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EndoS and SpeB from *Streptococcus pyogenes* Inhibit Immunoglobulin-Mediated Opsonophagocytosis

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The human pathogen *Streptococcus pyogenes* primarily infects the upper respiratory tract and skin, but occasionally it disseminates and causes severe invasive disease with high mortality. This study revealed that the activity of extracellular EndoS, which hydrolyzes the functionally important N-linked oligosaccharides on opsonizing immunoglobulin G (IgG), contributes to increased survival of *S. pyogenes* in human blood ex vivo. The inability to kill the bacteria is due to reduced binding of IgG to Fc receptors and impaired classical pathway-mediated activation of complement. In addition, the activity of extracellular SpeB, which cleaves IgG into Fc and Fab fragments, also increases bacterial survival. This suggests that *S. pyogenes* expresses two enzymes, EndoS and SpeB, which modulate IgG by different mechanisms in order to evade the adaptive immune system.

A prerequisite for a pathogen to successfully invade and colonize a host is to escape recognition by the immune system. Many host defense mechanisms are involved in the battle against bacterial infections, and two significant events are opsonization and phagocytosis. These events involve antigen recognition, complement deposition, and phagocytic cells that engulf and kill the microorganism after recognition by host receptors for the Fc portion of immunoglobulins, Fc receptors (FcR), and complement receptors (CR1 and CR3). In this process, the human host relies on the adaptive immune response that produces opsonizing antibodies directed towards surface-exposed antigens on the microorganism.

Human immunoglobulin G (IgG) is composed of two identical light chains and two identical heavy chains. These chains form three independent protein domains connected through the flexible and protease-sensitive hinge region. The two Fab fragments of IgG bind antigen, while the Fc region is the site of interaction with a number of effector molecules, including complement protein C1q. The Fc region also interacts with phagocytic cells, such as monocytes, macrophages, and neutrophils. A conserved complex biantennary oligosaccharide is attached to each Asn297 in the CH2 domain (29, 36). These oligosaccharides are located in the cavity between the CH2 domains and are thought to stabilize the molecule (10). The structural and biological significance of these oligosaccharides has been extensively investigated (11, 28, 30, 38, 43).

Many molecules involved in the innate and adaptive immune system are glycoproteins (31). Consequently, the immune evasion strategies used by pathogens may include interfering with oligosaccharides on glycoproteins. Potential targets could be the N-linked oligosaccharides on the heavy chains of immunoglobulins, which are known to play a role in effector functions, including complement activation and FeR binding on effector cells (4, 28).

Human pathogens have evolved many strategies to evade immune recognition. Examples include the IgA proteases found in many bacterial species (18, 23, 27). These IgA proteases are highly specific extracellular proteins that cleave IgA in the hinge region into Fc and monomeric Fab fragments, resulting in impairment of IgA effector functions (19). Specific IgG proteases are not common, but IgG-degrading proteases are produced by a variety of human pathogens, such as *Pseudomonas aeruginosa* (14), *Prevotella intermedia*, and *Prevotella nigrescens* (15), as well as by the helminth parasite *Paragonimus westermani* (32).

*Streptococcus pyogenes*, which is a significant human pathogen, causes infections such as impetigo, scarlatina, and pharyngitis. It also causes severe invasive diseases like necrotizing fasciitis and sepsis (9). *S. pyogenes* secretes a number of proteins that potentially can affect the human host. One abundant protein is the well-characterized streptococcal cysteine proteinase SpeB (17). Recently, the IgG-hydrolyzing activity of SpeB, which cleaves human IgG into Fc and monomeric Fab fragments, was described (6). In the same report, a novel extracellular enzyme from *S. pyogenes*, designated EndoS, was identified and characterized. EndoS hydrolyzes the conserved N-linked oligosaccharides on the heavy chains of human IgG. Interestingly, EndoS hydrolyzes only the glycan on native IgG and is the first enzyme from a bacterial pathogen with this unique specificity (5, 6).

Enzymatic activity against oligosaccharide structures on IgG or cleavage in the hinge region could have implications for IgG functionality in response to a streptococcal infection. In this study we showed that two extracellular enzymes from *S. pyogenes*, EndoS and SpeB, alter opsonizing antibodies, resulting in increased bacterial survival in human blood. Thus, expres-
tion of some of these enzymes might help the bacteria evade an attack by the immune system.

MATERIALS AND METHODS

Recombinant expression of EndoS. EndoS was expressed in Escherichia coli by using the glutathione S-transferase (GST) gene fusion system (Amersham-Phar- macia Biotech, Uppsala, Sweden) as described previously (5). A 2,929-bp PCR product including bases 304 to 3232 of the nldS sequence was amplified from S. pyogenes genomic DNA by using primer 5'-ACT-GGG-ATC-CGG-GAG- GAG-AGC-ACT-3' with a BamHI site (underlined) and primer 5'-TTA-ATC- TCC-AAG-GGT-GCT-3' with a Sall site (underlined). This fragment was digested with BamHI and XhoI and ligated into pGEX-5X-3, generating plasmid pGEXEndoS, which was used to transform E. coli BL21(DE3) pLysS. BL21(DE3)pLysSpGEXEndoS was induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside. Cleavage of GST from EndoS with factor Xa and removal of factor Xa were performed as described previously (5).

Spell and recombinant EndoS (rEndoS) treatment of human IgGop. To obtain immune sera, blood samples from healthy individuals were screened for the ability to kill the S. pyogenes API strain. For purification of opsonizing IgG (IgGop), serum was isolated from the volunteers whose blood S. pyogenes did not multiply. The IgG was purified by affinity chromatography by using HiTrap protein G-Sepharose according to the manufacturer’s instructions (Amersham-Phar- macia Biotech). Purified IgG was dialyzed against phosphate-buffered saline (PBS) prior to use.

SpecB was purified from strainAPI cells as described previously (3). The S. pyogenes strain API used in this study is a serotype M1 strain and has been well characterized in terms of expression of cysteine proteinase (7), M1 protein (2), the M-like IgG-binding protein H (1), and IdeS (41). For Spell treatment, 150 µg of purified IgG was incubated for 24 h at 37°C in 120 µl of PBS with 50 µg of SpelB and 0.2 mM dithiothreitol (DTT) or with DTT alone. For rEndoS treat- ment, 200 µg of purified IgG was incubated for 24 h with 10 µg of rEndoS or with PBS alone. SpecB-treated IgG was analyzed by nonreducing sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE), and rEndoS- treated IgG was analyzed by SDS-PAGE or lectin blot analysis with Galanthus nivalis lectin as described previously (6). Briefly, after separation, the IgG was electroblotted onto a polyvinylidene difluoride membrane (Millipore, Bedford, Mass.). The membrane was blocked with Tris-buffered saline containing 0.1% Tween 20 (TBST) and incubated with 0.5 µg of biotinylated G. nivalis lectin (Vector Laboratories, Burlingame, Calif.) per ml. After the membrane was washed in TBST, it was incubated with 1 µg of peroxidase-labeled streptavidin (Vector Laboratories) per ml. The membrane was washed in TBST and was developed by using 1.25 mM 5-amino-2,3-dihydro-1,4-phthalazinedione, 0.22 mM p-coumaric acid, and 0.09% H2O2 (Sigma, St. Louis, Mo.) according to the manufacturer’s instructions (Amersham-Phar- macia Biotech). Sequencing of the wells coated with native IgG or rEndoS-treated IgG. For quantitative analysis of C3 deposition, a reference curve was prepared based on direct adsorption of purified C3 (37) onto the ELISA plates. The C3 concentration in a preparation was determined by rocket electrophoresis (16) with rabbit anti-C3c in the gel and with a commercial calibrator as a reference (human serum protein calibrator code no. X0908; DAKO).

Flow cytometry. The promonocytic cell line U937 was maintained at 37°C in the presence of 5% CO2 in RPMI 1640 medium supplemented with Glutamax-I, 10% heat-inactivated fetal calf serum, 50 U of penicillin per ml, and 50 µg of streptomyacin (Gibco BRL, Bethesda, Md.) per ml. Cells were washed once in Hanks’ balanced salt solution (Gibco BRL), and 106 cells were pelleted in each well of a round-bottom 96-well plate (Becton Dickinson, Franklin Lakes, N.J.). The pellets were resuspended in 100 µl of fluorescence-assisted cell sorting buffer (PBS containing 2% fetal calf serum and 0.05% NaN3) containing 1 µg of untreated IgG per ml or 1 µg of rEndoS-treated IgG per ml, incubated at 20°C for 10 min, and washed once. The cells were then resuspended in 100 µl of fluorescence-assisted cell sorting buffer containing 1 µg of the fluorescein isothiocyanate (FITC)-conjugated F(ab')2 fragment of rabbit anti-human IgG specific for γ-chains (DAKO) per ml, incubated at 20°C for 10 min, and immediately analyzed with a FACS Calibur flow cytometer (Becton Dickin- son). In the experiments in which EndoS-treated IgG was used together with U937 cells, both GST-EndoS-treated IgG and rEndoS-treated IgG were exam- ined. When GST-EndoS-treated IgG was used, excess GST-EndoS was removed by using glutathione-Sepharose and was analyzed by using G. nivalis lectin as described previously (6) before addition to U937 cells. Comparable results were obtained when GST-EndoS was removed by affinity chromatography (see Fig. 3; also data not shown). Thus, we concluded that EndoS per se did not have any effect on U937 cells.

RESULTS

EndoS hydrolysis of IgG impairs killing of bacteria in blood due to diminished FcgRII binding and complement activation. The enzymatic activity of EndoS, which hydrolyzes the β1-4-di-N-acetylglucosamine core of the N-linked glycan of human IgG, during streptococcal growth in whole blood was investi- gated. By using the opsonophagocytic killing assay described by Lancefield (20), the capacity of EndoS to alter the functionality of an opsonizing antibody was examined. In this analysis, polyclonal IgG containing opsonizing antibodies was isolated from a healthy donor. The specificity of the IgGop was compared with that of control IgG for reaction against M1 protein pu- rified from the indicator strain used by immunoblotting. This revealed that the IgGop reacted with M1 protein, while the control IgG from the nonimmune donors bound M1 to a sig- nificantly lesser extent (Fig. 1). The IgGop was treated with
purified rEndoS before addition to whole blood from three different donors lacking opsonizing antibodies against S. pyogenes. Bacteria were inoculated into the whole blood-IgG mixtures, and bacterial survival was monitored over time. rEndoS activity on IgG was confirmed by SDS-PAGE analysis, which showed that the rEndoS-treated IgG migrated as a protein that was approximately 4 kDa smaller than the untreated IgG protein (Fig. 2A). Removal of the oligosaccharide was confirmed by using G. nivalis lectin, which recognizes the α-1,3 mannose residue on the γ-chain (Fig. 2A).

Subsequently, the rEndoS-treated IgG ops were analyzed in the opsonophagocytic killing assay. This assay revealed that after incubation in human blood for 3 h, untreated IgG ops inhibited growth to a significantly greater extent than rEndoS-treated IgG ops inhibited growth. Addition of rEndoS alone without any antibodies did not influence growth (Fig. 2B). These data show that pretreatment of human IgG ops with rEndoS leads to significantly increased bacterial survival, demonstrating the importance of the oligosaccharides for IgG function.

IgG oligosaccharides are important for stability and function of the molecule. To determine if reduced killing of bacteria is due to impaired IgG binding to FcR on leukocytes, rEndoS-treated IgG was analyzed for binding to promonocytic U937 cells. Flow cytometry with FITC-labeled rabbit F(ab′)2 directed against human IgG was used to detect IgG binding to the cells. Three separate experiments revealed that 81.8% ± 2% of the cells incubated with intact IgG were positive for IgG binding, while only 9.1% ± 0.2% of the cells incubated with rEndoS-treated IgG were positive (Fig. 3). This demonstrates that the N-linked oligosaccharides of IgG are important for the interaction with FcR on leukocytes.

Another possible explanation for the impaired opsonizing activity is that oligosaccharides are important for activation of complement through one of its three pathways, the classical, the alternative, and the MBL pathways (40). MBL binds directly to certain carbohydrate structures and has an opsonizing function per se in addition to its capacity to trigger complement activation through the MBL pathway (26). The activation of complement was assessed by comparing the capacities of native IgG and rEndoS-treated IgG to support deposition of serum C3 in an ELISA system. Microtiter plate wells were coated with intact native IgG or rEndoS-treated IgG and were then incubated with serum at 37°C for 30 min. C3 deposition was measured with enzyme-conjugated rabbit anti-C3c. MBL-deficient serum from which C1q, factor D, and properdin had been removed was used as the complement source. Purified complement proteins were added to the serum to differentiate among the complement pathways (Fig. 4). Quite clearly, rEndoS treatment destroyed the capacity of IgG to activate the classical pathway. There was no evidence of MBL pathway activation, and both IgG preparations supported alternative pathway-mediated C3 deposition under the conditions used.

These data suggest that impaired binding of IgG to FcR is a major reason for the reduced ability of rEndoS-treated IgG to support opsonophagocytic killing of the bacteria. In addition,
the loss of classical pathway-activating properties may be expected to severely limit protective functions of the antibodies. Taken together, the data emphasize the role of N-linked oligosaccharides on IgG in opsonization and killing of *S. pyogenes* in blood.

**SpeB-mediated cleavage of purified opsonizing human antibodies results in enhanced growth of *S. pyogenes* in blood.** SpeB, an abundant extracellular protein from *S. pyogenes*, can cleave IgG in a papain-like manner into Fc and monomeric Fab fragments (6). Thus, a preparation of IgG<sub>ops</sub> was incubated with SpeB and analyzed in the opsonophagocytic killing assay as described above. To obtain the highest activity of the enzyme without altering the structure of IgG and its disulfide bonds, the concentration of the reducing agent, DTT, was adjusted to 0.2 mM by titration. Reducing conditions are necessary for activation of SpeB (21). The cleavage of IgG was analyzed by SDS-PAGE under nonreducing conditions. This analysis revealed that SpeB partially cleaved the F<sub>H</sub>253 chains, generating 45- to 55-kDa fragments (Fig. 5A). In contrast, when IgG was incubated with DTT alone, no dissociation of the IgG molecule was detected (Fig. 5A). This suggested that no breakage of the disulfide bonds occurred. The variation in the sizes of the fragments generated was due to the heterogeneity of human IgG. Furthermore, the partial cleavage could be explained by the fact that the IgG2 subclass is relatively resistant to proteolysis by papain (13). This is also true for SpeB; IgG2 is significantly more resistant to proteolysis by this enzyme than the other IgG subclasses are (data not shown).

**DISCUSSION**

The host relies on opsonizing antibodies to trigger protective functions of the immune system. This study revealed two possible strategies that the human pathogen *S. pyogenes* may use to allow blood-borne bacteria to overcome the effects of opsonizing antibodies produced by the host. One strategy is rep-
killing assays performed with SpeB-treated IgG and added SpeB protein. (B) Representative results of opsonophagocytic Fab fragments generated. The arrow indicates the position of the Fc portion, resulting in a damaged IgG molecule. SpeB is the major protein secreted from S. pyogenes, and its role during infection has been shown in several in vivo models (22, 39). Interestingly, SpeB can cleave IgG in the hinge region when bacteria are grown in human plasma (6), suggesting that this also might occur during an infection.

Further support for a strategy in which streptococci attack immunoglobulins was recently obtained when a second secreted cysteine proteinase from S. pyogenes AP1 strain was identified (41). This IgG-degrading enzyme of S. pyogenes, IdeS, specifically cleaves the hinge region of human IgG and IgG subclasses but not other immunoglobulin isotypes. Interestingly, IdeS cleaves IgG at the same position as SpeB, and in vitro experiments revealed that the IdeS activity on opsonizing antibodies inhibits killing of S. pyogenes by phagocytic cells (41). A second strategy involves activity of EndoS that specifically hydrolyzes the conserved N-linked oligosaccharides on IgG. Removal of these oligosaccharides, as suggested by Radaev and Sun (28), most likely changes the structure of the Fc portion from an open conformation to a more closed conformation. This study showed that deglycosylated IgG had a 10- to 15-fold-lower affinity for FcR III than intact IgG had. Thus, EndoS-induced alterations in IgG structure may explain the reduced binding to cells expressing FcR.

EndoS cleaves between two N-acetylglucosamine (GlcNAc) molecules in the chitobiose core of IgG (6). This IgG-degrading enzyme cleaves a single GlcNAc with a fucose attached. Completely deglycosylated IgG binds significantly less efficiently to FcR than IgG containing, for example, the trimannose group (24). Thus, EndoS-mediated cleavage between the two innermost GlcNAc molecules results in decreased FcR binding. This suggests that there is an efficient bacterial strategy to decrease the effector function of IgG.

N-linked oligosaccharides did not appear to be important for the capacity of IgG to activate the alternative complement pathway. On the other hand, it is quite possible that specific opsonizing antibodies do not activate the alternative pathway on the surface of S. pyogenes. Judging from the present data, the MBL pathway does not play any role in IgG-mediated opsonophagocytic killing of the organism (Fig. 4). The functionality of IgG Fc glycosylation has been controversial. A human pathogen that is able to express a highly specific endoglycosidase which results in increased survival in human blood suggests that there is an adaptive feature with which the pathogen modulates a critical host defense molecule. In addition, our study highlights and strengthens the importance of the oligosaccharides in Fc-mediated killing of a microorganism.

In conclusion, we identified two different strategies by which an important human pathogen might interfere with the adaptive immune response. The first strategy is expression of a proteinase that cleaves IgG in the hinge region, and the second involves an IgG-oligosaccharide hydrolase, EndoS. These two activities promote bacterial survival in human blood containing opsonizing antibodies ex vivo. These findings expand our understanding of S. pyogenes pathogenesis and reveal new insights into how this human pathogen might evade immune recognition during infection.

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