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**Haemophilus influenzae** Interacts with the Human Complement Inhibitor Factor H

Teresia Hallström,* Peter F. Zipfel,‡ Anna M. Blom,† Nadine Lauer,‡ Arne Forsgren,* and Kristian Riesbeck‡‡

Pathogenic microbes acquire human complement inhibitors to circumvent the innate immune system. In this study, we identify two novel host-pathogen interactions, factor H (FH) and factor H-like protein 1 (FHL-1), the inhibitors of the alternative pathway that binds to Hib. A collection of clinical *Haemophilus influenzae* isolates was tested and the majority of encapsulated and unencapsulated bound FH. The isolate Hib 541 with a particularly high FH-binding was selected for detailed analysis. An increased survival in normal human serum was observed with Hib 541 as compared with the low FH-binding Hib 568. Interestingly, two binding domains were identified within FH; one binding site common to both FH and FHL-1 was located in the N-terminal short consensus repeat domains 6–7, whereas the other, specific for FH, was located in the C-terminal short consensus repeat domains 18–20. Importantly, both FH and FHL-1, when bound to the surface of Hib 541, retained cofactor activity as determined by analysis of C3b degradation. Two *H. influenzae* outer membrane proteins of ~32 and 40 kDa were detected with radiolabeled FH in Far Western blot. Taken together, in addition to interactions with the classical, lectin, and terminal pathways, *H. influenzae* interferes with the alternative complement activation pathway by binding FH and FHL-1, and thereby reducing the complement-mediated bactericidal activity resulting in an increased survival. In contrast to incubation with active complement, *H. influenzae* had a reduced survival in FH-depleted human serum, thus demonstrating that FH mediates a protective role at the bacterial surface. *The Journal of Immunology*, 2008, 181: 537–545.

**Haemophilus influenzae** is a Gram-negative human pathogen responsible for both local respiratory tract infections and serious invasive disease. Encapsulated *H. influenzae* belong to one of six serotypes (a-i), of which type b (Hib) is the most virulent (1, 2). The most serious and sometimes life-threatening conditions caused by encapsulated *H. influenzae* are septicaemia, meningitis, and epiglottitis (3). In contrast, nontypeable *Haemophilus influenzae* (NTHi), which lacks a capsule, commonly causes local disease in the upper and lower respiratory tract (e.g., bronchitis, sinusitis, and otitis media). NTHi is after pneumonia the second most common pathogen isolated from children with acute otitis media (1, 2). In addition, NTHi is also the main cause of acute exacerbations in patients with chronic obstructive pulmonary disease and bronchiectasis (4–6).

The polysaccharide capsule of the typeable *H. influenzae* is an important virulence factor that protects against complement attack and phagocytosis (7, 8). The capsule consists of several compounds of which lipooligosaccharide is important for colonization, bacterial persistence, and survival in the circulatory system (9, 10). Fimbriae and outer membrane proteins (OMPs) are other important virulence determinants. *Haemophilus* surface fibrils (Hsf), *Haemophilus influenzae* adhesin, hemagglutinating pili, *Haemophilus* adhesion and penetration protein, and high m.w. proteins 1 and 2 are major adhesins involved in colonization identified in Hib and/or NTHi (11, 12).

The complement system is the first line of defense against pathogenic microorganisms, and activation of the cascade leads to C3 deposition on the bacterial surface. Opsonization of the pathogen is followed by phagocytosis and formation of the membrane attack complex (MAC) (13). The classical pathway of the complement system is activated by target bound Abs and C-reactive protein (14), whereas the alternative pathway is spontaneously activated through direct contact with foreign particles or cells (15). Both pathways lead to the formation of C3 convertase, with subsequent cleavage of C3 to C3a and C3b. Thereafter the terminal pathway is activated, which is a key step in producing an inflammatory response. To prevent nonspecific damage from excess complement activation, the complement cascade is tightly regulated. Important regulators of the complement system are factor H (FH) and factor H-like protein-1 (FHL-1) (alternative pathway) (16), C4BP (C4b-binding protein) (classical/lectin pathway) (17), and vitronectin (terminal pathway) (18).

FH is a 150-kDa fluid phase protein that regulates the alternative pathway of the complement system (19). It is a glycoprotein in human plasma composed of 20 repetitive units of 60 aa, which is designated complement control protein domains or short consensus repeats (SCRs) (20). Another regulator of the alternative pathway is the FHL-1, which is a product of alternative splicing of the FH gene (21). FHL-1 is a 42-kDa protein composed of seven SCRs identical with the N-terminal SCRs of FH and includes an

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*Abbreviations used in this paper: Hib, *Haemophilus influenzae* type b; NTHi, nontypeable *Haemophilus influenzae*; OMP, outer membrane protein; Hsf, *Haemophilus* surface fibrils; MAC, membrane attack complex; FH, factor H; FHL-1, FH-like protein-1; C4BP, C4b-binding protein; SCR, short consensus repeats; BHI, brain heart infusion; RT, room temperature; pAb, polyclonal Ab.

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The pathogenesis of many microbial pathogens relies on the capacity to avoid, resist, or neutralize the complement system to promote survival in the host. Pathogens have evolved multiple mechanisms to conquer the complement cascade. Some pathogens destroy host complement activation components, whereas others mimic the complement inhibitors (22, 23). Another strategy used by some pathogens is binding of host complement inhibitors such as FH and/or FHL-1. In fact, a number of microorganisms, including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Borrelia burgdorferi*, *Neisseria meningitidis*, and *Candida albicans* have been reported to bind FH and FHL-1 (24–28). When these inhibitors are bound to the microbial surfaces, they maintain their complement regulatory activity and protect the pathogens against direct lysis. In general, the binding of FH contributes to serum resistance and prevention of opsonophagocytosis of the bacteria (29). The exact mechanism how *H. influenzae* evade host complement attack is unclear, but in a previous study we showed that NTHi binds C4BP and that this significantly contributed to bacterial serum resistance (30). In addition, we have recently reported that Hsf, which is expressed by encapsulated *H. influenzae*, is involved in serum resistance (31). Hsf-expressing *H. influenzae* binds vitronectin, an important regulator that inhibits the MAC formation in the terminal pathway of the complement system, and thereby prevents complement-induced lysis resulting in serum resistance.

The aim of the present study was to examine the complement resistance mechanisms of *H. influenzae* with focus on the alternative pathway. Most of the clinical Hib strains tested bound the complement inhibitor FH, and some of them bound FHL-1. The binding site of the model strain Hib 541 was located to SCRs 6–7 of FH and FHL-1, and to SCRs 18–20 that were unique for FH. Functional analyses revealed that both FH and FHL-1 maintain cofactor activity when bound to the surface of the bacterial cell. Furthermore, when Hib 541 was exposed to FH-depleted serum a significantly decreased survival was seen. Most importantly, the high FH-binding strain was more resistant to serum-mediated killing as compared with the low FH-binding strain.

**Materials and Methods**

**Bacterial strains and culture conditions**

Typeable *H. influenzae* (*n* = 24), non-typeable *H. influenzae* (*n* = 16), and *Escherichia coli* were clinical isolates obtained from Medical Microbiology (Malmo University Hospital, Lund University) and the State Serum Institute. Type strains were obtained from the Culture Collection University of Gothenburg (Department of Clinical Microbiology, Sahlgrenska Hospital). The clinical capsule-deficient *H. influenzae* isolate RM804 has been described in detail (32, 33). All strains were characterized by standard bacteriological techniques including oxidase, fermentation, satellite, and XV tests. Thereafter, the *H. influenzae* strains were typed by PCR using specific primers for each capsular type. Bacteria were routinely cultured in brain heart infusion (BHI) liquid broth supplemented with NAD and hemin (both at 10 μg/ml) or on chocolate agar plates at 37°C in a humid atmosphere containing 5% CO₂.

**Proteins and Abs**

Generation of the human FH-specific mAbs, mAb B22 against SCR5, mAb E14 against SCR20, and mAb C18 against SCR20, has been described previously (34). The HRP-conjugated donkey anti-goat polyclonal Ab (pAb) was obtained from Serotec. A polyclonal goat anti-human FH anti-serum was used for the detection of different regions in FH and was purchased from Calbiochem or Serotec. Human FH was from CompTech or purified from human plasma (35). The FITC-conjugated goat anti-rabbit pAb was obtained from Dakopatts. Recombinant fragments of FH (SCRs 1–5, 1–6, 8–11, 8–20, 15–20, 18–20, and 19–20) and FHL-1 (SCRs 1–7) were expressed in the baculovirus system as described (36). Briefly, *Sodoptera frugiperda* (Sp9) cells were grown in expression medium (Bio-Whittaker) supplemented with streptomycin (100 μg/ml), penicillin (100 U/ml), and amphotericin B (250 ng/ml) and infected with a recombinant baculovirus at a multiplicity of infection of 5. The culture supernatants were collected 9 days after infection, and recombinant proteins were purified by Ni²⁺-chelate chromatography as described (36). The proteins were concentrated using Centricon microconcentrators with a cut-off at 10 kDa (Millipore). Vitronectin from human plasma was obtained from Sigma-Aldrich. The FITC-conjugated goat anti-human vitronectin and donkey anti-goat pAb were purchased from Serotec.

**Protein labeling and direct binding assay**

Purified FH was labeled with 0.05 mol of iodine (GE Healthcare) per molecule of protein, using the chloramine-T method (37). The different *H. influenzae* strains were grown in BHI liquid broth overnight and washed in PBS containing 1% BSA (Saveen Werner). Bacteria (2 × 10⁷) were incubated with 125I-labeled FH at 37°C for 1 h. After incubation, the bacteria were centrifuged (10,000 × g) through a 20% sucrose column. The tubes were frozen and cut, and radioactivity in the pellet and supernatant were measured in a gamma counter. Binding was calculated as amount of bound radioactivity (pellet) vs total radioactivity (pellet + supernatant). In the competition assays, FH, heparin (Leo Pharma), or NaCl at increasing concentrations were added to the reactions.

**Serum binding assay**

To analyze whether Hib and NTHi bound FH and/or FHL-1 directly from normal human serum (NHS), bacteria were grown overnight in BHI broth. Bacteria (10⁸) were incubated with NHS and buffer (100 mM NaCl, 50 mM Tris-HCl (pH 7.4)) for 1 h at 37°C. To remove unbound proteins, bacteria were washed five times with the same buffer. Thereafter, the bacterial pellet was resuspended in 150 μl of 0.1 M glycine-HCl (pH 2.0) to elute bound proteins. Bacteria without NHS were used as a negative control. After 15 min of incubation at 37°C with shaking, the bacteria were centrifuged and the supernatants were subjected to SDS-PAGE (10%). Electrophoretical transfer of protein bands from the gel to an Immobilon-P membrane (Millipore) was done at 35 V for 2 h. After transfer, the Immobilon-P membrane was blocked in PBS with 0.1% Tween 20 and incubated (10⁸ CFU) for 1 h at room temperature (RT). After washing, HRP-conjugated donkey-anti-goat pAb was added. After incubation for 40 min at RT and additional washings in PBS-Tween, development was performed with ECL Western blotting detection reagents (Pierce).

**Flow cytometry**

The efficiency of Hib binding of FH purified from human plasma was analyzed by flow cytometry. Hib 541 was grown in BHI liquid broth overnight, washed once in PBS containing 2% BSA and incubated (10⁷ CFU) with increasing concentrations of FH (range: 10–100 μg/ml). After two washings, the bacteria were incubated with rabbit anti-SCRs 1–4 pAb for 30 min on ice. Thereafter, the bacteria were washed and incubated with FITC-conjugated goat anti-rabbit pAb. After additional washes, bacteria were analyzed by flow cytometry (EPICS XL-MCL, flow cytometer; Beckman Coulter). All incubations were kept in final volumes of 100 μl PBS-BSA and the washings were done with the same buffer. The anti-SCRs 1–4 pAb and FITC-conjugated goat anti-rabbit pAb were used as negative controls. To analyze Hib 541 and Hib 568 for vitronectin binding, bacteria were incubated with 10 μg/ml vitronectin for 1 h at 37°C. After washings, bacteria were incubated with goat anti-human vitronectin pAb for 30 min on ice, before incubation with the FITC-conjugated donkey anti-goat pAb. After additional washes, bacteria were analyzed by flow cytometry.

**ELISA**

To estimate which SCRs of FH were involved in the binding of Hib 541, microtiter plates (F96 Maxisorb, Nunc-Immuno Module) were coated with live bacteria (0.5 × 10⁷) for 1 h at 37°C. The plates were washed four times with PBS-Tween and blocked for 1 h with PBS supplemented with 2% BSA. After washings, the plates were incubated for 1 h at RT with the various FH constructs, each at a concentration of 20 μg/ml. Thereafter, the wells were washed and incubated with a goat anti-human FH pAb (Serotec or Quidel) in PBS-BSA for 1 h at RT. After additional washings, HRP-conjugated donkey anti-goat pAbs (Serotec) were added for 40 min at RT.
The wells were washed and developed, and the absorbance was measured at OD₅₅₀ with a MRX microplate reader (Dynatech Laboratories). In all experiments, samples were analyzed in duplicates. In addition, the non-FHL-1-binding Hib strain 539 was also analyzed to estimate which SCRs of FH were involved. In the competitive assay, mAbs B22, E14, and C18 were added at a concentration of 200 μg/ml to the reactions.

Co-factor assay

Hib 541 was incubated with FH (100 μg/ml) or FHL-1 (100 μg/ml) in 50 mM Tris-HCl (pH 7.4) supplemented with 150 mM NaCl for 1 h at 37°C. After thorough washing in the same buffer, the bacteria were mixed with 150 μg/ml C3m20, 20 μg/ml factor I, and trace amounts of 125I-labeled C3b in 50 μl of buffer. As a positive control, FH or FHL-1 was used in fluid phase instead of bacteria preincubated with FH. As a negative control, the Hib 541 without FH/FHL-1 preincubation was used. The samples were incubated for 2 h at 37°C and the reactions were terminated by the addition of SDS-PAGE sample buffer. The samples were analyzed on an SDS-PAGE (10%; Invitrogen). Thereafter, the gel was dried and the proteins were visualized with a Personal FX Imager (Bio-Rad) using intensifying screens.

Serum bactericidal assay

The Hib strains were diluted in DGVB⁻⁺ (2.5 mM Veronal buffer (pH 7.4) containing 70 mM NaCl, 140 mM glucose 0.1% (w/v) gelatin, 1 mM MgCl₂, and 0.15 mM CaCl₂). Bacteria (10⁵ CFU) were incubated in 5% NHS diluted either in DGVB⁺⁺ or 10% FH-depleted serum prepared as described previously (38) in a final volume of 100 μl. At different time points, 10-μl aliquots were removed and spread onto chocolate agar plates. After 18 h of incubation at 37°C, CFUs were determined. NHS was obtained from healthy blood donors with informed consent.

OMP preparations

Bacteria grown to stationary phase were washed with 50 mM Tris-HCl buffer (pH 8.0). The pellet was resuspended in Tris-HCl buffer containing 3% Tween 80 (Calbiochem) and protease inhibitors (Complete; Roche) (39). OMPS were extracted by rotating the mixture at 37°C for 2 h. The bacterial cells, stripped of their outer membranes, were centrifuged and supernatants collected. Thereafter, the supernatants were analyzed on SDS-PAGE and Far Western blots. OMPS were also extracted according to the rapid microprocedure for isolating detergent insoluble OMPS (40). Briefly, sodium N-lauroyl sarcosine (sarcosyl) insoluble proteins were obtained from the cell pellets from Hib 541 and Hib 568. Cell pellets were resuspended in 10 mM HEPES buffer (pH 7.4) and sonicated on ice. Samples were centrifuged to remove unbroken cells and debris followed by centrifugation of the supernatants to collect cell membranes. These were resuspended in 10 mM HEPES buffer and 2% sarcosyl and incubated for 30 min at RT to solubilize the cytoplasmic membranes. The outer cell membranes were pelleted by centrifugation and resuspended in 10 mM HEPES buffer. Thereafter, the pellets were analyzed on SDS-PAGE and Far Western blots.

SDS-PAGE and Far Western blots

OMPs were subjected to SDS-PAGE (10%) (41) and stained with Coo massie brilliant blue R-250 (Bio-Rad). Electrophoretical transfer of protein bands from the gel to an Immobilon-P membrane (Millipore) was done at 35 V for 2 h to transfer the proteins. After transfer, the Immobilon-P membrane was washed in PBS-Tween containing 1.5% OVA. After several washings in PBS-Tween, the membrane was incubated with 125I-labeled FH in PBS-Tween including 1.5% OVA overnight at room temperature. After the incubation and additional washings in PBS, the proteins were visualized with a Personal FX Imager (Bio-Rad) using intensifying screens.

Statistical analysis

Results were assessed by Student’s t test for paired data. p ≥ 0.05 was considered to be statistically significant.

Results

H. influenzae binds human FH

Several bacterial species have been shown to bind FH and thereby counteract the alternative pathway of the complement system (24–28). To determine whether the respiratory pathogen H. influenzae interacts with FH, a collection of both encapsulated and unencapsulated (NTHi) H. influenzae strains were incubated with 125I-labeled FH followed by separation of unbound ligand. Binding was calculated as the ratio of bound radioactivity vs total radioactivity added. Interestingly, the majority of the H. influenzae strains bound FH (Fig. 1). Among all the strains tested, H. influenzae types b and f showed the highest FH binding using the direct 125I-FH-binding assay, whereas the other H. influenzae strains bound FH to a varying extent. The clinical isolate Hib 541 showed the strongest binding (45%) of the Hib strains and was therefore chosen for further studies. In addition, Hib 568 was selected as a low-binding (12%) type b strain. To determine whether the growth phase influenced expression of the FH-binding protein, aliquots of Hib 541 were collected at different time points (Fig. 2A) and analyzed in the direct binding assay. No significant difference in FH binding was found when bacteria from various growth phases were analyzed (Fig. 2B). Therefore, additional experiments were performed with bacteria that had been cultured overnight.

To analyze whether the Hib strains and NTHi strains also bound FH directly from serum, bacteria were incubated with NHS for 1 h at 37°C. Thereafter, bound proteins were eluted, separated by SDS-PAGE, and analyzed by Western blot using an Ab directed against human FH. As can be seen in Fig. 3A, all ten different Hib

FIGURE 1. H. influenzae binds FH. A series of clinical isolates were tested for FH binding. The different H. influenzae strains were grown over night and incubated with 125I-labeled FH. Binding was determined as percentage of bound radioactivity vs added radioactivity measured after separation of free and bound 125I-labeled FH over a sucrose column. The mean values of three independent experiments are shown. SD: 0.74–10.7 (median 1.88). The two Hib strains used for detailed experiments in the present study are encircled.

FIGURE 2. FH binding does not differ during various growth phases. A growth curve is established (A) and the binding to FH at different time points is shown (B). Aliquots of Hib 541 were collected at each time point (1–8 h and overnight; o/n). OD₅₅₀ was measured, and an equal number of bacteria (2 × 10⁶) were incubated with 125I-labeled FH. Binding was determined as percentage of bound radioactivity vs added radioactivity measured after separation of free and bound 125I-labeled FH. The mean values of three independent experiments are shown. Error bars indicate SD.
strains bound FH and four of them also bound FHL-1. No cross-reactivity of the anti-FH pAb was seen with bacteria incubated in the absence of NHS. In addition, all 10 NTHi strains tested bound FH directly from NHS and two of them bound FHL-1 (Fig. 3B). In addition to binding FH and FHL-1 directly from serum, one FH/FHL-binding strain (Hib 541) and one FH-binding strain (Hib 539) were analyzed for binding of purified and recombinant FH and FHL-1, respectively. The results show that Hib 541 bound more of both FH and FHL-1 as compared with Hib 539 (Fig. 3C). To characterize the FH binding to *H. influenzae*, Hib 541 was incubated with increasing concentrations of purified FH. Hib 541 bound FH in a dose-dependent manner at FH concentrations 10–100 μg/ml reaching saturation at 75 μg/ml FH (below the serum concentration) (Fig. 3D). To further rule out the possibility that the capsule is involved in the interaction between Hib and FH, a capsule-deficient Hib was tested for FH binding. The clinical capsule-deficient *H. influenzae* isolate RM804 and six other Hib strains expressing the capsule were incubated with 125I-labeled FH followed by separation of unbound ligand. The results show that there is no significant difference in binding between the different Hib strains and the capsule-deficient Hib strain RM804 (Fig. 3E).

**FIGURE 3.** *H. influenzae* binds FH and FHL-1 irrespectively of the capsule. Hib (A) and NTHi (B) bound FH and FHL-1 from human serum. Hib strain 539 bound FHL-1 to a lesser extent compared with Hib strain 541 (C). Hib 541 bound FH in a dose-dependent manner (D). The capsule-deficient Hib strain RM804 bound FH to the same level as the encapsulated Hib strains (E). In A and B, 10 different Hib strains and 10 NTHi strains, respectively, were incubated with heat-inactivated serum. Purified FH and 0.1 μl of NHS was run in parallel as positive controls. Bacteria without serum were used as a negative control (A, lane 11; buffer). Bacteria were washed and bound proteins were eluted. The eluates were separated on SDS-PAGE gels, transferred to a polyvinylidene difluoride membrane. FH/FHL-1 was detected using an anti-FH pAb. One representative experiment of three independent ones performed is shown. C, Hib 539 and 541 immobilized in microtiter plates were incubated with purified FH and recombinant FHL-1, and bound fragments were detected with goat anti-human FH pAb and HRP-conjugated donkey anti-goat pAb in an ELISA. The bacteria were incubated with increasing concentrations (10–100 μg/ml) of FH followed by detection with an anti-FH (SCRs 1–4) pAb (D). FITC-conjugated anti-rabbit pAb was added followed by flow cytometry analysis. The different Hib strains were grown overnight and incubated with 125I-labeled FH (E). Binding was determined as percentage of bound radioactivity vs added radioactivity measured after separation of free and bound 125I-labeled FH over a sucrose column. The mean values of three independent experiments are shown. Error bars indicate SD.

**FIGURE 4.** The *H. influenzae* FH binding is specific and is based on ionic interactions. FH (A) and heparin (B) inhibited the binding of 125I-labeled FH to Hib 541. C, Hib 541 bound FH in a non-covalent manner and the binding was decreased with increasing concentrations of NaCl. Hib 541 was incubated with 125I-labeled FH and increasing amounts of unlabeled FH, heparin, or NaCl, followed by centrifugation through a sucrose gradient and determination of radioactivity associated with the bacterial pellet. Binding was determined as percentage of bound radioactivity vs added radioactivity. The FH binding of *H. influenzae* in the absence of competitor was defined as 100%. The mean values of three experiments are shown with error bars indicating SD.

The ionic interaction between Hib 541 and FH is mediated by SCRs 6–7 and 18–20

To test the specificity of the FH binding to Hib 541, bacteria were incubated with increasing concentrations of unlabeled FH (0–1000 nM) in addition to 125I-labeled FH. As can be seen in Fig. 4A, FH inhibited the binding of 125I-FH to Hib 541 in a dose-dependent manner (Fig. 4A). To analyze whether heparin, which has previously been shown to bind to SCRs 7, 13, and 19–20 in FH and to SCR 7 in FHL-1 (42), could inhibit the interaction, increasing concentrations of heparin were added to the mixture containing bacteria and radiolabeled FH. Heparin (0–100 nM) inhibited the interaction between Hib 541 and 125I-FH (Fig. 4B) in a dose-dependent manner. At a concentration of 50 mM heparin the binding was inhibited >75%. The binding between Hib 541 and FH was...
FH binds Hib with SCRs 6–7 and SCRs 18–20. A, Hib 541 and Hib 539 were immobilized in microtiter plates and incubated with recombinant FH fragments. Bound fragments were detected with goat anti-human FH pAb and HRP-conjugated donkey anti-goat pAb. Hib 541 bound SCRs 1–6, SCRs 15–20, and SCRs 18–20, while reduced binding to SCRs 1–7 and SCRs 19–20 could be detected. A similar pattern as with Hib 541 was seen with Hib 539. B, In the blocking assay, Hib 541 were incubated with FH and mAbs B22 (SCR5), E14 (SCR20), or C18 (SCR20), and the FH binding was detected as described above. Over 50% of the binding was inhibited when mAb C18 was added. A, Heparin-binding domains are indicated by the shaded areas. Bacteria incubated with anti-FH and HRP-conjugated donkey anti-goat pAb was used as a negative control. The mean values of three independent experiments with duplicates are shown. Error bars indicate SD. *** p<0.001.

FIGURE 5. FH binds Hib with SCRs 6–7 and SCRs 18–20. A, Hib 541 and Hib 539 were immobilized in microtiter plates and incubated with recombinant FH fragments. Bound fragments were detected with goat anti-human FH pAb and HRP-conjugated donkey anti-goat pAb. Hib 541 bound SCRs 1–6, SCRs 15–20, and SCRs 18–20, while reduced binding to SCRs 1–7 and SCRs 19–20 could be detected. A similar pattern as with Hib 541 was seen with Hib 539. B, In the blocking assay, Hib 541 were incubated with FH and mAbs B22 (SCR5), E14 (SCR20), or C18 (SCR20), and the FH binding was detected as described above. Over 50% of the binding was inhibited when mAb C18 was added. A, Heparin-binding domains are indicated by the shaded areas. Bacteria incubated with anti-FH and HRP-conjugated donkey anti-goat pAb was used as a negative control. The mean values of three independent experiments with duplicates are shown. Error bars indicate SD. *** p<0.001.

FIGURE 6. FH and FHL-1 retained its cofactor activity when bound to the surface of H. influenzae. Hib 541 with or without FH (A) or FHL-1 (B) was incubated with 125I-labeled C3b and factor I, followed by separation of the proteins from the supernatants in SDS-PAGE. FH or FHL-1 in fluid phase was used as a positive control. The gel was dried and visualized by a phosphor imager. Data shown are representative of three independent experiments.

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FH regulates the alternative pathway activity by serving as a cofactor for factor I in the degradation of C3b, which generates the cleaving fragments α’ 68, 46, and 43 kDa. To analyze the cofactor activity of FH and FHL-1 bound to H. influenzae, Hib 541 was incubated in the presence or absence of FH/FHL-1, and with factor I and 125I-labeled C3b. Following incubation for 1 h, all lysates were separated by SDS-PAGE, the gel was dried, and the cleavage of C3b was analyzed by autoradiography. The cleavage products generated in the presence of surface bound cofactors and factor I showed comparable size as the fragments generated by factor I in combination with FH/FHL-1 in fluid phase. When 125I-labeled C3b was incubated in the presence of FH or FHL-1-coated Hib 541, the cleavage products of α’ 68, 46 and/or 43 kDa appeared (Fig. 6). However, almost no cleavage products were seen with bacteria only. Taken together, FH and FHL-1 bound to Hib retain cofactor activity and inactivate C3b.

Hib-dependent FH binding promotes serum resistance

To test whether the difference in binding of FH had any effect on serum resistance, a high and a low FH-binding Hib strain were compared in a serum bactericidal assay. After incubation in human serum the high FH-binding Hib 541 strain showed significantly higher resistance to NHS as compared with the low FH-binding strain Hib 568 (Fig. 7A). After 5 min, 89% of Hib 541 survived as compared with Hib 568 that did not show any survival. Thus, the H. influenzae-dependent interaction with FH appears to be important for bacterial serum resistance. To further confirm that the binding of FH to Hib had an effect on the bacterial serum resistance, bacteria were incubated in FH-depleted complement-active
serum. Hib 541 showed reduced survival in the FH-depleted serum (Fig. 7B), indicating the importance for the bacteria to bind FH.

To analyze whether FHL-1 was involved in serum resistance, a Hib strain that did not bind FHL-1 in the elution assay (Fig. 3A) and showed weak binding in the ELISA (Figs. 3C and 5A) was analyzed for serum resistance, and compared with Hib 541 that interacts with both FH and FHL-1. As can be seen in Fig. 7C, there was no significant difference in survival between the FHL-1 binder Hib 539 and the non-FHL-1 binder Hib 541. The experiment was performed as described in A. For all panels (A–C), the mean values of three independent experiments are shown with error bars indicating SD. Statistical significance of differences was estimated using Student’s t test. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

The serum resistance of Hib 541 is not dependent on vitronectin

We have shown in a previous study that Hib interacts with vitronectin and that this interaction mediates serum resistance (31). To analyze the importance of vitronectin in serum resistance of Hib 541 and Hib 568, bacteria were incubated with purified vitronectin. The Hib strain RM804, with a well defined vitronectin binding capacity, was used as a positive control (31). Vitronectin was bound to the serum-sensitive Hib 568 and in lower amounts to the serum-resistant Hib 541 (Fig. 8). Despite the higher vitronectin binding of the Hib 568 as compared with the Hib 541, this strain was more sensitive to serum. We therefore concluded that the serum resistance of Hib 541 was dependent, among others, on its ability to bind FH.

Two Hib OMPs are responsible for the FH binding

To identify ligands at the bacterial surface, which mediate FH binding, a Far Western blot with OMPs was used. OMPs were extracted from eight different Hib strains and three NTHi strains using Empigen. The OMPs were separated by SDS-PAGE and blotted onto a membrane followed by incubation with 125I-labeled FH. All eight Hib strains showed two bands, one stronger and one weaker band of ~32 and 40 kDa (Fig. 9A). To further analyze the FH-binding ligand(s), the high FH-binding strain Hib 541 and the low FH-binding strain Hib 568 were chosen. OMPs extracted by two different methods were analyzed for FH binding by Far Western blot. Two different detergents were used to extract the OMPs. When Empigen was used to extract OMPs of Hib 541, the two bands (32 and 40 kDa) were identified and with sarkosyl the 40-kDa band was more prominent (Fig. 9B). The expression of both

FIGURE 7. A low FH binding H. influenzae is less protected against the bactericidal activity of human serum than a high FH-binding strain. A. The high and low FH-binding strains Hib 541 and Hib 568 were incubated in the presence of 5% NHS for the indicated times. Thereafter, bacteria were spread on chocolate agar plates to allow determination of the number of surviving bacteria. Numbers of bacteria (CFU) at the initiation of the experiment was defined as 100%. B. Hib 541 showed a reduced survival upon growth in FH-depleted complement-active human serum. The Hib 541 was incubated in the presence of NHS or FH-depleted serum both diluted in DGVB * * and spread on chocolate agar plates to allow determination of the number of surviving bacteria. C. The non-FHL-1 binder Hib 539 was incubated in the presence of NHS and the survival was compared with the FHL-1 binder Hib 541. The experiment was performed as described in A. For all panels (A–C), the mean values of three independent experiments are shown with error bars indicating SD. Statistical significance of differences was estimated using Student’s t test. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.

FIGURE 8. Hib 541 bound less human vitronectin as compared with Hib 568. Hib strain 541 and 568 were incubated with human vitronectin followed by detection with a sheep anti-vitronectin pAb. FITC-conjugated anti-goat pAb was added followed by flow cytometry analysis. RM804 was used as a positive control. The mean values from three independent experiments are shown. Error bars indicate SD.

FIGURE 9. Two putative ligands of Hib were identified for FH binding and the expression of these ligands was decreased in the low FH-binding strain Hib 568. A. Eight Hib strains, three NTHi strains were analyzed in Western blot analysis. B. OMP analysis of the high FH-binding strain Hib 541 compared with the low FH-binding strain Hib 568. Empigen (A) and Empigen and sarkosyl (B) were used to extract OMPs. Resulting proteins were analyzed by Far Western blots using 125I-labeled FH. A typical experiment of three is demonstrated. The arrows indicate the two FH-binding OMPs.
these proteins were of reduced intensity in the low FH-binding strain Hib 568, indicating different affinity of these bacterial ligands.

Discussion

Serum resistance is important for bacterial virulence. Binding of complement inhibitors such as FH, FHL-1, C4BP, and vitronectin are efficient survival strategies used by several pathogens (43–45). In the present study, we show specific binding of the human complement inhibitor FH to *H. influenzae*. The majority of both unencapsulated and encapsulated *H. influenzae* strains bound FH as demonstrated by a direct binding assay. This binding is specific, which is in contrast to a recent study in which no binding of FH to *H. influenzae* was found (46). However, that study focused on only two particular strains of NTHi. In our study we tested 16 different NTHi isolates and the FH binding varied among these strains. The Hib strains used in the present paper showed strong FH binding. In addition, when human serum was used as a source of FH, all the tested Hib and NTHi strains bound FH to a varying extent and also some of them bound FHL-1. There was a significant difference in surface-attached FH between two Hib strains (Hib 541 and Hib 568), which may explain why the low-binding strain Hib 568 was more sensitive to killing by human serum than the high FH-binding strain Hib 541. When bacteria were incubated in FH-depleted, complement-active human serum, a statistically significant decrease in survival was seen with Hib 541, suggesting that binding to FH indeed protects the bacteria against the attack of the complement system.

Several pathogens (e.g., *C. albicans*, *N. meningitidis*, *Neisseria gonorrhoeae*, *S. pneumoniae*, and *B. burgdorferi*) express surface molecules that bind FH and thereby protect the pathogen against the alternative pathway of complement activation (25–27, 47, 48). In addition to inhibition of the alternative pathway, several bacteria (e.g., *Moraxella catarrhalis*, *S. pyogenes*, *E. coli* K1, and *H. influenzae*) bind C4BP and are shielded against the classical and lectin pathways (49–51). Interestingly, many species, *C. albicans*, *Borrelia recurrentis*, and *S. pyogenes*, among others, have been shown to bind both FH and C4BP (26, 52–55). We have recently demonstrated that non-typeable *H. influenzae* binds C4BP (30). The NTHi-dependent interaction with C4BP, and consequently the classical pathway, significantly contributes to NTHi serum resistance. In yet another study, it was shown that Hib binds vitronectin, which is the main regulator of the terminal complement pathway (31). This interaction contributed to serum resistance of encapsulated type b strains and was linked to Hsf protein expression. Available data thus suggest that *H. influenzae* interacts with more than one complement inhibitor and uses several strategies to avoid complement-mediated attacks.

To analyze whether the vitronectin binding capacity contributed to the survival of the strains, Hib 541 and 568 were analyzed for vitronectin binding. Despite an increased vitronectin binding of Hib 568 as compared with Hib 541, Hib 568 was more sensitive to serum. These results further suggest that the survival in human serum depend to a large extent on the capacity of Hib 541 to bind FH.

The ability of *H. influenzae* to bind FH and FHL-1 suggests that this species uses FH and FHL-1 to inhibit complement-mediated attacks in several ways. Functional analysis showed that FH and FHL-1 maintained cofactor activity when bound to the surface of the bacteria and promoted factor I-mediated C3b inactivation. Consequently, this inactivation prevents C3b from opsonizing the pathogen. In addition, because surface bound FH binds C3b, the formation of C3 convertase (C3bBb) will most likely be inhibited and its decay accelerated, also promoting Hib to avoid MAC-induced lysis.

FH is composed of 20 SCR domains, and different regions of the FH molecule bind various bacterial species. The interaction between Hib and FH appears to be ionic because the binding decreased with increasing concentrations of NaCl. Using recombiant fragments spanning the FH molecule, two binding sites between Hib 541 and FH were found. Hib 541 bound to SCRs 6–7, which is common to both FH and FHL-1. Particularly, SCR 6 seems to be important for the interaction between Hib and FH, but SCR 7 also contributes to the binding. The second binding site for FH was located within SCRs 18–20, which is the C-terminal part of the molecule. SCRs 18–20 bound significantly stronger than the fragment encompassing SCRs 19–20, suggesting that SCR 18 has a stronger affinity for Hib as compared with SCRs 19–20. In addition, heparin inhibited the interaction between Hib 541 and FH, suggesting involvement of the heparin-binding domains. The heparin-binding domains of FH is located in SCRs 7, 13 and 19–20 and in FHL-1 in SCR 7 (19), further corroborating our results. In addition, >50% of the binding was inhibited between Hib 541 and FH when mAb C18 (against SCR20) was added to the reaction, confirming the involvement of SCR20 in the C-terminal end. The mAbs against SCR5 (B22) and SCR20 (E14) did not inhibit the binding, which excludes sterical hindrance. The two mAbs against SCR20 were directed against different epitopes in the same region. Only four of ten Hib strains bound FHL-1 directly from human serum. Thus, one non-FHL-1-binding strain (Hib 539) was analyzed for binding recombinant fragments spanning the FH molecule. This strain was shown to bind FHL-1 to a lesser extent compared with Hib 541. In addition, SCRs 18–20 in the C-terminal part of the FH are involved in the interaction between Hib 539 and FH. However, SCRs 1–6 also showed binding to the bacteria. Despite the differences in FHL-1-binding, the two Hib strains 541 and 539 showed similar survival when exposed to human serum, further suggesting the importance of FH binding in serum resistance. *C. albicans* is another pathogen interacting with FH via two binding sites (26). The first binding domain of *C. albicans* is located in SCRs 6 and 7 and the second in SCRs 19 and 20. Other microbial binding sites on FH have been located in SCR 7 and SCRs 8–11 for *S. pneumoniae*-Hic and PspC, and *S. pyogenes*-Fba, respectively (25, 56, 57). Furthermore, SCRs 13–15 and SCRs 16–20 are binding sites for *S. pneumoniae*-PspC and Hic and *N. gonorrhoeae*-lipooligosaccharide, respectively (58, 59). Intriguingly, these studies show involvement of different SCRs depending on the species.

*H. influenzae* expresses several adhesive proteins (11), which mediate adherence to host respiratory epithelium in the initial step in colonization of the human host. Different regulators of the complement system have been shown to mediate adherence and ingestion to epithelial and endothelial cells. The binding of C4BP by *C. albicans* has been shown to mediate adherence to endothelial cells, and bound FHL-1 to *S. pyogenes* mediates and enhances the ingestion of the pathogen into epithelial cells (54, 57). In fact, several studies have indicated that complement proteins and regulators are present in the respiratory tract (50, 60, 61). Whether FH and/or FHL-1 has any role in adhesion of Hib to epithelium remains to be studied.

To identify FH-binding ligands at the bacterial surface, a Far Western blot with OMPs proteins was used. When OMPs were extracted from Hib, two bands (32 and 40 kDa) were identified. The expression of both these proteins were of reduced intensity in the low FH-binding strain Hib 568, indicating different affinity of these bacterial OMPs. *H. influenzae* expresses >30 OMPs and there are several in the size ranging from 25 to 42 kDa that may be potential candidates for FH
binding. P1 (35–50 kDa) P2 (40 kDa), P4 (28 kDa), P5 (27–35 kDa), and protein D (42 kDa) are all OMPs of *H. influenzae* that has approximately the corresponding size to the ligands found in the Far Western blot and may be suggested as possible FH-binding ligands (62). P2 is the most abundant protein in the outer membrane, it is highly immunogenic, and the variability allows the bacteria to evade protective Abs (63). P2 and P5 have adhesive capacity, and protein D has been shown to be involved in the infection process; therefore, all these proteins are important virulence factors of *H. influenzae* (64). Finally, P4 plays a role in the bacterial growth (65). However, further studies have to be done in detail to characterize the different ligands.

In conclusion, *H. influenzae* interferes with the complement system in several ways. NTHI has been shown to interact with C4BP, the regulator of the classical/lectin pathways, and thereby uses this interaction as a protection against the complement-mediated attack (30). In addition, Hib has been reported to bind the regulator of the terminal pathway, vitronectin (31). In the present paper, we have identified a novel interaction between *H. influenzae* and FH and FHL-1, the inhibitors of the alternative pathway. The *H. influenzae*-attached FH renders the bacteria more resistant to serum killing, and when FH-deficient complement-active human serum was used, growth of the bacteria was significantly affected. Bacteria were more susceptible in this case and showed reduced survival. Thus, FH may consequently contribute to *H. influenzae* virulence and it remains to elucidate our novel findings in a proper in vivo model.

Disclosures

The authors have no financial conflict of interest.

References


