**Group G Streptococcal IgG Binding Molecules FOG and Protein G Have Different Impacts on Opsonization by C1q**

D. Patric Nitsche-Schmitz, Helena M. Johansson, Inka Sastalla, Silvana Reissmann, Inga-Maria Frick, and Gursharan S. Chhatwal

From the ‡Helmholtz Centre for Infection Research, Microbial Pathogenesis, Inhoffenstrasse 7, D-38124 Braunschweig, Germany and §Section for Clinical and Experimental Infection Medicine, Department of Clinical Sciences, Biomedical Center, Lund University, S-22184 Lund, Sweden

Recent epidemiological data on diseases caused by β-hemolytic streptococci belonging to Lancefield group C and G (GCS, GGS) underline that they are an emerging threat to human health. Among various virulence factors expressed by GCS and GGS isolates from human infections, M and M-like proteins are considered important because of their anti-phagocytic activity. In addition, protein G has been implicated in the accumulation of IgG on the bacterial surface through non-immune binding. The function of this interaction, however, is still unknown.

Using isogenic mutants lacking protein G or the M-like protein FOG (group G streptococci), respectively, we could show that FOG contributes substantially to IgG binding. A detailed characterization of the interaction between IgG and FOG revealed its ability to bind the Fc region of human IgG and its binding to the subclasses IgG1, IgG2, and IgG4. FOG was also found to bind IgG of several animal species. Surface plasmon resonance measurements indicate a high affinity to human IgG with a dissociation constant of 2.4 pM. The binding site was localized in a central motif of FOG.

It has long been speculated about anti-opsonic functions of streptococcal Fc-binding proteins. The presented data for the first time provide evidence and, furthermore, indicate functional differences between protein G and FOG. By obstructing the interaction between IgG and C1q, protein G prevented recognition by the classical pathway of the complement system. In contrast, IgG that was bound to FOG remained capable of binding C1q, an effect that may have important consequences in the pathogenesis of GGS infections.

Streptococci of the Lancefield group G and C (GGS2 and GCS) have long been considered as animal pathogens, being a minor threat to human carriers. Carriage of GCS and GGS in the throat and on the skin is often asymptomatic. However, reports from different parts of the world are accumulating demonstrating that they are emerging as important human pathogens (1–4). Individual processes contributing to the immune defense against GGS are still elusive, and the knowledge about how GGS undermine and obstruct the actions of the immune system is fragmentary. Before the development of a specific immune response, the innate immune system constitutes a first line of defense against intruding bacteria. Part of the innate immune defense is the complement system that has two major functions. One is to kill bacteria by forming lytic complexes on the bacterial membrane, referred to as membrane attack complex. The other one is to facilitate the actions of the cellular immune response. The complement cascade produces anaphylatoxins to attract phagocytes; other complement reactions opsonize the bacteria, promoting extracellular bactericidal actions and phagocytosis (5–8).

Bacteria have evolved diverse protective mechanisms to prevent the actions of the complement system. The membrane attack complex is thought to be inefficient in killing Gram-positive bacteria due to their resistant cell wall (9). C5a-peptide of *Streptococcus pyogenes*, which has a homologue in GGS, cleaves and thereby inactivates a potent anaphylatoxin (10, 11). M proteins of *S. pyogenes* prevent opsonization by C4b by recruiting plasma proteins like fibrinogen or C4b-binding protein. A recent study suggests that fibrinogen bound to M protein interferes with the deposition of C4b2a, the C3 convertase of the classical pathway, on the bacterial surface (12). A recently defined fibrinogen-binding M-like protein of GGS referred to as FOG was shown to exert anti-phagocytic activity on neutrophils by forming fibrinogen aggregates (13). The complement cascade can be initiated by three different pathways; that is, the classical, the lectin, or the alternative pathway. A triggering step of the classical pathway is the binding of factor C1q to aggregated or antigen-bound IgG. Factor C1q together with the serine proteases C1r and C1s forms the C1 complex. The interaction with IgG induces conformational changes in C1q that lead to the activation of the serine proteases of the C1 complex. This sets off the classical pathway by cleaving C2 and C4, thus leading to the formation of opsonins and anaphylatoxins (5). Moreover, C1q itself acts as an opsonin, eliciting antibacterial actions of phagocytes (7, 8, 14).

GCS and GGS accumulate IgG on their surface by non-immune binding. A major protein that is responsible for the high capacity accumulation of IgG on GGS is protein G (15). Here we demonstrate for the first time that FOG also contributes in
accumulating human IgG on the GGS surface and present a detailed characterization of the interaction between IgG and FOG. The influence of the IgG accumulations on recognition by human C1q was investigated. The data revealed an anti-opsonic function of protein G. In contrast to protein G, FOG did not interfere with the interaction between IgG and C1q. The results suggest opposed functions of the IgG interactions of FOG and of protein G in human infections.

**EXPERIMENTAL PROCEDURES**

**Growth Conditions and Bacterial Strains**—Strains G41 and G148 are throat isolates collected at the Department of Clinical Microbiology, Lund University Hospital, Sweden. Strain G45 was collected at the Royal Brisbane Hospital, Australia. G41 and G45 carry protein FOG on their surface, and G148 naturally lacks protein FOG. *Streptococci* were routinely grown in Todd-Hewitt (Difco) liquid medium in 5% CO₂ at 37 °C and then incubated with radiolabeled FOG (200,000 cpm) in PBS containing 0.05% Tween 20, and bound ligand was determined by incubation for 3 h. Membranes were washed 3 × 20 min in PBS containing 0.05% Tween 20, and bound ligand was detected using the Fuji Imaging System.

**Slot Blots**—Protein preparations were applied to polyvinylidene difluoride membranes using the MilliBlot-D system. Membranes were blocked in PBS containing 1% bovine serum albumin and 0.25% Tween 20 (block buffer) for 3 × 20 min at 22 °C and then incubated with radiolabeled FOG (200,000 cpm/ml block buffer) for 3 h. Membranes were washed 3 × 20 min in PBS containing 0.05% Tween 20, and bound ligand was detected using the Fuji Imaging System.

**Surface Plasmon Resonance (SPR) Measurements**—Protein interactions were studied in a BIAcore 2000 system (BIAcore AB) using 10 mM HEPES, 100 mM NaCl, pH 7.4, as the running buffer. A CM5 sensor chip was activated by a 4-min injection of 0.05 M n-hydroxyisoucuminide, 0.2 M N-ethyl-N-(3-dimethylaminopropyl)-carbodiimide hydrochloride in water. Human IgG (11 mg/ml; Jackson ImmunoResearch) was diluted 1:1000 in 10 mM sodium acetate, pH 4.5. Injection of 25 µl at a flow rate of 5 µl/min led to immobilization of 1000 response units of human IgG. Residual reactive groups were inactivated by a 6-min injection of 1 M ethanolamine, 0.1 M NaHCO₃, 0.5 M NaCl, 5 mM EDTA, pH 8.0. Interaction measurements were carried out at a flow rate of 60 µl/min. Surface regeneration was achieved by injection of two 30-s pulses of 0.2% SDS in water. The BIAevaluation 3.0 software was used for further analysis of the data. Shown curves represent the difference between the signal of the IgG-coupled surface and of a deactivated control surface devoid of protein. They were further corrected by subtraction of the curve that was obtained after injection of running buffer alone. Buffer injection led to responses less than five response units.

**Construction of the FOG Deletion and the Protein G Deletion Mutants**—Isolation of chromosomal DNA from *Streptococcus dysgalactiae* subsp. *equisimilis* strain G45 was performed using the BIORAD Instagene Matrix solution as described by the manufacturer. All restriction and modifying enzymes were purchased from New England Biolabs. The sequence of the *fog* gene was derived from accession number AF600861 and served as basis for the selection of *fog*-specific oligonucleotides. The sequence of the *protG* gene was derived from accession number X06173. For construction of the FOG deletion mutant, a 697-bp internal fragment of *fog* was amplified by PCR using oligonucleotides *Fog*_mut1 (GCGGAGAATACATACGATAAAAATTCGGACCA-3′ (BamHI B2), 5′-GCGGATCCGAAGCTG-AAAAGGCTAAAGAAGG-3′ (BamHI S), and 5′-GCGGATCCCGTCTATTATGCTTTGTGCTGCAACAC-3′(BamHI aa305). The sequences of non-coding strand primers were 5′-GCGGATCCCGTCTATTATGCTTTGTGCTGCAACAC-3′ (BamHI S), and 5′-GCGGATCCCGTCTATTATGCTTTGTGCTGCAACAC-3′ (BamHI aa305). The sequences of coding strand primers were 5′-GCGGATCCCGTCTATTATGCTTTGTGCTGCAACAC-3′ (BamHI S), and 5′-GCGGATCCCGTCTATTATGCTTTGTGCTGCAACAC-3′ (BamHI aa305). The sequence of the *aad9* gene was derived from accession number M96221, oligonucleotides *Sp*c1 *Mun*l_overlap (ccggccaatcggggAAATCAGATGAACACGTGCGGAC) and *aad9* were ligated into the cloning vector pCR2.1 (Invitrogen) generating vector-harboring restriction sites BamHI and XhoI, resulting in vector harboring restriction sites BamHI and XhoI, resulting in vector harboring restriction sites BamHI and XhoI, resulting in vector harboring restriction sites BamHI and XhoI, resulting in vector harboring restriction sites BamHI and XhoI, generating pCR2.1-fog-aad9 and pCR2.1-protG-aad9. The *aad9* was subsequently ligated into the thermosensitive shuttle vector pRS233 (17) using vector-harboring restriction sites BamHI and XhoI, resulting in vector pISA14 and vector pIF2, respectively. *E. coli* DH5α clones carrying the recombinant plasmids were selected on spectino- mycin as indicated above. Plasmid isolations were performed using the Qiagen Miniprep kit according to the manufacturer's recommendations.

The plasmids pISA14 and pIF2 were introduced into competent G45 bacteria by electroporation as described by McLaughlin and Ferretti (18). After selection of erythromycin-resistant GGS at 30 °C and verification of the presence of the plasmid by PCR using vector-priming oligonucleotides M13 forward and M13 reverse, a temperature shift to 37 °C led to transient inte-
Functional Differences between IgG-binding Proteins

Intracellular plasmids of bacteria/ml (wet weight/volume). A

PBS containing 0.05% Tween 20. Binding of radiolabeled IgG

bound to human IgG (Fig. 2

IgG binding to immobilized human, equine, murine, monkey, guinea pig, porcine, and rabbit IgG (Table 1). In contrast to protein G, which is

SDS-PAGE, the samples were neutralized by the addition of 1.5 mM Tris-HCl, pH 8.8.

SDS-Polyacrylamide Gel Electrophoresis and Immuno-

Mapping of Binding Regions on IgG Coupled to Immunebeads—

B

C

S

D

A

Figure 1. Recombinant FOG constructs and mapping of the IgG binding region of FOG. The modular composition of FOG is shown in a schematic representation of the mature protein at the top. Positions of the A, B, and C repeats of the non-repetitive S-region and of the D-domain are indicated. Recombinant FOG and the FOG fragments that were used to map the IgG binding region are depicted below together with their position in the mature full-length protein. The designations of shorter fragments and their ability to bind human IgG in ligand blots are given on the right. The results on IgG binding are concordant with the data of surface plasmon resonance measurements shown in Fig. 3. The proteins possessed an N-terminal glutathione S-transferase tag.

OG was shown to be a major surface protein that is common in clinical isolates of GGS (13, 22). Recombinant FOG showed affinity for the immunoglobulin IgG (13), but the interaction is virtually uncharacterized. The primary structure of FOG does not contain any sequences with significant homology to the IgG binding motif of protein G, the major IgG binding molecule on the surface of GGS (15). To allow a first comparison between these IgG-binding proteins, recombinant FOG that represents the mature protein after sortase cleavage (amino acids (aa) 1–557, Fig. 1) was tested for binding to IgG of different animal species (Table 1), to different subclasses of human IgG (Fig. 2A) and its papain fragments Fc and Fab (Fig. 2B). Radiolabeled FOG bound to immobilized human, equine, murine, monkey, guinea pig, porcine, and rabbit IgG in ligand blot experiments. A low but considerable signal was detected for canine IgG, but no binding was detectable for caprine, ovine, rat, or bovine IgG (Table 1). In contrast to protein G, which is known to bind all the four subclasses of human IgG (15), FOG bound to human IgG subclasses IgG1, IgG2, and IgG4 but not IgG3 (Fig. 2A). As opposed to a clear signal for binding to Fc fragment, the signal obtained for Fab fragment was faint (Fig. 2B). To further characterize the interaction with IgG, FOG was examined in SPR measurements for binding to immobilized human IgG, revealing a dissociation constant of 2.4 pm (Fig. 3A); thus, an interaction of high affinity.

Localization of the IgG Binding Region of FOG—It was described previously that a truncated form of FOG (aa 1–278 of the mature protein), which was referred to as FOG1-B, did not bind IgG, indicating that the C-terminal part (aa 279–578) of the bacterial protein is crucial for the interaction with the
immunoglobulin (13). To localize the responsible IgG binding region, suitable recombinant FOG fragments were expressed as glutathione S-transferase fusion proteins and tested in ligand blot experiments (Fig. 1) and SPR measurements (Fig. 3, B–G) for binding to human IgG. Both approaches gave identical results. In control experiments glutathione S-transferase did not show any binding to IgG either in ligand blots (data not shown) or in SPR measurements (Fig. 3H). In accordance with previous experiments (13), FOG1-B did not bind IgG (Fig. 3G). The minimal motif tested positive for IgG binding (aa 233–339) was the S-region of FOG (Fig. 3E). Truncation after amino acid 304 (B2-aa304) abolished the interaction completely (Fig. 3F). Constructs that started with amino acid 305 (aa305-S and aa305-C1) were also not capable of binding IgG (Fig. 1). For the interaction between the S-region and IgG, a dissociation constant of 890 pM was determined by SPR measurements. How-ever, when the FOG fragment comprised the S-region together with flanking regions like the B2 domain (B2-S, Fig. 3D), the C-repeats C1 and C2 (S-C2; Fig. 3C), or both the B2 and the C-repeats (B2-C2; Fig. 3B), its affinity for IgG was significantly increased. This was judged by the dissociation constants measured by SPR of 610, 480, and 220 pm, respectively. These data imply that the flanking regions contributed to the structural integrity of the binding motif that was localized in the S-region of FOG.

FOG Accumulates IgG on the Bacterial Surface—To test whether FOG contributes to binding IgG to the bacterial surface, the fog gene of strain G45 was interrupted to generate a FOG-deficient mutant (G45ΔFOG). Insertion of the destructive DNA element was tested by Southern blot, and in contrast to lysates of the wild type strain, lysates of the mutant did not contain the protein FOG (results not shown). Binding of fibrinogen was abolished in G45ΔFOG (Fig. 4A), corroborating previous data about FOG-mediated fibrinogen binding to GGS (13). Additionally, by disrupting the prtG gene, a protein G-deficient mutant (G45ΔproteinG) was generated. When binding of radiolabeled human IgG to whole bacteria was examined (Fig. 4B), the wild type strain had the highest capacity for IgG. It bound 69–80% of the labeled IgG. Strain G45ΔFOG had a clearly decreased capacity but was capable of binding more than 57% of the IgG even at the lowest bacterial concentration tested. Strain G45ΔproteinG had even lower capacity for IgG, but still, considerable binding of the immunoglobulin was measured (25–65%). The results indicate that FOG, like protein G, contributes substantially in accumulating IgG on the GGS surface.

Influence of Fc-binding GGS Proteins on the Interaction between IgG and the C1 Complex—To gain insight into the biological functions of the Fc-binding proteins of GGS, their interaction with the triggering component of the classical pathway of the complement system, the C1 complex, was examined. Because both FOG and protein G bind IgG to the surface of GGS (Fig. 4B) by interacting with its Fc-region, it was interesting to test if such surface-bound IgG is recognized by factor C1q or whether FOG or protein G interferes with C1q binding. To test binding of human C1q to whole bacteria, GGS isolates that were known to carry FOG and protein G (G41 and G45) or
protein G only (G148) were chosen. When comparative absorption experiments were performed, the FOG-positive strains G41 (Fig. 5) and G45 (see Fig. 7A), but not the FOG-negative strain G148 (Fig. 5), bound C1q from non-immune human blood plasma. The three subunits of C1q (25–30 kDa) were resolved and detected in Western blot using an antiserum specific against human C1q.

To dissect the system of occurring interactions, isolated C1q was added to the bacteria with or without preincubation with human IgG (Fig. 6A). Neither FOG-positive bacteria (G41) nor FOG-negative bacteria (G148) bound isolated C1q, indicating that direct binding of C1q to the GGS surface is of negligible magnitude. After preincubation, both GGS strains accumulated similar amounts of IgG, but only the FOG-positive strain G41 bound C1q. Experiments with the Fc fragment of IgG paralleled those results, demonstrating that a non-immune interaction with Fc-binding proteins is involved for the C1q binding (Fig. 6B, for Fc binding see also Fig. 2B). The quantity of bound C1q, however, was considerably lower, so that only the subunit with the strongest signal was detected in the Western blot.

To test FOG-dependent C1q binding in the same genetic background, experiments were carried out with strain G45 and its FOG-deficient mutant (G45/H9004FOG). Incubated with nonimmune plasma, G45 but not G45/H9004FOG bound C1q on its surface, unambiguously identifying FOG as the responsible protein (Fig. 7A). Consecutive incubation with IgG and C1q verified that the FOG-mediated C1q binding was IgG-dependent (Fig. 7A).

Moreover, the protein G-deficient mutant (G45/H9004protein G) retained binding of C1q to the bacterial surface, further emphasizing protein FOG as the responsible protein (Fig. 7B).

On the surface of G148 as well as on the FOG-deficient mutant G45ΔFOG, the major IgG binding molecule is protein G. The experiments, thus, demonstrate that binding of protein G to IgG efficiently prevents opsonization by C1q. On the sur-
face of G41 and G45, however, a second population of IgG, bound via FOG, was present. This population was capable of binding C1q. Competition experiments with IgG coupled to immunobeads demonstrated that binding of radiolabeled FOG could be inhibited by the addition of protein G (Fig. 8A). This indicates an overlap in the targeted binding motifs on IgG and suggests that simultaneous binding of FOG and protein G to one heavy chain of IgG did not occur. In summary, the experiments demonstrate that FOG facilitates an IgG-dependent binding of C1q on GGS under non-immune conditions and even in the presence of other plasma proteins (e.g., fibrinogen), whereas protein G counteracts opsonization by the first component of the classical pathway, the C1 complex. We, therefore, propose the model depicted in Fig. 8B.

### DISCUSSION

Binding of immunoglobulins is a key event in immune defense being crucial for the development of humoral immune response and contributing to innate immunity. Such host responses greatly depend on antigen binding. Streptococci have evolved a variety of proteins for non-immune binding of IgG. The role of these interactions is thought to be inactivation of IgG, but the consequences of such binding are widely understudied. On the bacterial surface non-immune binding of immunoglobulins and the mode of binding may have an important influence on the recognition by Fc receptors on phagocytes as well as on recognition by the first step of the classical pathway of the complement system, the C1 complex. The latter point has been addressed in this study. One major IgG-binding protein of GGS is protein G (15). Because it has been shown that soluble protein G does not interfere with the binding of sub-component C1q to IgG (23), a role of protein G in protection against the classical pathway was arguable. However, the inter-
Functional Differences between IgG-binding Proteins

actions in solution may not necessarily represent those occurring at the streptococcal surface where protein G is arrayed on the cell wall of GGS with a defined orientation and spacing and where protein G is present as a full-length protein. Also, it cannot be excluded that co-factors on the bacterial surface contribute to the ability of protein G to arrest IgG. Comparing protein G and FOG-expressing strains (G41, G45) with strains deficient for FOG (G148, G45AFOG) revealed that the IgG population attached to the GGS surface by non-immune binding to protein G does not promote or even allow considerable binding of C1q to the bacterial surface. Although contribution of hitherto unknown co-factors cannot be excluded, the presented results clearly indicate that interaction with protein G abolishes the capability of IgG to bind C1q. We, therefore, conclude that they clearly indicate that interaction with protein G abolishes the capability of IgG to bind C1q. We, therefore, conclude that they

The role of protein G is to act as an anti-opsonin that blocks an important interaction of the innate immune system. Protein FOG contributes to accumulating IgG molecules on the surface of GGS but does not interfere with the interaction between IgG and C1. A potential initiation of the classical pathway due to FOG may not be a threat for the bacteria since FOG is a potent anti-phagocytic factor (13). Production of anaphylatoxins due to a triggered complement cascade and possible induction of inflammatory responses, however, may play a crucial role in the pathogenesis of GGS infections.

Acknowledgments—We are indebted to Lars Björck and Dick Heinegård for valuable and encouraging discussions. We are grateful to Bianka Karge and Ulla Johannesson for expert technical assistance.

REFERENCES

1. Cohen-Poradosu, R., Jaffe, J., Lavi, D., Grisariu-Greenzaid, S., Nir-Paz, R., Valinsky, L., Dan-Goor, M., Block, C., Beall, B., and Moses, A. E. (2004) Emerg. Infect. Dis. 10, 1453–1460
2. Nohlgard, C., Björklind, A., and Hammar, H. (1992) Acta Derm. Venereol. 72, 128–130
3. Sylvestry, N., Raveh, D., Schlesinger, Y., Rudensky, B., and Yinnon, A. M. (2002) Am. J. Med. 112, 622–626
4. Mathur, P., Kapil, A., and Das, B. (2004) Indian J. Med. Res. 120, 199–200
5. Walport, M. J. (2001) N. Engl. J. Med. 344, 1058–1066
6. Walport, M. J. (2001) N. Engl. J. Med. 344, 1140–1144
7. Tenner, A. J., and Cooper, N. R. (1982) J. Immunol. 128, 2547–2552
8. Bobak, D. A., Gaither, T. A., Frank, M. M., and Tenner, A. J. (1987) J. Immunol. 138, 1150–1156
9. Joiner, K., Brown, E., Hammer, C., Warren, K., and Frank, M. (1983) J. Immunol. 130, 845–849
10. Wexler, D. E., Chenoweth, D. E., and Cleary, P. P. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8144–8148
11. Cleary, P. P., Peterson, J., Chen, C., and Nelson, C. (1991) Infect. Immun. 59, 2305–2310
12. Carlsson, F., Sandin, C., and Lindahl, G. (2005) Mol. Microbiol. 56, 28–39
13. Johansson, H. M., Mörgelin, M., and Frick, I. M. (2004) Microbiology 150, 4211–4221
14. Ishihara, U., and Reid, K. B. (2000) Immunoopharmacology 49, 159–170
15. Björck, L., and Kronvall, G. (1984) J. Immunol. 133, 969–974
16. Horton, R. M., Cai, Z. L., Ho, S. N., and Pease, L. R. (1990) Biotechniques 8, 528–535
17. Perez-Casal, J., Okada, N., Caparon, M. G., and Scott, J. R. (1995) Mol. Microbiol. 15, 907–916
18. McLaughlin, R. E., and Ferretti, J. J. (1995) Methods Mol. Biol. 47, 185–193
19. Dinkla, K., Rohde, M., Jansen, W. T., Carapetis, J. R., Chhatwal, G. S., and Talay, S. R. (2003) Mol. Microbiol. 47, 861–869
20. Laemmli, U. K. (1970) Nature 227, 680–685
21. Frick, I. M., Wikström, M., Forsén, S., Drakenberg, T., Gomi, H., Sjöbring, U., and Björck, L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 8532–8536
22. Nitsche, D. P., Johannson, H. M., Frick, I. M., and Mörgelin, M. (2006) J. Biol. Chem. 281, 1670–1679
23. Bergo, A., Kihlberg, B. M., Sjöholm, A. G., and Björck, L. (1997) J. Biol. Chem. 272, 20774–20781
24. Stone, G. C., Sjöbring, U., Björck, L., Sjöquist, J., Barber, C. V., and Nordella, F. A. (1989) J. Immunol. 143, 565–570
25. Reis, K. J., Ayoub, E. M., and Boyle, M. D. (1984) J. Immunol. 132, 3998–3102
26. Åkerström, B., and Björck, L. (1986) J. Biol. Chem. 261, 10240–10247
27. Duncan, A. R., and Winter, G. (1988) Nature 332, 738–740
28. Simpson, W. J., Robbins, J. C., and Cleary, P. P. (1987) Microb. Pathog. 3, 339–350