WTA-IgG as described in our previous study (12). Taken together, we successfully prepared the serum that does not contain S. aureus-recognizing serum-IgG and serum MBL by absorption with the S. aureus Δspa mutant.

Next, the relative complement C3 deposition obtained with intact MBL (+) serum and serum absorbed with the Δspa mutant cells were examined in the presence of Ca2+ and Mg2+ ions by flow cytometry (Fig. 4). The 2% Δspa mutant-treated serum lost most of its C3 deposition ability on the S. aureus M0107 (Δspa) cells compared with the 2% intact MBL (+) serum (Fig. 4, panels 4-1, 4-2). However, by addition of the proteins eluted from Δspa mutant bacteria into Δspa mutant-treated serum, the C3 deposition ability was clearly restored (Fig. 4, panel 4-3). This result suggests that triggering molecules for C3 deposition were depleted from MBL (+) intact serum by Δspa mutant treatment, and that the eluted fraction from Δspa mutant bacteria contains the essential trigger molecule(s) for C3 deposition on M0107 (Δspa) cells. We therefore examined the possibility that anti–WTA-IgG is a real trigger molecule for C3 deposition. The addition of purified anti–WTA-IgG also

Table II. Determination of IgG subclass for the purified anti-WTA IgG

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<tr>
<th>Samples</th>
<th>IgG Subclass Concentration (ng/100 ng Proteins)</th>
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<tbody>
<tr>
<td></td>
<td>IgG1</td>
</tr>
<tr>
<td>IVIG</td>
<td>62</td>
</tr>
<tr>
<td>Eluted fraction</td>
<td></td>
</tr>
<tr>
<td>1 M NaCl</td>
<td>33</td>
</tr>
<tr>
<td>0.1 M glycine</td>
<td>19</td>
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Table III. Determination of sera concentration of serum MBL and anti-WTA-IgG

<table>
<thead>
<tr>
<th>Sera</th>
<th>MBL (ng/μl)</th>
<th>Anti–WTA-IgG (ng/μl)</th>
</tr>
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<tbody>
<tr>
<td>MBL (+) serum</td>
<td>5.6</td>
<td>117</td>
</tr>
<tr>
<td>Δspa-treated serum</td>
<td>0</td>
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IgG (10 μg) instead of the eluted fraction from Δspa mutant bacteria into Δspa mutant-treated serum clearly restored C3 deposition (Fig. 4, panel 4-4). This demonstrates that anti–WTA-IgG is a real trigger molecule for the induction of complement C3 deposition on S. aureus cells. When we examined the dose dependency of anti–WTA-IgG, C3 deposition increased in a dose-dependent manner (data not shown). For further confirmation, 10 μg IVIG was added to the Δspa mutant-treated serum. The addition of the IVIG gave a slighter recovery of C3 deposition on M0107 (Δspa) cells (Fig. 4, panel 4-5), than an identical amount of anti–WTA-IgG did (Fig. 4, panel 4-4). Furthermore, when this C3 deposition was examined in the presence of Mg2+–EGTA buffer conditions, which inhibit the classical and lectin pathways, but not the alternative pathway, C3 deposition completely disappeared (Fig. 4, panel 4-6). These results suggest that the classical pathway may be involved in the anti–WTA-IgG–mediated C3 deposition.

Next, we examined anti–WTA-IgG–mediated C3 deposition on WTA-deficient ΔtagO mutant cells. Decreased C3 deposition on the ΔtagO mutant cells was observed in Δspa mutant-treated serum compared with MBL(+) intact serum (Fig. 4, panels 4-7, 4-8). However, addition of anti–WTA-IgG (10 μg) into Δspa mutant-treated serum did not fully restore C3 deposition on the ΔtagO mutant cells (Fig. 4, panel 4-9), confirming that the anti–WTA-IgG–mediated C4 deposition response occurs in a WTA-dependent manner.

Anti–WTA-IgG specifically induces C4 deposition via the classical pathway

Because anti–WTA-IgG–mediated C3 deposition was clearly observed on S. aureus parental cells, we next investigated anti–WTA-IgG–mediated C4 deposition. As shown in Fig. 5, although 2% Δspa mutant-treated serum showed little C4 deposition compared with intact MBL(+) serum (Fig. 5, panels 5-1, 5-2), C4 deposition was restored by the addition of anti–WTA-IgG (10 μg) into Δspa mutant-treated serum (Fig. 5, panel 5-3). However, this C4 deposition was abolished by the addition of C1 inhibitor (Fig. 5, panel 5-4), indicating that anti–WTA-IgG–dependent C4 deposition is mediated by C1-derived classical complement pathway. Furthermore, when the same amount of the eluted proteins (10 μg) from Δspa mutant bacteria was added into the Δspa mutant-treated serum, little amounts of C4 deposition were observed (Fig. 5, panel 5-5). However, with addition of the same amount of
Anti–WTA-IgG induces complement-dependent opsonophagocytic response against S. aureus

Because anti–WTA-IgG specifically induced C3 deposition on S. aureus, we assumed that anti–WTA-IgG-dependent C3-deposited bacteria are more easily engulfed by human PMNs. To examine anti–WTA-IgG–mediated opsonophagocytosis of S. aureus, we directly counted and indicated the numbers of ethanol-killed bacteria engulfed by 100 numbers of human PMNs (Fig. 6). In the absence of MBL(+), serum, anti–WTA-IgG increased the total numbers of S. aureus Δspa cells phagocytosed by 100 PMNs from 7 ± 4 (Fig. 6, column 1) to 174 ± 59 (column 3), indicating that IgG receptor (FcγR)-mediated phagocytosis may be induced on PMNs (21). When S. aureus Δspa cells were opsonized with 10% intact MBL(+) adult serum, the number of bacteria phagocytosed by 100 PMNs is increased to 667 ± 232 (Fig. 6, column 4), supporting that complement-mediated opsonophagocytosis was induced. Addition of IVIG or anti–WTA-IgG into column 4 (Fig. 6) did not increase the engulfed bacteria number (columns 5, 6), indicating that MBL(+) serum contains sufficient serum anti-S. aureus IgG. In contrast, when 10% Δspa mutant-treated serum was used for opsonization, bacteria were almost not phagocytosed by PMNs (Fig. 6, column 7), which is consistent with the absence of C3 deposition in this combination (Fig. 4, panel 4-2). However,
addition of purified anti–WTA-IgG (0.6 \mu g; Fig. 6, column 9) but not IVIG (column 8) into \( \Delta ppa \) mutant-treated serum (column 7) dramatically increased the number of engulfed bacteria per 100 PMNs from 13 \pm 7 (column 7) to 793 \pm 258 (column 9). These results strongly demonstrate that anti–WTA-IgG is a real trigger molecule for induction of complement-mediated opsonophagocytosis against \textit{S. aureus} cells.

To further confirm the specificity of anti–WTA-IgG-mediated opsonophagocytosis, we again counted the numbers of engulfed bacteria by 100 PMNs using both protein A and WTA-deficient \textit{S. aureus} \( \Delta ppa \), \( \Delta tagO \) double-mutant cells. In contrast with the case of \( \Delta ppa \) mutant cells (Fig. 6, column 3), anti–WTA-IgG itself did not increase the \( \Delta ppa \), \( \Delta tagO \) double-mutant cell number engulfed by PMNs (column 12). In addition, when these double-mutant cells were opsonized with MBL(+) serum, the phagocytosed bacteria by 100 PMNs were counted as 205 \pm 42 (Fig. 6, column 13). Also, when the \( \Delta ppa \), \( \Delta tagO \) double-mutant cells were opsonized with \( \Delta ppa \) mutant-treated serum, the number of engulfed bacteria was counted as 184 \pm 25 (Fig. 6, column 16), which is consistent with the obvious C3 deposition in this combination (Fig. 4, panel 4–8). Furthermore, addition of IVIG or anti–WTA-IgG into column 16 (Fig. 6) maintained the similar number of phagocytosed bacteria (columns 17, 18). These results strongly suggest that anti–WTA-IgG–mediated opsonophagocytosis is induced only after specific recognition of \textit{S. aureus} WTA by anti–WTA-IgG. Taken together, our results demonstrate that anti–WTA-IgG functions as a direct trigger molecule, leading to the induction of complement-dependent opsonophagocytosis of \textit{S. aureus}.

**Discussion**

The cell wall of most \textit{S. aureus} cells is composed of five major constituents: capsular polysaccharides (CPs), WTA, LTA, PGN, and proteins. Among them, three glycopolymers, WTA, LTA, and PGN, are not easy to purify because of their structural similarity and complexity (22). Furthermore, the purification of specific serum IgGs recognizing these glycopolymers and the determination of their binding specificities to their ligands are difficult. Recently available \textit{S. aureus} mutant strains that are deficient in cell-wall glycopolymers enable us to address the biological functions of \textit{S. aureus} cell-wall components during host innate immune responses and pathogen infection in hosts (12, 14, 15, 23). In this study, we confirmed the binding specificity of purified anti–WTA-IgG toward its ligand with \textit{S. aureus} mutants deficient for WTA or LTA. In addition, the serum depleted of both MBL and \textit{S. aureus}-recognizing IgG was prepared using \( \Delta ppa \) mutant cells. Recently developed DNA sequencing techniques, bacterial genomic information, and bacterial genetic technologies that enable the creation of several different bacterial mutant strains will be useful in the future to address the relationships between pathogens and hosts, and to screen ligand molecules of host receptors during host innate immune responses.

The membrane attack complex generated after complement activation readily lyses the plasma membranes of most Gram-negative bacteria, but it poorly lyases those of Gram-positive bacteria because of their thick PGN layers (24). Thus, many reports have suggested that serum Ab-mediated opsonophagocytotic bacterial killing is necessary to increase the bactericidal efficiency of the membrane attack complex against pathogenic Gram-positive bacterial infection (3). In this study, we identified a serum Ab specifically recognizing \textit{S. aureus} WTA, which can activate the complement cascade leading to opsonization and PMN-mediated opsonophagocytosis of \textit{S. aureus}.

Previous studies have suggested that the different human Ig subclasses differ in their ability to bind C1q and, therefore, have different abilities of activating the classical complement pathway (25–27). IgM, IgG1, and IgG3 activate it effectively, whereas IgG2 shows a much weaker ability, and IgG4, IgA, IgD, and IgE are not capable of activating the classical pathway (26). Bindon et al. (26) constructed a chimeric Ab consisting of the mouse L chain and an H chain assembled from different human constant regions each joined to the same mouse V region, specific for the hapten 5-iodo-4-hydroxy-3-nitrophenacetyl. However, in our work, human anti–WTA-IgG purified from human IVIG contained IgG2 subclass as a major component and clearly induced anti–WTA-IgG–dependent C4 and C3 deposition and opsonophagocytosis responses against \textit{S. aureus}. The inconsistency of IgG subclasses in the complement-activating abilities between chimeric mAbs and native serum anti-WTA Ab may be because of the different structures of IgGs and different Ags. These results provide the evidence that naturally existing serum anti–WTA-IgG containing mainly the IgG2 subclass can activate the complement system.

Our study showed that both specific \textit{S. aureus} WTA-recognizing IgGs and the complement system are efficient for the induction of opsonization and PMN-mediated opsonophagocytosis of \textit{S. aureus}. The anti–WTA-IgGs induced probable FcyR-mediated phagocytosis of \textit{S. aureus} cells via PMNs as well. A number of previous studies have suggested the need for specific serum Abs in the opsonization of encapsulated \textit{S. aureus} (28–31). In particular, Verbrugh et al. (31) found that although encapsulated \textit{S. aureus} bound significantly less C3 than unencapsulated strains in diluted normal serum, the C3 deposition ability was increased 4.7-fold by the addition of \textit{S. aureus}-challenged immune Ab. They also showed via immunoelectron microscopy that opsonized C3b was localized throughout on the capsule, as well as on the staphylococcal cell wall, when bacteria had been opsonized in human serum containing immune Abs against \textit{S. aureus}. However, they did not identify what kind of specific serum Ab was involved in this opsonization and PMN-mediated opsonophagocytosis. Based on our current data, it is possible that the unidentified Ab in the \textit{S. aureus}-challenged immune serum will include serum anti–WTA-IgG.

In vitro, \textit{S. aureus} binds to MBL with high affinity (32), and the MBL/MASP complex enhances complement activation and opsonophagocytosis after binding to \textit{S. aureus} (33). However, Eisen et al. (34) and Smithson et al. (35) observed no significant association between MBL deficiency and high mortality caused by \textit{S. aureus} infection. In contrast, MBL(−) infants are susceptible to \textit{S. aureus} infection (36–38). Although there is no clear explanation for why MBL(−) adults are not susceptible to \textit{S. aureus} infection, it can be assumed that if these MBL(−) adults have a high serum level of anti–WTA-IgG, significant differences in \textit{S. aureus} infectivity will not be observed between healthy MBL(−) adults and patients with \textit{S. aureus} bacteremia. This spontaneous production of serum anti–WTA-IgG by the adaptive immune system can compensate for the deficient MBL-mediated innate immunity. To our knowledge, this is the first biochemical and immunological study to demonstrate the importance of serum anti–WTA-IgG as a trigger of complement-mediated opsonophagocytosis of \textit{S. aureus}.

The serum levels of circulating IgGs in patients with \textit{S. aureus} infections and healthy individuals have been studied intensively (39–43). Among them, human serum IgG levels against 11 different purified Ags from \textit{S. aureus}, such as teichoic acid, clumping factor A and B, bone sialoprotein binding protein, \( \alpha \)-toxin, lipase, enterotoxin A, toxic shock syndrome toxin, scalded-skin syndrome toxin, fibrinogen binding protein, and extracellular adherence protein have been carefully examined by ELISA against 151 healthy individuals aged 15–89 y (43). The results of that study showed great individual variation in serum Ab levels in both young and elderly healthy subjects. Now, to understand the exact
biological significance of serum IgGs generated against S. aureus Ags, it would be necessary to establish more biocomptically reliable purification and characterization methods for specific S. aureus Ags, as well as to perform mechanistic studies of how S. aureus Ags can escape from host innate immunity and how serum IgGs can induce the activation of host innate and adaptive immune responses. In this respect, our current and previous studies provide a basis for how serum IgGs against S. aureus Ags molecules regulate host innate immunity against pathogenic microbe infections.

Finally, when bacterial CPs from several pathogenic bacteria, such as Haemophilus influenzae, Neisseria meningitidis, and Streptococcus pneumoniae, are conjugated to a carrier protein and then injected into a human host, they function as powerful vaccines, showing >90% protection efficacy against infection by these pathogenic bacteria (44). However, when staphylococcal CP type 5 and type 8 were coupled to a carrier protein (Pseudomonas exotoxin A toxoid) and then used a vaccine candidate (45), clinical phase III data showed that there was no significant difference between vaccinated and unvaccinated groups of hemodialysis patients. Currently, although it is not clear why staphylococcal CPs cannot be used as an effective vaccine, S. aureus could be particularly adept at obviating a vaccine developed to target a specific bacterial component by altering its expression of the vaccine target. Another possibility is that if the terminal part of staphylococcal WTA is exposed to the most outside surface rather than CPs, serum IgGs recognizing S. aureus CPs cannot easily bind to CPs, leading to a low efficacy of opsonophagocytic killing.

A plausible approach will use the purified WTA as a challenging vaccine candidate molecule that can function as a trigger of complement-dependent and probable FcYR-dependent phagocytosis of S. aureus.

Disclosures

The authors have no financial conflicts of interest.

References

Supplementary Fig. S1A. Structure of monomeric-PGN-conjugate WTA.

Because the phosphodiester linkage between WTA and MurNAc of monomeric-PGN is easily hydrolyzed by acid, the precipitates containing WTA-coupled PGN were suspended in 5% (w/v) trichloroacetic acid, kept at room temperature for 18 h and centrifuged at 20,000 x g for 10 min. The supernatants were mixed with two volumes of acetone, and the resulting precipitates were dissolved in water and centrifuged as above. The final supernatants were collected as crude WTA. To further purify WTA to homogeneity, we used an ion-exchange column (Hitrap-Q column) equilibrated with buffer B (20 mM Tris-HCl, pH 6.0). After loading of crude WTA, the column was washed, followed by elution with a 20 ml gradient from 0 to 1 M NaCl in buffer B. Fractions (1 ml) were collected and assayed for inorganic phosphate, and PAGE was performed with silver staining to detect WTA.
Supplementary Fig. S1B. Synthetic scheme of the purified WTA prepared by TCA treatment-conjugated Sepharose resin. We first prepared acetylene-conjugated Sepharose using CNBr-activated Sepharose and an azide conjugated WTA derivative using purified WTA. Then, these two compounds were conjugated by the method of Click-Chemistry. To prepare acetylene-conjugated Sepharose and the WTA-azide derivative, CNBr-activated Sepharose (dry weight 1 g, GE Healthcare) was activated with 1 mM HCl for 3 hrs at room temperature. To prepare the WTA-azide derivative, the purified WTA (5 mg) suspended in 450 µl of PBS and the azide linker (2,5-dioxopyrrolidin-1-yl 5-azidopentanoate, 4.3 mg dissolved in 50 µl of DMSO) were mixed in the reaction tube and then shaken with vortexing overnight at room temperature. To prepare WTA-conjugated Sepharose resins, acetylene-conjugated Sepharose resin was washed with 5 ml of 1 mM HCl three times, then washed with 5 ml of diethyl ether three time and finally washed with 5 ml of PBS three times. Then, the WTA-azide-containing aqueous layer was combined with acetylene-conjugated Sepharose. CuSO₄ (2.9 mg dissolved in 10 µl of distilled water), Tris[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl]amine (TBTA, 5.1 mg dissolved in 100 µl of DMSO) and Tris(2-carboxyethyl) phosphine hydrochloride (TCEP, 9.5 mg dissolved in 10 µl of distilled water) were added into the WTA-azide derivative and acetylene-conjugated Sepharose.
Supplementary Fig. S2. C4 deposition was analyzed using *S. aureus ΔtagO, Δspa*. (A) double mutant cells in the MBL(+) intact serum (S2-1), Δspa-treated serum (S2-2) and Δspa-treated serum plus anti-WTA-IgG (10 μg) (S2-3) in the presence of 10 mM Ca^{2+} and 10 mM Mg^{2+} buffer. (B) C3 deposition was examined using Δspa mutant cells in the C1q(-) serum (S2-4), Δspa-treated C1q(-) serum (S2-5) and Δspa-treated C1q(-) serum plus anti-WTA-IgG (10 μg) (S2-6) in the presence of 10 mM Ca^{2+} and 10 mM Mg^{2+} buffer (S2-6) or in the presence of 5 mM Mg^{2+}-10 mM EGTA buffer (S2-7). The serum concentration was 2%. C3b bound to *S. aureus* cells was detected by flow cytometry as described in the Materials and Methods. The *gray* area represents data from bacterial cells only.