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Factor H Binding as a Complement Evasion Mechanism for an Anaerobic Pathogen, *Fusobacterium necrophorum*

Nathalie Friberg,* Petteri Carlson,* Erna Kentala, † Petri S. Mattila, † Pentti Kuusela,* ‡ and Hanna Jarva2*‡

*Fusobacterium necrophorum* subspecies *funduliforme* is an obligate anaerobic Gram-negative rod causing invasive infections such as the life-threatening Lemierre’s syndrome (sore throat, septicemia, jugular vein thrombosis, and disseminated infection). The aim of our study was to understand if and how *F. necrophorum* avoids C activation. We studied 12 *F. necrophorum* subsp. *funduliforme* strains isolated from patients with sepsis. All strains were resistant to serum killing after a 1-h incubation in 20% serum. The bacteria bound, at different levels, the C inhibitor factor H (fH). Binding was ionic and specific in nature and occurred via sites on both the N terminus and the C terminus of fH. Bound fH remained functionally active as a cofactor for factor I in the cleavage of C3b. Interestingly, patients with the most severe symptoms carried strains with the strongest ability to bind fH. An increased C3b deposition and membrane attack complex formation on the surface of a weakly fH-binding strain was observed and its survival in serum at 3.5 h was impaired. This strain had not caused a typical Lemierre’s syndrome. These data, and the fact that fH-binding correlated with the severity of disease, suggest that the binding of fH contributes to virulence and survival of *F. necrophorum* subsp. *funduliforme* in the human host. Our data show, for the first time, that an anaerobic bacterium is able to bind the C inhibitor fH to evade C attack. The Journal of Immunology, 2008, 181: 8624 – 8632.

*Fusobacterium necrophorum* is an obligate anaerobic Gram-negative rod. Two subspecies are identified: *F. necrophorum* subsp. *necrophorum* (biovar A) involved in diseases of animals, and *F. necrophorum* subsp. *funduliforme* (biovar B) involved in human pathology. *F. necrophorum* subsp. *funduliforme* is often viewed in the literature as part of the normal oropharyngeal flora, but this point is still uncertain. *F. necrophorum* causes local infections (e.g., tooth infections, acute sore throat with tonsillitis and pharyngitis, persistent or chronic sore throat), but also invasive infections such as Lemierre’s syndrome (1, 2). Lemierre’s syndrome is life-threatening and affects healthy adolescents or young adults with a 2:1 male/female ratio (3). The syndrome is characterized by sore throat followed by jugular vein thrombosis, septicemia, and distant infections with multiple abscesses (4, 5). Lemierre’s syndrome is rare and its incidence varies from 0.8 to 1.5 cases per million per year (3, 6). In the preantibiotic era the mortality rate was 90%. Currently, mortality rates vary from 4% to 18% (5, 7, 8).

To cause disease, pathogens such as *F. necrophorum* must evade innate immune responses. Innate immunity, characterized by rapid and non-specific responses, is the first-line of human defense against microorganisms. An important and efficient part of the innate immune response is the C system. As C is able to discriminate self from non-self cells (e.g., microbes), it plays a major role in the opsonization and lysis of microbes. C activation products can also attract and activate phagocytic cells to the area of microbial invasion. The C system can become activated directly by the surface of pathogens via the alternative or the lectin pathway or by specific Abs via the classical pathway. C activation results in the formation and deposition of C3b on microbes’ surface and formation of the lytic membrane attack complex (MAC)3.

To protect host cells from destruction or inappropriate activation, C is inhibited by several specific proteins present in the fluid phase or on cell surfaces. Factor H (fH), a 150-kDa fluid phase protein, inhibits the alternative pathway (AP) by competing with factor B for binding to C3b, accelerating the decay of C3 convertase, and by acting as a cofactor for factor I-mediated degradation of C3b.

Pathogenic microorganisms have developed several sophisticated mechanisms to escape host defenses. To avoid C attack, pathogens can inactivate C components or mimic C proteins (9). Furthermore, several pathogenic microbes have been shown to bind C regulators such as fH or C4b binding protein (C4BP) from blood plasma. Bacteria (e.g., *Streptococcus pyogenes*, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Yersinia enterocolitica*, *Borreella burgdorferi*), some fungi such as *Aspergillus fumigatus* or *Candida albicans*, some viruses (e.g., HIV or NS1 filovirus), and also parasites such as *Echinococcus spp.* or *Onchocerca* spp. are able to recruit and bind the C regulator fH (9). For *S. pyogenes*, *S. pneumoniae*, *B. burgdorferi*, *N. meningitidis*, and *N. gonorrhoeae*, fH binding has been shown to correlate with resistance to opsonophagocytosis.
or serum killing (10–14). For these bacteria, fH binding appears to confer protection against opsonization and/or MAC formation. Binding of fH to the microbial surface can thus be considered as an important virulence factor.

In this study we aimed to understand how F. necrophorum avoids C activation. We observed that this anaerobic bacillus also binds human fH. The bound regulator remains functionally active as a cofactor for factor I in the cleavage of C3b. The strains that bound a higher amount of fH were more serum resistant than was a strain that bound less fH. Thus, our data show that binding of fH contributes to the serum resistance of F. necrophorum.

Materials and Methods

Bacterial strains and culture conditions

The F. necrophorum strains (n = 12) were clinical blood isolates from patients with septicaemia in the Helsinki University Central Hospital region. The strains were collected between 1994 and 2005. The strains were initially identified at the species level by classical methods such as by colony and Gram staining morphology as well as by growth characteristics. Further identification was done by real-time PCR according to the protocol previously described by Jensen et al. (15). This assay identified all the strains as F. necrophorum subsp. funduliforme.

Strains were grown on fastidious anaerobic agar (FAA) for 24 h at 37°C using AnaeroGen compact (Oxoid). Anaerobic BacT/ALERT SN culture bottles (bioMérieux) were inoculated with the bacterial suspension and bacteria were grown until mid-log phase. Bacteria were washed with Veronal-buffered saline (VBS; 142 mM NaCl, 1.8 mM sodium barbital, 3.3 mM barbital buffer, pH 7.4) and the concentration was adjusted.

S. pneumoniae strain PR 218 was used as a positive control for direct fH-binding assays (12). It was grown in Todd-Hewitt broth with 0.2% yeast extract supplement to mid-log phase. S. aureus ATCC (American Type Culture Collection) 25923 was grown in Todd-Hewitt broth.

Proteins and Abs

Pooled normal human serum (NHS) was obtained from healthy laboratory personnel and stored at −70°C until used. Factor H-related protein-1 (FHR-1)−deficient serum was provided by a healthy donor. Heat-inactivated serum (HIS) was obtained by incubating NHS at 56°C for 30 min. fH, C3b, and factor I were purchased from Calbiochem. fH and C3b were radiolabeled with 125I using the iodogen method (16). Heparin was obtained from Sigma-Aldrich. Polyclonal goat anti-human fH was provided by Calbiochem. Mouse mAb 196X against fH has been described previously (17). Polyclonal rabbit anti-human C3c Ab was obtained from Dako. Mouse Ab IgG against SC5b-9 was from Quidel. The peroxidase-conjugated donkey anti-goat IgG was provided by Jackson ImmunoResearch Laboratories. Alexa Fluor 488-conjugated donkey anti-rabbit and goat anti-mouse IgG Abs were from Invitrogen. Recombinant constructs of fH (short consensus repeat (SCR) 1–5, 1– 6, 1– 7, 8–11, 11–15, 15–20, and 18–20) were expressed using the baculovirus system and purified using Ni-Sepharose as described previously (18). Recombinant SCR5–7 and SCR9–20 constructs were expressed in Pichia pastoris as described previously (19, 20).

Serum resistance of the strains

Freshly grown bacteria were washed three times in sterile VBS. The concentrations were adjusted to 2 × 10^8 bacteria/ml and 3 × 10^8 bacteria (150 μl) were incubated in 20% or 75% NHS or Mg-EGTA serum at 37°C under anaerobic conditions with gentle agitation. Fifty-microliter portions of the suspensions were removed from the reaction tubes at 0, 1, 2, and 3.5 h time points and diluted with VBS. To stop C activation, EDTA (final concentration 10 mM) was added to the first dilution tube. After the last dilution, 100 μl of the solutions were plated on FAA plates and incubated for 48 h at 37°C under anaerobic atmosphere. Bacteria incubated in 20% or 75% HIS were used as reference. Survival was counted as percentage of survival of bacteria incubated with HIS at the corresponding time points.

Direct 125I-fH binding assays

The F. necrophorum, S. pneumoniae, and S. aureus strains were grown to mid-log phase. Bacteria were washed in VBS, resuspended in 0.01% gelatin in VBS (GVB), and the concentration was adjusted to 2 × 10^8 cells/ml. Twenty microliters of bacteria (−4 × 10^7 cells/assay) was incubated with 20 μl of 125I-fH (−20,000 cpm/sample) for 30 min at 37°C with agitation. After incubation, the samples were centrifuged through 20% sucrose in GVB at 10,000 × g to separate free protein from bacteria-bound proteins. After separation, activities in the pellet and in the supernatant were measured in a gamma counter. The ratio of bound to total radioactivities was calculated. In these assays S. pneumoniae PR 218 was used as positive control (12). S. aureus was the negative control (S. Meri and H. Jarva, unpublished observation).

The effect of ionic strength on the binding of 125I-fH to fusobacteria was studied by using increasing concentrations of NaCl in GVB (final concentrations of 150, 300, 450, and 600 mM). In a competition assay, unlabeled fH was added to the reaction at the same time as 125I-fH. The concentration of unlabeled fH varied between 1 and 300 nM. BSA, at equimolar concentrations, was used as a negative control.

Elution of bound proteins

Bacteria were grown to mid-log phase, washed, and the concentration was adjusted to 5 × 10^8 bacteria/ml in VBS. The cells (10^8 cells/assay) were incubated in HIS (final concentrations of 0.1, 1, 2.5, 5 or 10%) or with equimolar amounts of recombinant constructs of fH for 30 min at 37°C with agitation. Bacteria were washed with VBS. After centrifugation, the supernatant of the last wash was saved. To elute surface-bound proteins, the pellet was resuspended in 50 μl of 0.1 M glycine-HCl (pH 2.5) and incubated for 20 min at 37°C. Samples were centrifuged and the supernatants (eluates) were saved and neutralized with 1 M of Tris-HCl (pH 9.5). Eluates and last washes were subjected to SDS-PAGE under nonreducing conditions. The proteins were transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked with 5% milk-PBS and the membrane was incubated overnight at 4°C with a goat polyclonal anti-fH Ab (diluted 1/5000). After washing, the membrane was incubated for 1 h with peroxidase-conjugated anti-goat IgG (diluted 1/5000). After washing, proteins were detected by ECL. The F. necrophorum strains incubated in VBS instead of HIS were used as negative controls.

Flow cytometry

Bacteria were grown to mid-log phase, washed with VBS, and the concentration was adjusted to 5 × 10^8 bacteria/ml in GVB. The cells (10^8 cells/assay) were incubated in HIS (final concentration 10%) for 30 min at 37°C with agitation. After washing with GVB, the bacteria were incubated with a mAb against fH (196X) at the concentration of 5 μg/ml in 1% BSA-VBS. We used an Alexa Fluor-488 conjugated anti-mouse IgG as second Ab (final concentration 1/200). After washing, the bacteria were resuspended in GVB and examined by flow cytometry (FACScan; BD Biosciences).

Cofactor assay for C3b inactivation

To study the functional activity of the bound fH, we used a C3b cofactor assay. Physiologically, fH is a cofactor for factor I in the cleavage of C3b. To study the cofactor activity of bacteria-bound fH, bacteria were incubated with 0.1, 1, or 10% HIS. After washing, the pellet was resuspended in VBS with 125I-C3b (100,000 cpm/sample) and factor I (final concentration 15 μg/ml). The incubation time was 1 h at 37°C. Samples were centrifuged and the supernatants run into an SDS-PAGE gel under reducing conditions to detect the cleavage of C3b. The gels were dried and subjected to autoradiography. A positive control, consisting of 125I-C3b (100,000 cpm/sample), factor I (15 μg/ml), and fH (10 μg/ml), was included in each series. 125I-C3b incubated without factor I and fH was used as a negative control.

C3b deposition

To study C3b deposition on bacterial surfaces, F. necrophorum strains were grown to mid-log phase and incubated with 50% NHS or Mg-EGTA-NHS (final concentration 5 mM MgCl₂, 10 mM EGTA) at 37°C with agitation. The mixtures were incubated for 1, 2, 3, 4, 5, 15, and 30 min. Bacteria were analyzed for C3b deposition with a rabbit anti-human C3c Ab and an Alexa Fluor 488-conjugated anti-rabbit IgG Ab was used as the secondary Ab. Deposited C3b was detected by flow cytometry.

MAC formation

MAC formation on fusobacterial membrane was studied by flow cytometry. The strains, grown to mid-log phase, were incubated in 50% normal human serum at 37°C with agitation for 3, 5, 10, and 30 min. MAC was detected by a monoclonal anti-SC5b-9 Ab and Alexa Fluor-conjugated anti-mouse IgG.
Results

Description of the patients

Clinical data were collected from 12 patients with a *F. necrophorum* infection. From each patient, age, sex, primary infection, and the presence of complications such as abscess, distant infections, and intravascular thrombosis were recorded. Except for one patient, aged 55 years, the ages of the patients ranged between 15 and 28 years. Male/female ratio was 1:1. At admission to hospital, all patients presented with a *F. necrophorum* bacteremia. In all patients, except patient 12, infection was initially located in the oropharynx (dental infection, tonsillitis, or pharyngeal infection). Six patients (nos. 2, 6, 7, 9, 10, and 11) developed abscesses. In three cases, jugular vein thrombosis was documented (nos. 3, 4, and 5), and patient 6 presented with a disseminated intravascular coagulation. Distant infection was observed in seven cases. The most frequent location of the distant infections was the lungs (five patients). Patient 5 developed a severe cardiorespiratory failure, and patient 6 died 7 days after his arrival to hospital due to multiorgan failure.

Patient 12 differed from the other cases. The final diagnosis for this 15-year-old adolescent was a facial skin cellulitis without any oropharyngeal infection, abscess, or intravascular complication.

Ability of *F. necrophorum* strains to resist serum killing

All strains were isolated from the bloods of the patients. The fact that all the strains had survived in human blood suggests that they had developed effective protection against immune responses. To study the ability of the bacteria to resist serum killing, the strains were incubated in 20% NHS or HIS for 1 h. After incubation, fusobacteria were plated on FAA plates and incubated under anaerobic conditions for 48 h. Survival was counted as a percentage of survival of bacteria incubated in the presence of HIS. At 1 h, the percentage of survival in 20% NHS ranged between 53% and >100% (data not shown). This result indicates that all strains are able to resist complement attack and serum killing to various extents.

Direct binding of 125I-labeled fH to *F. necrophorum*

Bacteria have developed several mechanisms to evade complement activation. One of them is the binding of the AP inhibitor fH. Using a binding assay with whole bacteria and 125I-fH, we observed that 11 fusobacteria out of 12 strains bound fH and FHR-1 and FHR-4. Strain 12 bound clearly less fH than did the other strains, and it did not bind FHR-1 or FHR-4. Diluted HIS (1/1000) was run for comparison. At this dilution, no FHR proteins could be detected. After incubation in 5% HIS deficient in FHR-1, it appears that strain 5 bound strongly FHL-1 and fH, but strain 4 showed weaker FHL-1 binding. Strain 12 bound hardly any fH and no FHL-1.

F. necrophorum binds fH from serum

To see if fH bound to fusobacteria directly from serum, *F. necrophorum* strains were incubated with 0.1–10% serum and, after washing, bound proteins were eluted with 0.1 M glycine-HCl. The eluates were run into an SDS-PAGE gel. A representative experiment is shown in Fig. 1B. In all elution experiments, a large amount of fH was eluted from the surface of all strains except strain 12, to which a smaller amount of fH had become bound. For the strongly fH-binding strains, it was still possible to elute bound fH after incubation in 0.1% of HIS (data not shown).

In these experiments, it could be seen that *F. necrophorum* was not only able to bind fH but also FHR-related proteins-1 and -4 (FHR-1, FHR-4) (Fig. 1B). In this experiment, however, it was not possible to analyze binding of fH-like protein-1 (FHL-1) because
of its comigration with FHR-1β. Therefore, we used FHR-1-deficient HIS to detect binding of FHL-1. By incubating bacteria in FHR-1-deficient HIS, we observed that *F. necrophorum* was able to bind FHL-1 (Fig. 1C). No FHL-1 binding was detected in strain 12.

**Flow cytometry analysis of fH binding**

To study if binding to the bacterial population was homogeneous, bacteria were incubated in 10% HIS and analyzed by flow cytometry. Binding was detected by mAb 196X against fH and Alexa Fluor 488-labeled secondary Ab. The flow cytometry assay results were in line with the direct binding and serum elution assays. All strains bound fH, but strain 12 bound clearly less than the other strains. The representative strains 3, 5, 10, and 12 are shown in Fig. 2.

**Binding of 125I-fH is ionic strength-dependent**

To study the salt sensitivity of the fH-*F. necrophorum* interaction, we used in a direct binding assay increasing concentrations (150–600 mM) of NaCl in the buffer. As shown in Fig. 3A, the binding of fH to *F. necrophorum* strain 5 decreased with increasing NaCl concentrations. When NaCl concentration increased from 150 to 300, 450, and 600 mM, we obtained 41%, 62%, and 78% inhibition of 125I-fH binding, respectively. This result indicates that the interaction between *F. necrophorum* and fH is ionic in nature.

**Binding of fH to *F. necrophorum* is specific**

To study the specificity of the interaction between *F. necrophorum* and fH, a competition assay with unlabeled fH was employed. Increasing concentrations of unlabeled fH (100 and 300 nM) inhibited binding of radiolabeled fH to *F. necrophorum* strain 5 by 36% and 70%, respectively (Fig. 3B). In the negative control, BSA was unable to inhibit 125I-fH binding. This result suggests that the interaction between *F. necrophorum* and fH is specific in nature.

*F. necrophorum* has two binding sites on fH

To further localize the sites on fH needed for binding to fusobacteria, recombinant fH constructs were used. Equimolar amounts (0.33 μM) of recombinantly expressed SCR1–5, 1–6, 1–7, 8–11, 11–15, 15–20, and 8–20 were incubated with strain 5, and bound proteins were eluted with an acid buffer. Eluates were separated by SDS-PAGE gel and analyzed by Western blotting using a polyclonal anti-fH Ab. With this method, no binding of SCR8–11 or SCR11–15 was seen, but SCR1–5, 1–6, 1–7, 8–20, and 15–20 fragments were bound by *F. necrophorum* (Fig. 4A). These data, in addition to the results of the binding of FHL-1, FHR-1, and FHR-4, pointed to two distinct binding domains, one located in the N-terminal part and the second in the C-terminal part of fH. The N-terminal binding site is located on SCR5–7 and the second site on SCR15 and SCR20. To confirm this result and further map the C-terminal site, *F. necrophorum* strain 5 was incubated with recombinant SCR5–7 and SCR19–20. Elution and analysis by Western blotting confirmed the presence of two binding sites: one at SCR5–7 and a second at SCR19–20.
of fH (Fig. 4B). Binding of the SCR15–18 region could not be excluded but was considered unlikely.

**Fusobacteria-bound fH remains functionally active**

Factor H inhibits the AP of C by three different mechanisms: it accelerates the decay of AP convertase, it competes with factor B for binding to C3b on self-cell surfaces, and cell-bound fH is also acting as a cofactor for factor I-mediated degradation of C3b. In a cofactor assay, we studied the functional activity of *F. necrophorum*-bound fH. The inactivation of C3b by factor I, in the presence of fH, was observed by the cleavage of the C3b α′-chain into α′67-, α′43-, and α′41-kDa bands. For the strongly fH-binding strain 5, cleavage of C3b was observed after incubation in 10%, 1%, and 0.1% HIS. For the weakly and intermediately binding strains, cleavage was seen only after incubation in 10% HIS. A positive control, consisting of 125I-C3b, factor I, and fH, was included in each series. 125I-C3b incubated without factor I, and fH was used as a negative control.

**Protection of *F. necrophorum* against C attack**

To test whether fH binding offered any survival advantage to *F. necrophorum*, we studied the kinetics of the bacterial survival in NHS. Because all strains were resistant to killing after a 1-h incubation in 20% NHS and also because all strains bound fH, we increased the percentage of NHS to 75. We tested four *F. necrophorum* strains: 3 and 5 (strongly fH-binding strains), 10 (intermediately fH-binding strain), and 12 (weakly fH-binding strain). Bacteria were incubated in 75% NHS or HIS. At 0, 1, 2, and 3.5 h, the bacteria were removed and cultivated on FAA plates for 48 h under anaerobic conditions. No clear difference was observed between the groups after 1 h (Fig. 6A). No clear difference was observed between the strongly and intermediately fH-binding groups at any time point. However, strain 12 was the most sensitive to serum killing: the survival at 2 and 3.5 h was 27% and 11%, respectively.

**FIGURE 5.** Bound fH is active as a cofactor for C3b inactivation. Three strains were tested: the weakly fH-binding strain 12, the intermediately fH-binding strain 10, and the strongly binding strain 5. Bacteria were incubated with HIS (final concentrations 10%, 1%, or 0.1%) and, after washes, with factor I and 125I-C3b. The cleavage of C3b is visualized by the disappearance of the 110-kDa α′ band and by the appearance of α′67-, α′43-, and α′41-kDa bands. For the strongly fH-binding strain 5, cleavage of C3b was observed after incubation in 10%, 1%, and 0.1% HIS. A positive control, consisting of 125I-C3b, factor I, and fH, was included in each series. 125I-C3b incubated without factor I, and fH was used as a negative control.

**FIGURE 7.** Binding of fH hinders the deposition of C3b on the surface of *F. necrophorum*. *A*, Strains 5 and 12 were incubated for 5 min in 50% NHS. After washing, bacteria were stained with a polyclonal anti-C3cAb and an Alexa Flur 488-labeled secondary Ab and analyzed by flow cytometry. Less C3b was deposited on the strongly fH-binding strain 5. *B*, A similar experiment was done in the presence of Mg-EGTA, which blocks the classical and lectin pathways. A higher amount of C3b was deposited on the weakly fH-binding strain surface. The results are shown as mean values ± SD of two separate experiments.
To eliminate the role of the classical pathway, we blocked it by using Mg-EGTA in the serum (final concentration 5 mM MgCl₂, 10 mM EGTA). Under these conditions AP is the only active C pathway. Two strains (5 and 12) were tested (Fig. 6B). Both strains were more resistant to killing in Mg-EGTA serum than in NHS, indicating that the classical pathway plays also a role in the killing of *F. necrophorum*. However, strain 12 was less resistant than the strongly fH-binding strain 5 to AP killing. At 3.5 h, only 34% of strain 12 survived vs 67% for strain 5. The correlation between survival and the fH binding suggests that the binding of fH contributes to the protection of *F. necrophorum* against C attack.

**Binding of fH prevents the deposition of C3b on the surface of *F. necrophorum***

To further investigate whether fH binding confers protection against C activation and specifically against C3b deposition, we compared the deposition of C3b between strains 5 and 12. After incubation in 50% NHS for 1, 2, 3, 4, 5, 10, and 30 min, the fusobacteria were washed and stained with a polyclonal anti-C3c Ab and an Alexa Fluor 488-conjugated secondary Ab. C3b deposition on the fusobacterial surfaces was fast and complete in 5 min (data not shown). The weakly fH-binding strain 12 was more susceptible to C3b deposition than was the strongly fH-binding strain 5 (Fig. 7A). The difference was observed at each time point. In Mg-EGTA serum more C3b was deposited on the surface of strain 12 than on strain 5 (Fig. 7B). This suggests that bound fH on strain 5 inhibits C activation and protects the bacteria against C3b deposition.

**Kinetics of MAC formation on the surface of *F. necrophorum***

The formation of MAC on *F. necrophorum* surface was studied by flow cytometry. *F. necrophorum* strains 5 and 12 were incubated in NHS for 3, 5, 10, and 30 min at 37°C with agitation. After washes, they were stained with a mAb against C5b-9 neoepitope and an Alexa Fluor 488 detection Ab. FACS analysis showed that MAC formation on the surface of both *F. necrophorum* strains was slower than C3b deposition. C3b deposition was complete in 5 min, but MAC formation started at 5 min and the process still continued at 30 min (Fig. 8). The mean fluorescence intensity (MFI) observed for strain 5 at 30 min was similar to the MFI observed for strain 12 at 10 min. At each time point a clear difference between strains 5 and 12 was observed. Consistently, less MAC was formed on strain 5.

**Clinical correlation***

The analysis of the clinical data and fH-binding tests pointed to a correlation between the severity of the disease and the ability of the strains to bind the AP inhibitor. Interestingly, patients presenting the most severe disease were carriers of strongly fH-binding strains (Table I). All patients who developed a distant infection or an intravascular complication carried a strongly fH-binding strain. Strains 5 and 6, involved in the most severe cases (cardiorespiratory failure, disseminated intravascular coagulation, and multiorgan failure followed by death), bound also strongly to fH.

Compared with others, the weakly fH-binding strain 12 was involved in a less severe disease. The patient had facial cellulitis. For all of the other strains, the port of entry was clearly the oropharynx. However, for strain 12 no primary infection in the oropharynx was reported. No complications, such as abscess, distant infection, or intravascular thrombosis, were detected. This strain was less resistant than the other strains in the serum killing assay. Thus, the decreased capacity to bind fH may contribute to the reduced serum resistance of strain 12 and a milder clinical picture.

### Table I. Clinical characteristics of patients with *F. necrophorum* sepsis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Age</th>
<th>Gender</th>
<th>Primary Infection</th>
<th>Complications</th>
<th>Intravascular Thrombosis</th>
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</tr>
<tr>
<td>1</td>
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<td></td>
</tr>
<tr>
<td>12</td>
<td>15</td>
<td>Male</td>
<td>Facial cellulitis</td>
<td>None</td>
<td>None</td>
</tr>
</tbody>
</table>

*After collection of clinical data, each patient was considered by age, sex, primary infection, and presence of complications such as abscess, distant infections, and intravascular thrombosis. The strains are ranked into three groups: strong, intermediate, or weak binding of fH. The carriers of strongly fH-binding strains were more susceptible to develop a severe infection complicated by a distant infection.

⁵ Disseminated intravascular coagulation. Patient died 7 days after arrival to hospital due to multi-organ failure.
Discussion

Fusobacteria are potentially invasive anaerobic bacteria that can cause severe infections. In this study we have shown that F. necrophorum subsp. funduliforme is able to resist cytolytic C attack in serum and to bind the fluid phase C inhibitor fH. Strains that had caused severe infections bound fH more strongly. Factor H-binding occurred through two separate sites, one located on the N-terminal part and another on the C terminus of fH. F. necrophorum-bound fH remained active as a cofactor for factor I in the cleavage of C3b, thereby promoting the survival of F. necrophorum in serum. Less C3b deposition and a delay in MAC assembly were observed on a strain that bound fH strongly (no. 5), compared with a weakly binding strain (no. 12).

F. necrophorum subsp. necrophorum is an animal pathogen. It has been only rarely isolated from human beings (21). F. necrophorum subsp. funduliforme, related to both animal and human infections, has been less studied than the subspecies funduliforme (22, 23). In our study, all strains belonged to the subspecies funduliforme. For a long time, F. necrophorum has been considered as part of the normal oropharyngeal flora. However, there is actually no supporting data and this is under reevaluation (24). A recent study, using molecular biology tools for the detection of F. necrophorum, showed that fusobacteria could be part of normal flora with a low carriage percentage in the control cohort (15). This raises the question of why certain fusobacterial strains become pathogenic and cause invasive infections.

A strictly anaerobic pathogen usually needs special environmental conditions, such as tissue hypoxia caused by trauma, concomitant infection, or neoplasia, to invade the host and cause disease. F. necrophorum seems to be different and can, on its own without these environmental conditions, be pathogenic (25). It is a strictly anaerobic primary pathogen causing local infections, such as acute or chronic tonsillitis, but also life-threatening diseases, such as Lemierre’s syndrome. The invasive and severe diseases affect most commonly healthy young adults. The mechanisms used by F. necrophorum to escape innate immunity and to invade into tissues of young previously healthy adults are unknown.

Complement evasion mechanisms of microorganisms have been actively studied during the last decade. One well-known mechanism is the binding of fluid phase C inhibitors, fH and C4BP. The AP of C is continuously activated at a low rate and a small amount of C3b is constantly deposited on surfaces. The AP is also efficiently recruited via activation of the classical pathway, for example, on the surfaces of microbes. Factor H down-regulates the AP in a highly effective way. It acts as a cofactor for factor I-mediated degradation of C3b, accelerates the decay of the AP C3 convertase, and competes with factor B by exhibiting a strong affinity for C3b deposited on surfaces rich in polyanions. Thus, fH prevents the formation and decays the AP C3 convertase on self surfaces. Pathogenic microbes, on the other hand, express fH-binding molecules and thereby utilize fH binding as a C escape mechanism to improve their survival in the host.

Several aerobic bacteria, parasites, and some fungi have been described to bind fH (9). Little is known about the C escape mechanisms of anaerobic bacteria. In our experiments, most F. necrophorum strains were able to bind fH to their surfaces. This result was confirmed by three independent techniques using human serum or purified fH protein. The level of fH binding by F. necrophorum was in the same range as that by S. pneumoniae, which has previously been shown in several studies to bind fH (12). However, there was variation in the strength of fH binding between strains. One of the strains (strain 12) differed from the other 11 strains by binding clearly less fH. This strain was isolated from a patient with a less severe disease (facial cellulitis). These data suggest variability in the surface components between the strains and correlation of fH binding to virulence of the bacteria.

Previously studied interactions between fH and bacteria point to one or more binding sites. Binding sites have been identified on the N-terminal (SCRs 1–7), C-terminal (SCRs 15–20), or in the middle part of fH (SCRs 8–15) (14, 26, 27). To localize the binding site for F. necrophorum on fH, we used three different assays. First, fH, FHR-1, and FHR-4 binding from serum was observed in the fH-binding assay. By using FHR-1-deficient serum in the same technique, binding of FHL-1 was also detected. Domains 1–5 of FHR-1 are homologous with SCR 6, 7, 18, 19, and 20 of fH and those of FHR-4 with SCRs 6, 8, 9, 19, and 20 of fH (28). A new member of the fH protein family, called FHR-4A, was recently described (29). It expresses the same homology with fH than does FHR-4 and possesses four more SCR domains than FHR-4, the alternatively spliced product of the same gene. FHL-1 is identical to the seven most N-terminal SCRs of fH and possesses four unique amino acids at the C terminus (30). The binding of FHL-1 by F. necrophorum suggests the possibility of an N-terminal binding site, whereas the binding of FHR-1 and FHR-4 suggests the possibility of a C-terminal binding site. Confirmation of these results was obtained by experiments with recombinant constructs of fH. F. necrophorum was found to bind fH via two separate sites. An N-terminal binding site is located within SCRs 5–7. No binding was observed in the middle part (SCRs 8–15) of fH. Of the C-terminal constructs, F. necrophorum bound to SCR fragments 15–20 and 19–20. Thus, two separate binding sites on fH were clearly identified for F. necrophorum: one at the N terminus (SCRs 5–7) and a second one at the C terminus (SCRs 19–20). However, we cannot totally exclude further binding sites on SCRs 1–4 and SCRs 15–18. Factor H thus appears to bind to unknown receptor(s) on F. necrophorum in a multipoint fashion.

Factor H plays an important role in the inactivation of deposited C3b. Factor I is the mediator of the reaction but it requires a cofactor: fH, FHL-1, membrane cofactor protein (MCP; CD46), or C receptor type 1 (CR1; CD35). The site on fH for cofactor activity for factor I-mediated degradation of C3b is located on the N-terminal of fH on SCRs 1–4 (31). The C3b binding sites are located on SCRs 1–4 but also on SCRs 8–15 and 20 (17, 32). Our experiments showed that F. necrophorum-bound fH remained functionally active as a cofactor for factor I. This result indicates that even if the F. necrophorum binding site at SCRs 5–7 is located near the active site, at the CR1–4 region, bound fH can be still active.

When fusobacteria were incubated in NHS and in Mg-EGTA serum to focus on AP activation, less C3b was deposited on strain 5 than on strain 12. This is in accordance with the fact that strain 5 exhibited a strong binding to fH and strain 12 bound fH only weakly. In addition to its cofactor activity in the inactivation of deposited C3b, fH competes with factor B for binding to C3b. Both mechanisms inhibit the formation of AP C3 convertase and the amplification loop. The consequences are that less C3 is cleaved and thus less C3b is deposited, as observed for strain 5.

As fusobacteria were observed to survive in human serum, we also wanted to explore if the terminal C pathway and MAC formation were affected by the binding of fH to F. necrophorum. When a strongly fH-binding strain (strain 5) was compared with a weakly binding strain (strain 12), a delay in the kinetics of MAC formation on strain 5 was observed in the FACS assay. Because other regulators (e.g., clusterin and vitronectin) are involved in the regulation of the terminal pathway, we cannot connect these results exclusively to the binding of fH (33, 34). Inhibition of AP C3 convertase generation would inhibit MAC formation, but further
work is needed to fully determine the role of fH binding in the delay of MAC assembly.

All 12 F. necrophorum strains studied were isolated from blood. This shows their capacity to survive in an extremely inhospitable environment. In our serum killing assay, all strains were serum resistant and had an excellent survival at 1 h. However, at 2 and 3.5 h, the weakly fH-binding strain 12 died more readily than did the other strains. The protective role of fH binding has been previously described for many pathogens (9), and it clearly appears that fH binding confers protection also to F. necrophorum against C attack. However, bacteria can have multiple serum resistance mechanisms (9). The fact that all strains, even strain 12, were resistant to killing in fresh serum points out the possible additional role of other C evasion mechanisms.

F. necrophorum is a pathogen causing local infections (e.g., dental infections, acute or chronic tonsillitis, and invasive pathology) (1, 15, 35, 36). Its role has also been discussed in Noma that affects malarious young children as a unilateral ulcer of the cheek rapidly progressing to perforation (37). In 1936 Lemierre described a severe postanginal sepsis, which was almost always fatal (2). In this preantibiotic area, the syndrome was described in a complete form with a primary oropharyngeal infection followed by a thrombophlebitis of the tonsillar vein followed by internal jugular vein thrombophlebitis, septicemia, and distant infections in lungs, joints, muscles, kidneys, spleen or liver. Nowadays, incomplete syndromes have been described without distant infections and/or internal jugular vein thrombophlebitis. These cases have still been recognized as Lemierre’s syndrome, and the definition of Lemierre’s syndrome 80 years after the first description is still confusing (5, 8). In our data, three cases presented with all the symptoms (cases 3, 4, and 5). However, four cases could be named Lemierre’s syndrome (cases 1, 2, 6 and 9). The strains involved in these seven cases exhibited a strong fH-binding ability. Even if Lemierre’s syndrome could not have been confirmed, also the other strains had invasive capacity and had caused a severe disease with sepsis.

Interestingly, clinical data showed that the carrier of strain 12 developed an atypical disease when compared with other patients. The final diagnosis was facial skin cellulitis. No primary infection was identified by microbiological tests. The carrier of strain 12 developed an atypical disease when compared with other patients. The final diagnosis was facial skin cellulitis. No primary infection was identified by microbiological tests.

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Disclosures

The authors have no financial conflicts of interest.

References


