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Interaction with C4b-Binding Protein Contributes to Nontypeable *Haemophilus influenzae* Serum Resistance

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Complement evasion by various mechanisms is important for microbial virulence and survival in the host. One strategy used by some pathogenic bacteria is to bind the complement inhibitor of the classical pathway, C4b-binding protein (C4BP). In this study, we have identified a novel interaction between nontypeable *Haemophilus influenzae* (NTHi) and C4BP, whereas the majority of the typeable *H. influenzae* (a-f) tested showed no binding. One of the clinical isolates, NTHi 506, displayed a particularly high binding of C4BP and was used for detailed analysis of the interaction. Importantly, a low C4BP-binding isolate (NTHi 69) showed an increased deposition of C3b followed by reduced survival as compared with NTHi 506 when exposed to normal human serum. The main isoform of C4BP contains seven identical α-chains and one β-chain linked together with disulfide bridges. Each α-chain is composed of eight complement control protein (CCP) modules and we have found that the NTHi 506 strain did not interact with rC4BP lacking CCP2 or CCP7 showing that these two CCPs are important for the binding. Importantly, C4BP bound to the surface of *H. influenzae* retained its cofactor activity as determined by analysis of C3b and C4b degradation. Taken together, NTHi interferes with the classical complement activation pathway by binding to C4BP. *The Journal of Immunology*, 2007, 178: 6359–6366.

*Haemophilus influenzae* is a Gram-negative human specific pathogen responsible for a variety of infections. Encapsulated *H. influenzae* strains belong to one of six serotypes (a-f), of which type b (Hib) is the most virulent (1). Invasive disease (bacteremia and meningitis) and epiglottitis are the most serious and sometimes life-threatening conditions, which are caused by encapsulated *H. influenzae*, mainly serotype b (2). In contrast, nontypeable *H. influenzae* (NTHi) accounts for the majority of local manifestations and respiratory tract infections (e.g., bronchitis, sinusitis, and acute otitis media) and is, after pneumococci, the second most common pathogen isolated from children with acute otitis media (1). NTHi is also the main cause of acute exacerbations in patients with chronic obstructive pulmonary disease and bronchiectasis (3). In addition, NTHi is also frequently found in patients with cystic fibrosis (4, 5).

The polysaccharide capsule of the typeable *H. influenzae* is an important virulence factor that protects against complement and phagocytosis (6, 7). LPS is another major virulence factor in *H. influenzae*, which is important for colonization, bacterial persistence, and survival in the circulatory system (8, 9). Other important virulence determinants are fimbriae and outer membrane proteins. *Haemophilus* adhesion and penetration protein, high molecular weight proteins 1 and 2, *Haemophilus* surface fibrils (Hsf), *H. influenzae* adhesin, and hemagglutinating pil are major adhesins involved in colonization identified in Hib and/or NTHi (10, 11). In addition, Hsf that is expressed by encapsulated *H. influenzae* has been reported to be involved in serum resistance (12). Hsf binds vitronectin, which is a regulator of the terminal complement pathway. Vitronectin inhibits membrane attack complex (MAC) formation and thereby prevents complement-induced lysis.

The complement system is the first line of defense against pathogenic microorganisms. Activation of complement leads to a cascade of protein activation and deposition on the surface of the pathogen, resulting in formation of the MAC and opsonization of the pathogen followed by phagocytosis. Invading pathogens activate complement either spontaneously due to differences in envelope/membrane composition compared with host (alternative and lectin pathways) or through Ab binding (classical pathway). All three pathways lead to the formation of complement factor 3 (C3) convertase, with subsequent cleavage of C3 to C3a (anaphylatoxin) and C3b (opsonin). Thereafter, they follow the same terminal pathway, which is a key step in producing an inflammatory response.

C4b-binding protein (C4BP) is a fluid-phase inhibitor of the classical and the lectin pathways of complement and inhibits the formation and accelerates the decay of C3 convertase (C4bC2a). It also serves as a cofactor to factor I in the proteolytic degradation of C4b and C3b (13–15). C4BP is a large plasma glycoprotein that exists in several forms varying in subunit composition. The major form consists of seven identical α-chains (70-kDa subunits) and one β-chain (45 kDa) (16). The α- and β-chains are composed of repeating domains of ~60 aa known as complement control protein (CCP) domains (17, 18). C4BP is also linked to the coagulation system because the β-chain is bound to the vitamin K-dependent anticoagulant protein S. Most C4BP in blood circulates in a 1:1 high-affinity noncovalent complex with protein S (16).
The pathogenesis of many microorganisms relies on the capacity of pathogens to avoid, resist, or neutralize the host defense including the complement system. Therefore, many pathogens have evolved different mechanisms to conquer complement. Some pathogens are able to inactivate complement components, whereas others produce proteins or other components that mimic complement inhibitors (19). Another frequent strategy used by some pathogens is binding of complement inhibitors such as C4BP, which protects from complement attacks. In fact, a number of microorganisms, including Streptococcus pyogenes, Escherichia coli K1, Neisseria meningitidis, Bordetella pertussis, and Moraxella catarrhalis have been reported to bind C4BP (20–24).

The aim of the present study was to examine complement resistance mechanisms in H. influenzae. We have found that most of the NTHi strains bound C4BP, whereas the majority of the typeable strains showed no binding. The binding site for our model NTHi strain 506 was localized within the CCP2 and CCP7 domains of the C4BP α-chain. By investigating the degradation of C3b and C4b, C4BP bound to the surface of NTHi was found to have preserved cofactor activity. Most importantly, the C4BP-binding strain was more resistant to killing by serum than the non-binding strain.

Materials and Methods

Bacterial strains and culture conditions

A total of 37 bacterial strains were included in the present study: 21 typeable H. influenzae, 14 NTHi, and 2 Haemophilus parainfluenzae. The bacterial strains were isolated from clinical specimens of human origin. All strains were characterized by standard bacteriological techniques including the oxidative test, the fermentation test, gram staining, the satellite test, and the nitroblue tetrazolium adenine dinucleotide and hemin (XV) test. Thereafter, the H. influenzae strains were typed by PCR using specific primers for each capsular type. Clinical isolates were obtained from the Department of Medical Microbiology (University Hospital Malmö, Lund University, Malmö, Sweden) and the State Serum Institute (Copenhagen, Denmark). Type strains were obtained from the Culture Collection University of Gothenburg (CCUG: Department of Clinical Bacteriology, Sahlgrenska Hospital, Gothenburg, Sweden). Bacteria were routinely cultured in brain heart infusion (BHI) liquid broth supplemented with nicotinamide adenine dinucleotide 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

Human C4BP was purified from human plasma (25). Recombinant wild-type C4BP (rC4BP) was expressed in human kidney cells 293 (ATCC CRL-1573, American Type Culture Collection), and purified using affinity chromatography with a mAb directed against the α-chain of C4BP, C3b-like molecules (C3met) and C4b-like molecules (C4met) were prepared by incubation of purified C3 or C3 with 100 mM methanolamine (pH 7.6) for 1 h at 37°C and subsequent dialysis against 100 mM Tris-HCl and 150 mM NaCl (pH 7.5). It has been demonstrated previously that C4 molecules treated this way are functionally equivalent to the C4b that results from cleavage by C1s component of the complement pathway (26). Throughout the study, the C3met and C4met derivatives are used but will be referred to as C3b and C4b for reasons of clarity. The construction and characterization of recombinant mutants of C4BP lacking individual CCP domains has been described previously (27). The polyclonal rabbit anti-human C4BP polyclonal Ab (pAb), the mouse mAb (mAb 104) against CCP1 of C4BP, and the mouse mAb (mAb 67) against CCP4 were a gift of Prof. B. Dahlbäck (Lund University, Malmö, Sweden).

Flow cytometry analysis

The capacity for different Haemophilus strains to bind C4BP purified from human plasma was analyzed by flow cytometry. Bacteria were grown in BHI liquid broth overnight and washed once in PBS containing 2% BSA (PBS-BSA) (Saveen Werner). Bacteria (10^7) were incubated with 50 μg/ml C4BP in PBS-BSA for 1 h at 37°C. After two washes, the bacteria were incubated with 0.5 μg/ml anti-C4BP/Ab pAb for 30 min on ice. Thereafter, the bacteria were washed twice and incubated for 30 min on ice with FITC-conjugated goat anti-rabbit pAb (Dakopatts). After two additional washes, bacteria were analyzed in the flow cytometer (EPICS XL-MCL; Coulter). All incubations were kept in a final volume of 100 μl of PBS-BSA and the washings were done with the same buffer. The anti-C4BP pAb and FITC-conjugated anti-rabbit pAb were used as a negative control for each strain analyzed. To determine the efficiency of NTHi binding to C4BP, two of the nontypeable strains, NTHi 506 and NTHi 69 (a high C4BP binder and a low C4BP binder, respectively), were incubated with increasing concentrations of C4BP (range: 1–100 μg/ml).

To monitor deposition of Abs and complement on bacterial surfaces during the course of the bactericidal kinetic assay, bacteria removed from the serum mixture were stained for the presence of IgG, IgM, and complement component C3, and analyzed by flow cytometry. The cell-serum mixture (0.2 ml) was after an incubation for 7.5, 15, or 60 min diluted in 1 ml of PBS containing 3% fish gelatin (Sigma-Aldrich). Bacteria were centrifuged and incubated on ice for 30 min either with FITC-conjugated anti-human IgG pAb (Dakopatts), rabbit anti-human IgM pAb (Dakopatts), or with rabbit anti-human C3 pAb (Dakopatts). After two additional washes, bacteria were analyzed by flow cytometry.

Protein labeling and direct binding assay using 125I-labeled C4BP

Purified C4BP was labeled with 0.05 M iodine (GE Healthcare) per mole of protein, using the chloramine T method (28). The specific activity of 125I-labeled C4BP was 1.5 × 10^7cpm/μg. The different H. influenzae strains were grown overnight in BHI liquid broth and washed in PBS-BSA. Bacteria (2 × 10^7) were incubated with 125I-labeled C4BP at 37°C for 1 h with shaking. After incubation, the bacteria were centrifuged (10,000 × g) through 20% sucrose. The tubes were frozen, cut, and radioactivity in pellets and supernatants was measured in a gamma counter. Binding was calculated as amount of bound radioactivity (pellet) vs total radioactivity (pellet plus supernatant). In the competitive binding assays, rC4BP, BSA, heparin (Sigma-Aldrich), or C4met were added to the reaction.

Determination of the time course of C4BP binding

A 50-ml tube containing 20 ml of BHI was inoculated with 500 μl of an overnight culture of the NTHi 506 in the same medium. The tube was incubated at 37°C with slow-over-end rotation and the first sample was collected after 2 h and subsequently after every hour. The OD650 of each sample was measured to establish a growth curve, followed by analysis of C4BP binding by the direct binding assay using 125I-labeled C4BP.

Binding of C4BP from serum

NTHi 506 was grown overnight and washed with Veronal-buffered saline (VBS) (gelatin-Veronal buffer (GVB): 142 mM NaCl, 1.8 mM sodium barbital, 3.3 mM barbituric acid (pH 7.4)) containing 0.1% fish gelatin (Sigma-Aldrich). Bacteria (10^7) were incubated with 1% serum or GVB at 37°C for 30 min under shaking. After washing five times with GVB to remove unbound proteins, the bacterial pellet was resuspended in 50 μl of 0.1 M glycine-HCl (pH 2.5) to elute bound proteins. After 20 min of incubation at 37°C with shaking, the bacteria were centrifuged; supernatants were transferred to a new tube and neutralized with 1 M Tris-HCl (pH 9.5). The supernatants were run into 5% SDS-PAGE gel under nonreducing conditions. The proteins were transferred to a polyvinyldiene difluoride membrane (Pall Life Sciences). Unspecific binding sites were blocked using 3% fish gelatin (Nordic) and the membrane was incubated with the primary Ab mAb 104 directed against CCP1 of C4BP. After washing, an alkaline phosphatase-conjugated rabbit-anti-mouse Ab (Dokopatts) was added and the blots were developed using a chromogenic substrate.

C4b-degradation assay

NTHI 506 (5 × 10^8) was incubated with 300 μg/ml C4BP 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 250 nM C4met or 150 μg/ml C3met, 60 mM factor I, and trace amounts of 125I-labeled C4b or C3b in 50 μl of buffer. As a positive control, 100 nM C4BP was used in fluid phase instead of the preincubated bacteria. As a negative control, the NTHI 506 without preincubation with C4BP 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 run on a SDS-PAGE. Thereafter, the gel was dried and the proteins were visualized with a Personal FX (Bio-Rad) using intensifying screens.

ELISA

To estimate which CCP domain of C4BP that is involved in the binding of NTHI 506, microtiter plates (F96 Maxisorp; Nunc-Immuno Module) were coated with bacteria (10^5) for 1 h at 37°C. The plates were washed four
times with PBS containing 0.05% Tween 20 (PBS-Tween) and blocked for 1 h with PBS-Tween 20 supplemented with 1.5% OVA (PBS-OVA). After washing, the plates were incubated for 1 h at room temperature (RT) with rC4BP mutants (each lacking one CCP) at a concentration of 45 μg/ml each or a set of mutants with mutations affecting positively charged residues at the CCP1–2 domain interface at a concentration of 10 μg/ml. Thereafter, the wells were washed and incubated with mAb 104 and/or mAb 67 in PBS-OA for 1 h at RT. After additional washings, HRP-conjugated and anti-mouse pAbs (Dakopatts) were added for 40 min at RT. The wells were washed, developed with 20 mM tetramethylbenzidine, and the absorbance was measured at 450 nm with a MRX Microplate reader (Dynatech Laboratories). For each experiment, samples were analyzed in triplicate.

Deposition of complement factors on NTHi

Normal human serum (NHS) was prepared and pooled from five healthy volunteers. The blood was allowed to clot for 30 min at RT and thereafter incubated on ice for 60 min. After centrifugation, the sera were pooled, aliquoted, and stored at −70°C. Blood samples were taken from blood donors after an informed consent was obtained.

Bacteria were grown overnight (10^6 CFU/ml). The bacterial suspension (0.2 ml) was added to a mixture of 0.2 ml of NHS and 0.4 ml of sterile GVB (2.5 mM Veronal buffer (pH 7.3), 150 mM NaCl, 0.1% gelatin, 1 mM MgCl2, and 0.15 mM CaCl2). Aliquots of this mixture were removed after 7.5, 15, or 60 min of incubation and the bacteria were stained for deposition of Abs or complement factor C3 and analyzed by flow cytometry. To compare C3b deposition generated by the classical/lectin and the alternative pathways, the bacteria were incubated with 1% NHS diluted in either GVB (allows the classical and lectin pathways) or Mg-EGTA (2.5 mM Veronal buffer (pH 7.4), containing 70 mM NaCl, 140 mM glucose, 0.1% gelatin, 7 mM MgCl2, and 10 mM EGTA) (allows the alternative pathway only due to lack of calcium) for 1 h at 37°C. Deposited C3b was detected by 1 h incubation on ice with specific pAbs (Dakopatts) followed by flow cytometry.

Serum bactericidal assay

Human serum deficient in the C4BP was prepared by passing fresh serum through a HiTrap column (GE Healthcare) coupled with mAb 104 against C4BP and the α-chain of C4BP (29). The flow-through was collected and the depleted serum was analyzed by ELISA for the absence of C4BP and stored in aliquots at −70°C. The NTHi strains were diluted in dextrose-GVB (DGVB) (2.5 mM Veronal buffer (pH 7.3) containing 70 mM NaCl, 140 mM glucose 0.1% (w/v) gelatin, 1 mM MgCl2, and 0.15 mM CaCl2) or Mg-EGTA buffers. Bacteria (10^6 CFU) were incubated in 10% of NHS diluted either in either DGVB (allows the classical and lectin pathways) or Mg-EGTA (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, CFU were determined.

Results

NTHi strains bind human C4BP

Several bacterial species have been shown to bind C4BP (22, 24, 30). To determine whether H. influenzae interacts with C4BP, different H. influenzae strains were incubated with C4BP followed by flow cytometry analysis using a polyclonal anti-C4BP antiserum. Interestingly, most of the NTHi strains bound C4BP in contrast to typeable strains, which displayed significant binding only in a few cases (Fig. 1A). To further confirm the interaction between C4BP and H. influenzae by a different method, 125I-labeled C4BP was added to the different strains and bound protein was separated from the one remaining in a solution. Binding was calculated as ratio of bound radioactivity vs total radioactivity added. The results obtained with this assay were in a good agreement with data obtained with flow cytometry (Fig. 1B). In our collection, the NTHi 506 showed the highest C4BP binding in both flow cytometry (84%) and in the direct binding assay using 125I-labeled C4BP (26%), whereas the other NTHi strains bound C4BP to a varying extent. The majority of the encapsulated H. influenzae serotypes (a-f) were low or nonbinders. The NTHi bound between 15 and 79 C4BP molecules/cell (Fig. 1B, inset). To determine the expression kinetics of the C4BP-binding protein in H. influenzae, aliquots were taken from the culture medium of NTHi 506 at different time points and analyzed by a direct binding assay. The highest C4BP binding was seen in stationary phase, after culturing bacteria overnight (Fig. 2). The NTHi 506 bound C4BP in a dose-dependent manner at concentrations of 1–100 μg/ml C4BP reaching saturation at 10 μg/ml C4BP (Fig. 3A). In contrast, no significant binding of C4BP was observed even at the highest concentration used with NTHi 69, which was chosen as a nonbinding NTHi strain for additional experiments. The experiments shown in Figs. 1 and 3A were performed using purified C4BP in the absence of serum. To analyze whether NTHi 506 also bound C4BP directly from serum in the presence of all serum proteins, we incubated the bacteria with 1% NHS or VBS buffer. Thereafter, bound proteins were eluted, separated by SDS-PAGE gel, and analyzed by Western blot using a mAb against C4BP. As can be seen in Fig. 3B, NTHi 506 bound C4BP from 1% human serum.
The ionic interaction between NTHi 506 and C4BP is mediated by CCP2 and CCP7 of C4BP α-chain

To test the specificity of the C4BP binding to NTHi 506, bacteria were incubated with increasing amounts of unlabeled rC4BP or BSA (0–1000 nM) in addition to 125I-labeled C4BP. rC4BP inhibited the binding of 125I-labeled-C4BP to NTHi 506 in a dose-dependent manner, whereas BSA did not affect the binding (Fig. 4A). C4met, which corresponds to the natural ligand of C4BP (i.e., C4b), also inhibited the binding of 125I-labeled C4BP to NTHi 506 in a dose-dependent manner (Fig. 4B). The binding was inhibited by 60% when C4met at a concentration of 1 nM was used.

To test whether heparin, another ligand of C4BP, could inhibit the interaction, increasing amounts of heparin were added to the incubation reaction with radiolabeled C4BP and bacteria. Heparin (0–100 nM) did not interfere with the interaction between NTHi 506 and 125I-labeled-C4BP (Fig. 4C). The binding between NTHi 506 and C4BP was based to a large extent on ionic interactions as increased concentrations of NaCl inhibited the interaction (Fig. 4D). When the salt concentration was increased from 50 to 150 mM, a 28% decreased binding was found, and a further NaCl increase from 150 to 500 mM resulted in a 65% decrease. Similar results were obtained by flow cytometry analysis (data not shown).

NTHi binds α-chains of C4BP as shown by the fact that recombinantly expressed C4BP, composed exclusively of α-chains and lacking the β-chain and consequently bound protein S, was efficiently bound by the bacteria (Fig. 5A; wild type (wt)). To evaluate which CCP domain of the C4BP α-chain was involved in the NTHi 506-C4BP interaction, eight mutant rC4BP proteins, each lacking individual CCP domains were analyzed for binding. Immobilized bacteria were incubated with equal amounts of the rC4BP mutants. Interestingly, binding of rC4BP lacking either CCP2 or CCP7 to NTHi 506 was most strongly and significantly (p < 0.001) decreased as compared with other mutants (Fig. 5A). All other CCP mutants showed significant binding. This binding test suggests that NTHi 506 binds to the same or overlapping region of the C4BP α-chain as C4b. To further analyze the binding to CCP2, a set of mutants with mutations affecting positively charged residues at the CCP1–2 domain were used. These mutants have previously been used to identify residues in C4BP important for binding of C4b and streptococcal M proteins (31, 32). Six single mutants and two multiple mutants representing combinations of the single mutations were used. In all mutants, one or more positively charged residues were replaced with polar Gln, which was expected and experimentally shown to be structurally well-tolerated. The binding abilities of all the mutants were significantly decreased.

The effect of increasing concentrations of unlabeled C4BP, C4b, heparin and ionic strength, on the binding of 125I-labeled C4BP to Haemophilus. NTHi 506 was incubated with 125I-labeled C4BP and increasing amounts of rC4BP, C4met, heparin, NaCl, or BSA, followed by centrifugation through a sucrose and determination of radioactivity associated with the bacterial pellet in a gamma counter. The C4BP binding of NTHi 506 in the absence of competitor was defined as 100%. The mean values of three experiments are shown with error bars indicating SD. A, rC4BP but not BSA inhibited the binding of 125I-labeled C4BP to NTHi 506. B, Increasing amounts of C4met inhibited the binding of 125I-labeled C4BP to NTHi 506. C, Heparin did not affect the binding of 125I-labeled C4BP to NTHi 506. D, NTHi 506 bound C4BP in a noncovalent manner and the interaction decreased with increasing concentrations of NaCl, implying that it is to a large extent based on ionic interactions. The binding is expressed as the percentage of binding observed in 50 mM NaCl.
Reduced as compared with the wild-type C4BP (Fig. 5B). The binding of the double (R64Q-R66Q) and the triple mutant (R39Q-R64Q-R66Q) was strongly reduced. These data indicate that the electrostatic cluster at the CCP1–2 domain interface is important for the interaction between NTHi 506 and C4BP.

C4BP exhibits factor I cofactor activity at the NTHi cell surface

C4BP regulates the classical pathway activity by serving as a cofactor for factor I in the degradation of C4b, which results in the appearance of the two fragments C4d and C4c. Upon cleavage, it has been demonstrated that C4d remains bound to the surface of the bacteria, but that C4c is released to the surrounding medium resulting in an increased C4d:C4c ratio at the bacterial surface (33). To analyze the cofactor activity of C4BP bound to H. influenzae, the NTHi 506 with or without addition of C4BP was incubated with factor I and 125I-labeled C4b or C3b. Soluble C4BP was used as a positive control and the density of the degradation product (C4d) was set to 100%. When 125I-labeled C4b was incubated in the presence of C4BP-coated NTHi 506, the degradation product C4d appeared (38% of the positive control) (Fig. 6A). However, very little C4d degradation (i.e., C4d was almost not formed) was seen with bacteria only (14% of the positive control). Thus, C4BP retained its cofactor capacity to degrade C4b when it was bound to NTHi. C4BP is also a cofactor for factor I-mediated cleavage of C3b (34). When 125I-labeled C3b was incubated in the presence of C4BP-coated NTHi 506, a 43-kDa degradation product was formed (41% of the positive control), which did not occur in the presence of bacteria alone (17% of the positive control) (Fig. 6B).

C3b is deposited on the surface of the low C4BP binding NTHi 69

To investigate whether the capacity of NTHi 506 to bind C4BP resulted in increased inhibition of the complement cascade as compared with nonbinding strain NTHi 69, C3b deposition on the bacteria was analyzed after incubation with NHS. NTHi 506 and 69 were incubated with NHS for 7.5 and 15 min. Thereafter, deposited C3b was detected with FITC-conjugated rabbit anti-C3 pAb and flow cytometry analysis. Interestingly, less C3b was deposited on the high C4BP binder NTHi 506 as compared with the low binder NTHi 69 at both time points (Fig. 7A). This implied that when NTHi 506 bound C4BP, the complement cascade was partly inhibited yielding less C3b deposition on the surface.

NTHi 506 is more serum resistant than NTHi 69

To test whether the difference in binding of C4BP had effect on serum resistance of the two strains, NTHi 506 and NTHi 69 were compared in a serum bactericidal assay in conditions allowing all complement pathways. The high C4BP-binding NTHi 506 strain was more resistant to NHS than the low C4BP-binding strain NTHi 69 (Fig. 7B). No survival was seen with the NTHi 69 after 10 min of exposure to NHS, whereas >80% of the NTHi 506 survived under identical conditions. To evaluate which complement pathways recognize H. influenzae, the survival of NTHi 506 was studied in serum diluted in DGVB++ or Mg-EGTA buffers, as the Mg-EGTA buffer inhibits the classical and the lectin pathways. When the classical and lectin pathways were inhibited, a significant increase in the survival of the bacteria was seen. After 20 min, 30% of the bacteria survived in NHS diluted in DGVB++ (Fig. 7B), while >70% survived in EGTA-treated serum (Fig. 7C). In addition, significantly less C3b was deposited on NTHi 506 when the serum was diluted in Mg-EGTA (mean fluorescence intensity 12.23) as compared with serum diluted in GVB++ (mean fluorescence intensity 60.79) (Fig. 7D). It appears that the classical/lectin pathways are the main ones recognizing NTHi 506 and therefore binding of C4BP should be of the major importance. To test whether the binding of C4BP indeed had effect on the serum resistance of the NTHi 506, C4BP-depleted serum was used. NTHi 506 was more serum sensitive when C4BP was depleted from the serum (Fig. 7E), indicating the importance for the bacteria to bind C4BP.

To investigate whether the observed difference in serum resistance between NTHi 506 and NTHi 69 could be dependent on differential binding of Abs, we analyzed deposition of IgG and IgM from the serum used in the bactericidal assay on these two strains. Both IgG and IgM were deposited on the high C4BP binder NTHi 506 and in lower amounts on the low binder NTHi 69 at both time points (Fig. 7F). Despite the high Ab deposition on the NTHi 506 as compared with the NTHi 69, this strain is more resistant to serum, which we propose is dependent on others on its ability to bind C4BP.
Discussion

In this study, we describe an interaction between NTHi strains and the complement inhibitor C4BP. Complement resistance is crucial for bacterial virulence and binding of complement inhibitors such as vitronectin, C4BP and factor H (FH) is an efficient strategy used by several serum resistant pathogens (12, 35). M. catarrhalis, S. pyogenes, and E. coli K1 are bacteria which express surface molecules binding C4BP protecting the bacteria against the classical/lectin pathways (22–24). In addition to inhibition of the classical/lectin pathways, several bacteria (e.g., Borrelia burgdorferi, Streptococcus pneumoniae, Borrelia hermsii; for reviews, see Refs. 36 and 37) bind FH and FH-like protein-1 molecules and hence are partially protected against the alternative complement pathway (38–40). Some microbes, e.g., group A streptococci, gonococci, B. pertussis and Candida albicans have been shown to bind both C4BP and FH (20, 30, 41, 42). Several studies have indicated that both complement factors and inhibitors are present in the respiratory tract (43, 44). Therefore, respiratory pathogens must protect themselves from complement in similarity to blood-borne pathogens and consequently these microbes also have access to complement inhibitors. NTHi and H. influenzae type b strains have earlier been shown to bind immobilized vitronectin, an inhibitor of MAC (45). Recently, it was shown that H. influenzae type b-dependent binds soluble vitronectin, which contributes to serum resistance of encapsulated strains (12). Although the complement-resistance mechanisms of the Haemophilus species have not yet been resolved, it has previously been shown that serum-resistant NTHi strains prevent MAC accumulation by delaying the synthesis of C3b through the classical pathway (3). By using two independent methods, we showed that most of the NTHi strains bound C4BP, whereas the majority of the typeable strains did not. This finding is in contrast to a recent study, in which no binding of H. influenzae to C4BP was found (46). However, this study focused on one particular strain of NTHi: R3392. The binding to strains used in the current study also occurred when serum was used as a source of C4BP. There was a very large difference in C4BP binding between two NTHi strains (506 and 69) and this difference may explain why C4BP-binding strain 506 is serum resistant while nonbinding strain 69 is sensitive to killing by human serum. When C4BP was depleted from serum, a statistically significant decrease in survival was seen with NTHi strain 506, suggesting that binding to C4BP indeed protects the bacteria against the complement system. We also showed a significant difference in C3b deposition on the bacterial surface between the two strains. Importantly, the two strains acquired an equal but very low amount of C1q, which acts as initiator of the complement cascade on bacterial surfaces (data not shown). It appears that NTHi 506 bound C4BP and this led to deposition of lower amounts of C3b on the bacterial surface and an overall decrease of complement attack. In contrast, NTHi 69 had much higher amounts of C3b on the surface.

The ability of H. influenzae to bind C4BP suggests that the species uses the capacity of C4BP to inhibit complement-mediated attacks in several ways. First, C4BP bound to the surface of H. influenzae maintains its activity to degrade C4b and C3b cleavage, which affects all three pathways of complement. Consequently, such degradation prevents C4b and C3b from participating in the opsonization of the pathogen. Second, because surface-bound C4BP binds C4b, the formation of enzymatic complex activating C3-C3 convertase (C4b2a) will most likely be inhibited and its decay accelerated. This may help NTHi to avoid MAC-induced lysis. C4BP is able to mainly inhibit the classical and lectin pathways and therefore it was important to ascertain whether these
pathways are relevant for the strains investigated. To study which complement pathways are recognizing *H. influenzae*, the survival of NTHi 506 was evaluated in serum diluted in DGVB+ that allows all complement pathways or Mg-EGTA, which inhibits the classical and the lectin pathways because it depletes calcium. When the classical and lectin pathways were inhibited, a significant increase in the survival of the bacteria was seen implying that they contribute significantly to recognition of NTHi 506 by complement. A significant decrease in C3b deposition was also seen when the classical pathway was inhibited further corroborating this hypothesis. The classical pathway can among others be initiated by binding of Abs to the bacteria. We found that both IgG and IgM were deposited on the high C4BP binder NTHi 506 and in a lower concentration on the low binder NTHi 69. Despite the high Ab deposition on the NTHi 506 as compared with the NTHi 69, this strain is more resistant to serum, which we propose depend to a large extent on the ability of NTHi 506 to bind C4BP.

All known ligands of C4BP, except for protein S, are known to interact with C4BP α-chains each being composed of eight homologous CCP domains. The complement inhibitory activity of C4BP and binding of C4b requires CCPs 1–3 of the homologous CCP domains. The complement inhibitory activity of C4BP and binding of C4b requires CCPs 1–3 of the α-chain. In the direct binding assay, C4met inhibited the binding between the bacterium and C4BP suggesting involvement of these CCP domains in the binding between the bacteria and C4BP. The interaction between NTHi 506 and C4BP appears to be ionic because the binding decreased with increasing concentrations of NaCl. The previously studied binding interactions of C4BP with microbes have been either ionic or hydrophobic in nature (42). Some of the ionic interactions were mediated by a patch of positively charged amino acids on the interface of CCP1 and 2. Current binding experiments with C4BP mutants, each deficient in one CCP domain, suggested that CCP2 and CCP7 are involved in the binding. It is possible that there are two ligands for C4BP on the surface of NTHi 506, one interacting with charged amino acids on CCP2 and the other binding CCP7. Several pathogens interact with CCP2, whereas *M. catarrhalis* is the only bacteria which has previously been described to bind CCP7 (22). NTHi 506-C4BP is sensitive to ionic strength on an extent on the ability of NTHi 506 to bind C4BP.

In conclusion, we demonstrate a novel interaction between NTHi strains and inhibitor of the complement system C4BP. The capacity to bind C4BP appears to render the bacteria more resistant to serum-mediated killing and may consequently contribute to their virulence. However, more studies are required to fully establish the significance of the C4BP binding in NTHi pathogenesis.

**Disclosures**

The authors have no financial conflict of interest.

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