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The bacterium *Streptococcus agalactiae* is an etiologic agent in the pathogenesis of endocarditis in humans. FbsA, a fibrinogen-binding protein produced by this pathogen, is considered an important virulence factor. In the present study we provide evidence that *S. agalactiae* clinical isolates bearing FbsA attach to fibrinogen and elicit a fibrinogen-dependent aggregation of platelets. Mutants of *S. agalactiae* lacking the *fbsA* gene lost the ability to attach to fibrinogen and to aggregate platelets. Plasmid-mediated expression of *fbsA* restored the capability for fibrinogen binding and platelet aggregation in *S. agalactiae fbsA* mutants, and allowed *Lactococcus lactis* to interact with fibrinogen and to aggregate human platelets. Moreover, a monoclonal anti-FbsA antibody inhibited bacterial adherence to fibrinogen and *S. agalactiae*-induced platelet aggregation. Platelet aggregation was inhibited by aspirin, prostaglandin E1, the peptide RGDS, and the antibody abciximab, demonstrating the specificity of platelet aggregation by *S. agalactiae* and indicating an involvement of integrin glycoprotein IIb/IIIa in the induction of platelet aggregation. Aggregation was also dependent on anti-FbsA IgG and could be inhibited by an antibody against the platelet FcγRIIa receptor. These findings indicate that FbsA is a crucial factor in *S. agalactiae*-induced platelet aggregation and may therefore play an important role in *S. agalactiae*-induced endocarditis. (Blood. 2005;105:1052-1059)

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**Introduction**

*Streptococcus agalactiae* (group B streptococcus [GBS]) is an important human pathogen that causes pneumonia, sepsis, and meningitis in neonates. It also poses a significant threat to immunocompromised adults in whom it is responsible for cellulitis, arthritis, urinary tract infections, and endocarditis.1,2 Adherence to extracellular matrix components and invasion of pulmonary epithelium may be a prerequisite for infection. In fact, like other pathogens, *S. agalactiae* appears to attach to host extracellular matrix proteins such as fibronectin3,4 and laminin.5 Several studies have also demonstrated the binding of *S. agalactiae* to fibrinogen (Fbg)6,7 and recently, the isolation of the *fbsA* gene has been reported, which encodes an Fbg receptor from *S. agalactiae*.8 The *fbsA* gene encodes a protein that possesses structural similarity to many cell surface-associated proteins from gram-positive bacteria including a typical cell wall attachment region comprising an LPKTG motif, an hydrophobic transmembrane sequence, and an intracytoplasmic C-terminus. In addition, the protein contains a signal sequence for secretion and a domain just outside the cell wall attachment region. Different *S. agalactiae* strains possess various numbers of repetitive units in the FbsA protein, being the basis of size heterogeneity of FbsA.

The repeat region of FbsA was demonstrated to bind to Fbg and to inhibit the attachment of the bacteria to human Fbg. Even a single repeat unit was shown to bind to Fbg and to interfere with the binding of *S. agalactiae* to Fbg. The repeat units of the FbsA are highly similar to each other and contain a consensus sequence for Fbg binding. The consensus motif could not be identified in Fbg-binding proteins from other organisms, indicating that it represents a novel type of Fbg-binding site.9 Numerous Fbg-binding proteins have so far been identified from different bacterial species, in particular staphylococci10-13 and streptococci.14-16 The ability of surface proteins from *Staphylococcus aureus* to bind Fbg is believed to be important in promoting the bacterial adherence to host tissues during an infection and may serve as a mechanism for colonization of the cardiac tissue in infective endocarditis.17,18 In line with this, Fbg-immobilized *S. aureus* or *Streptococcus pyogenes* cells induce aggregation of flowing platelets and thrombus formation.19

Cases of *S. agalactiae*-induced endocarditis in adults have been reported more frequently in recent years and are usually associated with predisposing conditions such as alcoholism and diabetes mellitus although endocarditis caused by *S. agalactiae* in healthy adults has also been described.20-22 *S. agalactiae* strains, possessing Fbg-binding activity, were previously shown to induce platelet aggregation and to be involved in disseminated intravascular coagulation in mice.24 Because crude extracts of *S. agalactiae* strains, exhibiting significant Fbg-binding activity, were able to...
inhibit platelet aggregation, it was suggested that the Fbg-binding activity of S agalactiae participates in platelet aggregation. Subsequent studies revealed that S agalactiae uses a proteinaceous factor for platelet aggregation; however, the molecular basis for this interaction remained unknown. These findings raise the question whether the S agalactiae Fbg receptor, FbsA, is involved in bacterial platelet aggregation and thereby plays a role in S agalactiae-triggered cardiovascular diseases such as endocarditis.

In the present report, we show that S agalactiae strains bearing FbsA attach to Fbg and induce a Fbg-dependent platelet aggregation. In addition, we provide evidence that specific antibodies bound to surface-immobilized FbsA promote platelet aggregation through an independent interaction with the FcγRIIA receptor.

Materials and methods

Proteins

Human Fbg (Calbiochem, San Diego, CA) was made free of contaminating fibronectin by purification over a gelatin-Sepharose column. IgG contaminants from fibronectin-free Fbg were removed by affinity chromatography through a protein G-Sepharose column. The monoclonal antibody (mAb) IV.3 was isolated from the supernatant of an hybridoma clone obtained from the American Type Culture Collection (Manassas, VA). RecPro was generously provided by Dr A. Barattini (Eli Lilly, Indianapolis, IN). RecPro contains abxcinaxab, a Fab fragment of the mAb 7E3 against glycoprotein (GP) IIa/IIIb. IgG from human sera were purified on a protein G-Sepharose column. FbsA-specific antibodies from human IgG were removed by absorption on Sepharose coupled with FbsA. Other laboratory reagents were from Sigma (St Louis, MO).

Bacterial strains and culture conditions

The S agalactiae strains 706 S2 (serotype Ia), 176 H4A (serotype II), 6313 (serotype III), and SS 1169 (serotype V) have been described previously. Strain 7805 (serotype Ib) was kindly provided by G. S. Chhatwal (Department of Microbial Pathogenesis and Vaccine Research, German Research Center for Biotechnology [GBF], Braunschweig, Germany), and strain 1504 (serotype V) was obtained from the National Reference Center for Streptococci in Aachen, Germany. S agalactiae was cultivated at 37°C in Todd-Hewitt broth containing 1% yeast extract. The recombinant S agalactiae clone, carrying the plasmid pOrf23 was selected with erythromycin (5 μg/mL). Lactococcus lactis strain MG1363 was used for heterologous gene expression. Lactococcus lactis strain MG1363 was used for heterologous gene expression. Lactococcus lactis strain MG1363 was used for heterologous gene expression. E. coli BL21 was generously provided by Dr A. Barattini (Eli Lilly, Indianapolis, IN). RecPro contains abxcinaxab, a Fab fragment of the mAb 7E3 against glycoprotein (GP) IIa/IIIb. IgG from human sera were purified on a protein G-Sepharose column.

Construction of fbsA deletion mutants

The fbsA gene was deleted in the chromosome of various S agalactiae strains as previously described by Schubert et al. The promoterless fbsA gene, including its ribosomal binding site, was cloned into the expression vector pOrf23. The construction of plasmid pOrf23 has been described previously. The plasmid pOrf23-derived plasmid was transformed by electroporation into S agalactiae and L lactis with subsequent erythromycin selection. Lactococcus lactis cells were used as host for the production of FbsA fusion proteins. E. coli was grown at 37°C in Luria broth (LB) and clones carrying plasmid pET28a were selected in the presence of kanamycin (50 μg/mL).

Plasmid-mediated expression of fbsA

The hexahistidyl-tagged FbsA fusion protein, carrying 19 internal repeats, was produced and purified as described previously.

Synthesis of the repeated unit of FbsA

A synthetic peptide corresponding to the repeat unit of FbsA (GNVLERRQRDAENRSQ) was synthesized by Primm (San Raffaele Biomedical Science Park, Milan, Italy). The peptide was analyzed by high-performance liquid chromatography (HPLC) using a C18 column and mass spectrometry. During the peptide synthesis a cysteine was added to the C-terminal end of the amino acid sequence that served for the coupling of ovalbumin (OVA) or keyhole limpet hemocyanin (KLH). The peptide was coupled to the carrier protein using sulfoaldehyde isothiocyanate (N-maleimidomethyl)cyclohexane-1-carboxylate (sulfoSMCC; Pierce Chemical, Rockford, IL) following the instructions of the manufacturer.

Generation of mAbs

The mAbs against the synthetic FbsA repeated unit were produced essentially as described by Köhler and Milstein with minor modifications. BALB/c mice were immunized by injection with the repeat unit conjugated with KLH. Hybridoma supernatants were screened for reactivity with the synthetic peptide-OVA conjugate immobilized on microtiter plates and positive clones were further characterized by enzyme-linked immunosorbent assay (ELISA) and Western blot.

Antibody purification and isotyping

The antibodies were purified from supernatants of hybridomas by using ammonium sulfate precipitation, followed by affinity chromatography on protein G-Sepharose column according to the recommendations of the manufacturer (Amersham Pharmacia Biotech, Piscataway, NJ). Isotyping of the produced mAbs was performed using a Mouse-¶ype subclassotyping kit (Bio-Rad, Hercules, CA). The mAb 5H2 was found to belong to subtype IgG1k and the mAb 2B1 to subtype IgG2b-k.

ELISAs

Assays were performed as detailed elsewhere.

Electrophoresis and blotting

Plasma proteins absorbed by and eluted from S agalactiae cells were fractionated on 7.5% polyacrylamide gels and then electroblotted onto a nitrocellulose membrane (Amersham Pharmacia Biotech) as previously reported.

Attachment of S agalactiae strains to Fbg

Microtiter plates were coated overnight at 4°C with 1 μg human purified Fbg in 100 μL 50-mM Na2CO3, pH 9.5. The wells were washed 3 times with phosphate-buffered saline (PBS) and then blocked with 1% bovine serum albumin for 2 hours. Then, 5 × 104 cells of S agalactiae were added to each well and the plates were incubated for 2 hours at 22°C. After extensive washing of the plates, 1 μg rabbit anti–S agalactiae IgG was added to each well, followed by a further incubation for 90 minutes. Subsequently, peroxidase-conjugated goat anti–rabbit IgG was added, and a color reaction developed after the addition of o-phenylenediamine. Absorbance at 490 nm was quantitated in a microplate reader (Bio-Rad).

Platelet preparation

Blood was collected from informed healthy volunteers who had not taken any nonsteroidal anti-inflammatory drugs during the previous 10 days. Nine volumes of blood were added to 1 volume of 3.8% sodium citrate (130 mM citric acid, 152 mM Na citrate, and 112 mM glucose). Platelet-rich plasma (PRP) was obtained by centrifugation of anticoagulated whole blood at 120g for 10 minutes at 22°C. Approval was obtained from the University of Pavia. Informed consent was provided according to the Declaration of Helsinki.
Platelet aggregation

PRP (0.4 mL) was incubated at 37°C in an aggregometer (Chrono-Log, Havertown, PA) under constant stirring and stimulated with 10 μM adenosine diphosphate (ADP) or 50 μl of a S agalactiae 6313 suspension (1 × 10⁹ cells/mL). To examine the effect of various inhibitors of platelet aggregation, PRP was pretreated for 5 minutes with 1 mM Arg-Gly-Asp-Ser (RGDS), the antibody abiciximab (10 μg/mL), 1 μM prostaglandin E₁ (PGE₁), or apyrase (10 U/mL), or for 20 minutes with 1 μM aspirin (ASA) prior to the addition of ADP or S agalactiae 6313 cells.

In experiments with gel-filtered platelets (GFPs), 1 × 10⁹ cells of S agalactiae 6313 were preincubated for 30 minutes in an end-over-end mixer with 1 mL human plasma or physiologic plasma concentrations of purified Fbg (3 mg/mL), fibronectin (300 μg/mL), or albumin (35 mg/mL).

After washing, bacteria were suspended in 1 mL HEPES buffer containing 1.25 mM CaCl₂ and 5.5 mM glucose, pH 7.4. Then, 50 μL of each streptococcal suspension was added to 0.4 mL (4.125 mM CaCl₂ and 5.5 mM glucose, pH 7.4. After washing, bacteria were suspended in 1 mL HEPES buffer containing 1.25 mM CaCl₂ and 5.5 mM glucose, pH 7.4. Then, 50 μL of each streptococcal suspension was added to 0.4 mL (4.125 mM CaCl₂ and 5.5 mM glucose, pH 7.4, and aggregation was monitored in the reaction mixture. The rate and extent of platelet aggregation was detected as the percentage of light transmission and presented as aggregation tracings.

Measurement of cytosolic Ca²⁺ concentration

Cytosolic Ca²⁺ concentration in Fura-2/AM–loaded GFPs was determined as previously described.³⁶ Measurement of Ca²⁺ was performed on 0.4-mL platelet suspensions (2 × 10⁸/mL) prewarmed at 37°C under gentle stirring in a Perkin-Elmer (Norwalk, CT) LS3 spectrofluorometer in the presence of 1 mM CaCl₂. Fura-2 fluorescence signals were calibrated according to the method of Pollock et al.³⁷

Results

S agalactiae cells expressing FbsA selectively absorb Fbg from human plasma

To determine the importance of FbsA in the interaction of S agalactiae with Fbg, the FbsA-expressing S agalactiae strain 6313 and its isogenic mutant 6313 AΔfbsA were tested for their ability to sequester Fbg from human plasma. Bacteria-bound plasma proteins were eluted at low pH, separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under either reducing or nonreducing conditions and subsequently stained with Coomassie (Figure 1A). Separation of bacteria-bound plasma proteins revealed for S agalactiae 6313 the internal sequences of the proteins absorbed by 6313 and its isogenic mutant 6313 AΔfbsA. Platelets were isolated on a 10-mL column of Sepharose Cl-2B that was pre-equilibrated with HEPES buffer. Platelet number was adjusted to 1 × 10⁸ cells/mL with the same buffer.

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In experiments with gel-filtered platelets (GFPs), 1 × 10⁹ cells belonging to different serotypes. After extensive washes, 1 μg rabbit anti–GBS IgG was added to each well, followed by an incubation for 90 minutes. Attachment of the bacteria was quantified by addition of peroxidase-conjugated goat anti-rabbit IgG and the plates were developed with o-phenylenediamine. The bars show SDs of triplicate samples.

FbsA mediates binding of S agalactiae to Fbg

In strain 6313 the FbsA protein represents the major Fbg-receptor of these bacteria.⁸ To test the importance of FbsA for the Fbg-binding of different S agalactiae strains, the fbsA gene was deleted in the genome of 6 clinical S agalactiae isolates, belonging to the medically relevant serologic groups Ia, Ib, II, III, and V, respectively. The parental strains and their isogenic fbsA deletion mutants were subsequently tested for their attachment to immobilized Fbg. As shown in Figure 1C, the various S agalactiae strains differed significantly in their interaction with human Fbg. However, in all of the analyzed strains, the deletion of the fbsA gene reduced the Fbg binding to background levels. For complementation studies, we used the L lactis/Streptococcus expression vector pOrNi23,³⁰ which replicates in high copy number in gram-positive bacteria and possesses the strong promoter P23 from L lactis.⁸

For platelet isolation, PRP was centrifuged at 300g for 10 minutes and resuspended in 0.4 mL 10 mM HEPES (N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) containing 137 mM NaCl, 2.9 mM KCl, 12 mM NaHCO₃, pH 7.4 (HEPES buffer). Platelets were isolated on a 10-mL column of Sepharose Cl-2B that was pre-equilibrated with HEPES buffer.
Vector pOri23 was transformed into *S agalactiae* 6313, and the plasmids pOri23 and pOri/fbsA were introduced into mutants 6313 ΔfbsA. The resultant strains were subsequently tested for their Fbg-binding capability. The presence of the pure vector pOri23 did not influence the binding of the *S agalactiae* strains 6313 and 6313 ΔfbsA to immobilized Fbg, whereas plasmid-mediated expression of fbsA increased the Fbg-binding of strain 6313 ΔfbsA pOri/fbsA to about 80% of wild-type level. These findings show that plasmid-mediated expression of fbsA can restore the Fbg binding of *S agalactiae* 6313 ΔfbsA. Our results also demonstrate that the impaired Fbg binding of the fbsA mutant is caused by its fbsA deficiency and not by unrelated mutations in its chromosome. To test if FbsA alone is sufficient for Fbg binding, we transformed the plasmids pOri23 and pOri/fbsA into *L lactis*, a gram-positive organism that naturally does not interact with human Fbg. Whereas strain *L lactis* pOri23 did not interact with Fbg, strain *L lactis* pOri/fbsA revealed significant binding to Fbg, being in the same magnitude as that of *S agalactiae* 6313 ΔfbsA pOri/fbsA. These results demonstrate that the fbsA gene is sufficient to confer Fbg-binding activity to different gram-positive bacteria.

An mAb against the repeat unit of FbsA blocks adherence of *S agalactiae* to Fbg

A panel of mouse mAbs was generated against a synthetic analog of the repetitive unit of FbsA conjugated with KLH. Two of the generated mAbs, 5H2 and 2B1, were further characterized. As expected, both the mAbs recognized the synthetic peptide or the peptide conjugated to KLH or OVA, whereas no reactivity was detected with KLH or OVA alone or with an unrelated peptide of similar size (data not shown). As shown in Figure 2A, mAbs 5H2 and 2B1 bound to FbsA-coated wells in a concentration-dependent manner and exhibited similar saturation isotherms. The mAbs were examined for their ability to interfere with the binding of biotin-labeled Fbg to immobilized FbsA. The mAb 5H2 substantially inhibited the interaction of FbsA with human Fbg when added to the microtiter wells at a concentration of 2 μg/well (Figure 2B). In contrast, less than 20% inhibition was obtained with up to 7.5 μg/well mAb 2B1. Likewise, in dose-response experiments, 2 μg/well mAb 5H2 inhibited the attachment of *S agalactiae* 6313 to immobilized Fbg by more than 95%, whereas up to 7.5 μg/well of control mAb 2B1 reduced the bacterial adherence by only 15% (Figure 2C). Taken together, these data demonstrate the crucial role of the internal repeat units of FbsA in mediating the attachment of *S agalactiae* to human Fbg.

FbsA plays a critical role in *S agalactiae*-induced platelet aggregation

To investigate the role of FbsA in platelet aggregation by *S agalactiae*, various *S agalactiae* strains and their isogenic fbsA-deletion mutants were tested for their ability to induce platelet aggregation. Typical examples of traces during platelet aggregation are shown in Figure 3A. The streptococcal strains 706 S2, 6313, and 1504 strongly supported platelet aggregation (lag time, 90 seconds). In contrast, strains 7805, SS1169, and 176 H4A revealed significantly longer lag times (4, 10, and 15 minutes, respectively). In general, the ability of these strains to induce platelet aggregation correlates with their capability to interact with human Fbg. However, it is unclear why strains SS1169 and 176 H4A, which exhibit comparable Fbg-binding activity, presented different lag times. The deletion of the fbsA gene completely abolished the ability of all the strains to aggregate platelets, suggesting that FbsA is required by *S agalactiae* to induce platelet aggregation. Moreover, plasmid-mediated expression of fbsA in mutant 6313 ΔfbsA restored its capability to mediate platelet aggregation to about the wild-type level (Figure 3B).

To further investigate the role of FbsA in the aggregation process, *L lactis* pOri23 and *L lactis* pOri/fbsA were tested for their ability to induce platelet aggregation. As shown in Figure 3C, *L lactis* pOri23, carrying the empty vector, did not cause any aggregation of platelets, whereas *L lactis* pOri/fbsA aggregated platelets at levels that were similar to those of *S agalactiae* 6313. The results presented unambiguously demonstrate that FbsA expression is a prerequisite for platelet aggregation by *S agalactiae* cells.

**S agalactiae**-induced platelet aggregation is a genuine aggregation

To demonstrate that the observed aggregation is the effect of a true platelet activation and is thus a genuine aggregation, *S agalactiae*-induced aggregation was performed in the presence of specific platelet activation inhibitors (Figure 4). PGE1 is a substance that elevates the intracellular cyclic adenosine monophosphate (cAMP).
and thereby inhibits platelet aggregation. Preincubation of platelets with PGE₁ completely inhibited *S agalactiae*-induced platelet aggregation, verifying that *S agalactiae* activates platelets prior to aggregation and that this process is thus a genuine aggregation. Aggregation was also inhibited by ASA, a cyclooxygenase inhibitor, suggesting a role of thromboxane A₂ in the aggregation process. However, apyrase (ADPase), an adenosine diphosphatase, failed to inhibit *S agalactiae*-induced aggregation, indicating that aggregation is not dependent on ADP secretion during activation. In contrast, apyrase completely inhibited ADP-induced platelet aggregation. Interestingly, peptide RGDS and the GPIIb/IIIa antibody inhibitor abciximab prevented bacteria-triggered aggregation suggesting that GPIIb/IIIa is involved in *S agalactiae*-induced platelet aggregation. Activation is the prerequisite of platelet aggregation. Among the earliest detectable biochemical responses of platelet activation is cytosolic calcium increase. To clarify whether *S agalactiae* cells drive an increase of intracellular calcium levels, GFPs were loaded with Fura-2 and then incubated with bacteria in the presence or absence of Fbg (Figure 4B). *S agalactiae* preincubated with Fbg induced a significant increase in cytosolic Ca²⁺, whereas no signal was observed with platelets incubated with bacteria or Fbg alone.

**mAb 5H2 interferes with platelet aggregation induced by *S agalactiae***

The mAb 5H2, which blocks the binding of *S agalactiae* to Fbg, was tested for its impact on *S agalactiae*-induced platelet aggregation. As depicted in Figure 5, *S agalactiae*-induced platelet aggregation was significantly blocked by mAb 5H2. In contrast, isotype-matched mAb 2B8, an mAb raised against the *S aureus* protein CNA, did not interfere with bacteria-induced platelet aggregation, suggesting that the inhibition of mAb 5H2 was specific. Moreover, mAb 2B1, which recognizes the internal repeats of FbsA without blocking the attachment of *S agalactiae* to Fbg, did not affect *S agalactiae*-induced platelet aggregation (data not shown).

**Fbg is required for *S agalactiae*-induced platelet aggregation**

To investigate the role of human Fbg in *S agalactiae*-induced platelet aggregation, aggregation experiments were performed with platelets in human plasma and in plasma-free medium. As depicted in Figure 6A, *S agalactiae* aggregated GFPs suspended in plasma, but not in plasma-free medium, suggesting the involvement of a plasma factor in the aggregation process. Because GFPs do not aggregate in the presence of serum (Figure 6A) and Fbg is present in plasma and is the natural ligand for the platelet receptor GPIIb/IIIa, we investigated the role of Fbg in *S agalactiae*-induced platelet aggregation (Figure 6B). As a matter of fact, the addition of Fbg triggered a prompt and specific aggregation of GFP by *S agalactiae*, whereas other plasma proteins such as fibronectin or albumin had no effect on platelet aggregation. Taken together, our
aggregation percent was related to the anti-FbsA antibody titer (data not shown). In the absence of Fbg, mouse IgG did not affect GFP aggregation elicited by \textit{S. agalactiae} and, unlike human immunoglobulins, poorly supported platelet adherence (data not shown), suggesting a low affinity of mouse IgG for human FcγRIIA. Preincubation of GFPs with mAb IV.3 neutralized the IgG-mediated effect on platelet aggregation elicited by \textit{S. agalactiae} (Figure 7C).

Figure 7. Role of specific anti-FbsA antibodies in \textit{S. agalactiae}–induced platelet aggregation. (A) \textit{S. agalactiae} cells (1 ¥ 10^9) were mixed for 1 hour with 3 mg/mL unfractionated Fbg. Fbg from which IgG had been removed, or with 625 μg/mL affinity-purified antibodies. Bacteria were harvested by centrifugation, washed, and adjusted to 1 ¥ 10^9 cells/mL. Then 50 μL of the bacterial suspensions was added to 0.4 mL GFPs (4 ¥ 10^9) in HEPES buffer supplemented with 1.25 mM CaCl_2 and 5.5 mM glucose, pH 7.4, and the suspensions were incubated for 10 minutes with the anti-FcγRIIA antibody IV.3 (20 μg/mL), washed, and adjusted to 1 ¥ 10^9 cells/mL. Then, 50 μL of the bacterial suspension was added to 0.4 mL GFPs (4 ¥ 10^9) and tested for platelet aggregation as reported in Figure 6. To examine the effect of FcγRIIA on platelet aggregation, 0.4 mL GFPs was preincubated for 10 minutes with the anti-FcγRIIA antibody IV.3 (20 μg/mL), mixed with 50 μL of a \textit{S. agalactiae} suspension previously treated with unfractionated Fbg or IgG-free Fbg and then tested for aggregation. (A inset) ELISAs were performed to quantify the level of anti-FbsA antibodies in unfractionated Fbg, IgG-depleted Fbg, or affinity-purified IgG. The assay was performed by incubating FbsA-coated microtiter wells (2 μg/well) with 7.5 μg protein of each fraction followed by the addition of peroxidase-conjugated rabbit antihuman IgG. (B) Cells of \textit{S. agalactiae} (1 ¥ 10^9) were preincubated for 1 hour with 100 μg/mL IgG from an individual with a high anti-FbsA antibody titer (donor no. 19), washed, and adjusted to 1 ¥ 10^9 cells/mL. Then, 50 μL of the bacterial suspension was added to 0.4 mL GFPs (4 ¥ 10^9), previously incubated with or without the anti-FcγRIIA antibody IV.3 according to the conditions described in panel A, and tested for platelet aggregation as reported. The effects of IgG from donor no. 19 depleted of anti-FbsA antibodies and of donor no. 20, who lacks anti-FbsA antibodies on GFP aggregation by \textit{S. agalactiae}, are also reported. All the results are representative of those observed in 3 separate experiments.
To examine the specificity of IgG-mediated platelet aggregation in more detail, IgG from donor no. 19 was passed through an FbsA-Sepharose affinity column. The flow-through, which was depleted of anti-FbsA IgG, did not stimulate platelet-aggregation in the presence of S agalactiae (Figure 7C), suggesting a role of anti-FbsA antibodies in S agalactiae–induced platelet aggregation. Taken together, our findings suggest an involvement of human anti-FbsA IgG and the platelet receptor FcγRIIA in S agalactiae–induced platelet aggregation.

Discussion

In the general interaction between pathogenic bacteria and the host, the ability of microbes to activate and aggregate platelets represents an emerging topic. Platelet binding and aggregation in infective endocarditis pathogenesis was initially described in Streptococcus sanguis and shown to be the effect of a direct interaction between platelet GPIb protein and S sanguis. It also has been reported that Helicobacter pylori, the causative agent of peptic ulcers and gastric carcinoma, induces platelet aggregation via GPIb and that anti- Hp pylori antibodies play a direct role in platelet response. S aureus produces 4 surface proteins that can recognize Fbg, namely, ClfA, ClfB, FnbpA, and FnbpB. Expressing individual determinants such as ClfA in the nonpathogenic bacterium L lactis, O'Brien et al demonstrated multiple mechanisms for the activation of platelets by S aureus and identified redundant, Fbg-dependent roles of ClfA and ClfB in platelet aggregation. A mechanism of platelet aggregation similar to that observed in S aureus has been described in S pyogenes strains expressing the Fbg-binding proteins M1, M3, and M5. Furthermore, if antibodies against bacteria are present in the blood, IgG may bind to surface-expressed bacterial antigens and aggregate platelets through an interaction with FcγRIIA.

In this work, we demonstrate that S agalactiae strains, belonging to different serotypes, promoted platelet aggregation, suggesting that the aggregation process is independent of the S agalactiae serotype. S agalactiae–induced platelet aggregation was inhibited by ASA and by antagonists of integrin GPIIb/IIIa, such as the peptide S agalactiae–induced platelet aggregation was inhibited by ASA and aggregation process is independent of the different serotypes, promoted platelet aggregation, suggesting that the RGDS and the antibody abciximab, indicating that such as ClfA in the nonpathogenic bacterium S pyogenes, as critical inducers of experimental endocarditis in rats. Thus, it is plausible that FbsA as an etiologic agent of platelet aggregation contributes to thrombus formation and consequently to the pathogenesis of S agalactiae-associated endocarditis. Due to the unique and critical role of FbsA in platelet aggregation, the development of anti-FbsA strategies could represent an important achievement for the prevention and treatment of such a life-threatening infective disease.

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