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*J Immunol* 2005; 175:3628-3636; doi: 10.4049/jimmunol.175.6.3628
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Ionic Binding of C3 to the Human Pathogen Moraxella catarrhalis Is a Unique Mechanism for Combating Innate Immunity

Therése Nordström,* Anna M. Blom,† Thuan Tong Tan,** Arne Forsgren,* and Kristian Riesbeck2**

Moraxella catarrhalis ubiquitous surface proteins A1 and A2 (UspA1/A2) interfere with the classical pathway of the complement system by binding C4b-binding protein. In this study we demonstrate that M. catarrhalis UspA1 and A2 noncovalently and in a dose-dependent manner bind both the third component of complement (C3) from EDTA-treated serum and methylamine-treated C3. In contrast, related Moraxella subspecies (n = 13) or other human pathogenic bacteria (n = 13) do not bind C3 or methylamine-treated C3. Experiments with recombinant proteins and M. catarrhalis mutants devoid of UspA1/A2 revealed that UspA1/A2 exert their actions by absorbing and neutralizing C3 from serum and restrain complement activation. UspA2 was responsible for most of the effect, and the Moraxella mutant lacking UspA2 was more sensitive to the lytic effect of human serum compared with the wild type. Interestingly, among the large number of bacteria analyzed, only M. catarrhalis has this unique ability to interfere with the innate immune system of complement by binding C3. The Journal of Immunology, 2005, 175: 3628–3636.

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M. catarrhalis is a Gram-negative diplococcus that for a long time was considered as a relatively harmless commensal in the respiratory tract. At present, it is the third most frequent cause of otitis media in children and is a significant agent in sinusitis and lower respiratory tract infections in adults with pulmonary disease (1–4). M. catarrhalis is also one of the most common inhabitants of the pharynx of healthy children. Despite the fact that M. catarrhalis is acknowledged as a human pathogen, only a few reports exist on its specific virulence factors. In recent years the focus of research on Moraxella has been on the outer membrane proteins and their interactions with the human host (5). Some of these outer membrane proteins appear to have adhesive functions, including, among others, M. catarrhalis IgD-binding protein (MID),3 also designated Hag for hemagglutinin), protein CD, M. catarrhalis adherence protein, and the ubiquitous surface proteins (UspA) (6–11).

The UspA family consists of UspA1 (88 kDa), UspA2 (62 kDa), and the hybrid protein UspA2H (92 kDa) (12, 13). In a series of 108 M. catarrhalis nasopharyngeal isolates from young children with otitis media, uspA1 and uspA2/2/A2H genes were detected in >99% of the isolates (14). The amino acid sequences of UspA1 and UspA2 are 43% identical and contain a stretch of 140 aa residues that is 93% identical (12). Moreover, it is known that naturally acquired Abs to UspA1 and UspA2 are bactericidal (15). Several functions have been attributed to the UspA family of proteins. UspA1 expression is essential for the attachment of M. catarrhalis to epithelial and laryngeal epithelial cells by binding carcinoembryonic Ag-related cell adhesion molecule 1 (13, 16, 17). In addition, both UspA1 and UspA2 play important roles in M. catarrhalis serum resistance (11, 18).

The complement system is one of the first lines of innate defense against pathogenic microorganisms, and activation of this system leads to a cascade of protein deposition on the bacterial surface, resulting in formation of the membrane attack complex and opsonization of the pathogen, followed by phagocytosis (for reviews, see Refs. 19 and 20). One of the most important complement proteins is C3, which is present in the circulation in a concentration similar to some Igs (1–1.2 mg/ml). C3 not only plays a crucial role as an opsonin, but also is the common link among the classical, lectin, and alternative pathways of complement activation. The alternative pathway functions as an amplification loop for the classical and lectin pathways and can also be spontaneously activated by covalent attachment of C3 to the surface of a microbe in the absence of complement inhibitors. C3 deposition requires the presence of an internal thioester bond, formed in the native protein by the proximity of a sulfhydryl group (Cys1012) on the C3 cleavage of a 77-residue peptide from the N terminus of the C3 α-chain (Fig. 1A) (21). Proteolytic cleavage of a 77-residue peptide from the N terminus of the C3 α-chain generates C3a (anaphylatoxin) and C3b (Fig. 1B). Attachment of C3b is then accomplished through a covalent link between the carboxyl group of the metastable thioester and either -NH2 or -OH groups of proteins or carbohydrate structures on the activator surface (22, 23). C3b-like molecules can also be generated from C3 by treatment with amines, chaotropes, or repeating freezing and

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0022-1767/05/$02.00

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Received for publication April 20, 2005. Accepted for publication July 1, 2005.

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1 This work was supported by grants from the Alfred O¨ sterlund Foundation, the Anna and Edwin Berger Foundation, the Crafoord Foundation, the Greta and Johan Kock Foundation, the Swedish Medical Research Council, the Swedish Society of Medicine, and the Cancer Foundation at University Hospital, Malmo.

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3 Abbreviations used in this paper: MID, M. catarrhalis IgD-binding protein; BHI, brain heart infusion; C3, third component of complement; C3met, methylamine-treated C3; C4BP, C4b-binding protein; CbpA, pneumococcal choline-binding protein; Efb, extracellular fibrinogen-binding protein; iC3b, inactivated C3b; mfi, mean fluorescence intensity; NHS, normal human serum; pAb, polyclonal Ab; PMN, polymorphonuclear leukocyte; PsPa, pneumococcal surface protein A; Usp, ubiquitous surface protein.
thawing, which results in cleavage of the internal thioester bond without liberation of C3a (Fig. 1C) (24).

We have recently demonstrated an interaction between the human complement fluid phase inhibitor of the classical pathway, C4b-binding protein (C4BP), and M. catarrhalis UspA1 and UspA2 (18). M. catarrhalis mutants devoid of UspA1 and UspA2 showed strongly reduced C4BP binding compared with the wild type. UspA1 and UspA2 bound to the α-chain of C4BP, and in particular, complement control protein domains 2, 5, and 7 were important for the interaction. Finally, C4BP bound to the M. catarrhalis cell surface retained its cofactor activity, as determined by analysis of C4b degradation.

The goal of the present study was to investigate whether M. catarrhalis interferes with the alternative pathway. Interestingly, we found that M. catarrhalis readily absorbed C3 from human serum in which complement was inactivated. Furthermore, M. catarrhalis noncovalently bound purified methylamine-treated C3 (C3met) in a dose-dependent and nonfunctional manner via the α-chain, which becomes covalently attached to the thioester.

Figure 1. Schematic illustration of C3, covalent-bound C3b, and C3met. A. The C3 molecule in serum consists of one α-chain and one β-chain. The α-chain contains an internal thioester site that, after activation, can covalently attach to a microbial surface. C3 has been treated with methylamine, which becomes covalently attached to the thioester.

Materials and Methods

Bacterial strains and culture conditions

The clinical M. catarrhalis isolates and related subspecies have recently been described in detail (6, 25). Type strains were obtained from the Culture Collection, University of Gothenburg, or American Type Culture Collection (ATCC): Neisseria gonorrhoeae CCUG 15821, Streptococcus pyogenes CCUG 25570 and 25571, Streptococcus agalactiae CCUG 4208, Streptococcus pneumoniae ATCC 49619, Legionella pneumophila ATCC 33152, Pseudomonas aeruginosa ATCC 10145, Staphylococcus aureus ATCC 29213, and, finally, S. aureus ATCC 29283. The remaining strains (Table I) were clinical isolates from our department (Medical Microbiology). The different non-Moraxella species were grown on appropriate standard culture media. M. catarrhalis strains were routinely cultured in brain heart infusion (BHI) liquid broth or on BHI agar plates at 37°C. M. catarrhalis BBH18 and RH4 mutants were manufactured as previously described (7, 18, 26). The MID-deficient mutants were grown in BHI supplemented with 1.5 μg/ml chloramphenicol (Sigma-Aldrich), and UspA2-deficient mutants were incubated with 7 μg/ml zeocin (Invitrogen Life Technologies). Both chloramphenicol and zeocin were used for growth of the UspA1/A2 double mutants.

Antibodies

Rabbits were immunized i.m. with 200 μg of recombinant full-length UspA1 emulsified in CFA (Difco), and boosted on days 18 and 36 with the same dose of protein in IFA (7). Blood was drawn 3 wk later. To increase specificity, the anti-UspA1 antiserum was affinity purified with Sepharose-bound UspA1 and UspA2 and was thus designated anti-UspA1/A2 polyclonal Ab (pAb). Rabbit anti-human C3d pAb and FITC-conjugated swine anti-rabbit pAb were purchased from Dakopatts, and goat anti-human C3d pAb was purchased from Advanced Research Technologies. HRP-conjugated donkey anti-goat pAb was obtained from Serotec.

Proteins and iodine labeling

The manufacture of recombinant UspA150–770 and UspA230–539 which are devoid of their hydrophobic C termini, has recently been described (18). The truncated UspA1 and UspA2 proteins were manufactured as described in detail by Tan et al. (27). C3b was purchased from Advanced Research Technologies. C3(H2O) was obtained by freezing and thawing purified C3. The C3b-like molecule (C3met) was made by incubation of purified C3 with 100 mM methylamine (pH 8.0) for 2 h at 37°C and subsequent dialysis against 100 mM Tris-HCl (pH 7.5) and 150 mM NaCl. For binding studies, C3met was labeled with 0.05 mol of 125I (Amersham Biosciences) mol protein, using the chloramine T method (28).

Flow cytometric analysis

Binding of C3 to M. catarrhalis and other species was analyzed by flow cytometry. Bacteria were grown on solid medium overnight and washed.
twice in PBS containing 2% BSA (Sigma-Aldrich; PBS-BSA). Thereafter, bacteria (10^6 CFU) were incubated with C3mert, C3h, C3H2(O), or 10% normal human serum (NHS) with or without 10 mM EDTA or 4 mM MgCl2 and 10 mM EGTA (Mg-EGTA) in PBS-BSA for 30 min at 37°C. After washings, the bacteria were incubated with anti-human C3d pAb for 30 min on ice, followed by washings and incubation for another 30 min on ice with FITC-conjugated goat anti-rabbit pAb. After three additional washings, bacteria were analyzed by flow cytometry (EPICS, XL-MCL: Coulter). All incubations were kept in a final volume of 100 μl of PBS-BSA, and the washings were performed with the same buffer. Anti-human C3d pAb and FITC-conjugated anti-rabbit pAb were added separately as a negative control for each strain analyzed. In the inhibition studies, serum was preincubated with 100 nM recombinant UspA150–770 and UspA250–539 proteins for 30 min at 37°C. To analyze the characteristics of the M. catarrhalis C3 receptor, increasing concentrations of NaCl (0–1.0 M) were added to bacteria and C3mert. To analyze UspA1/A2 expression, bacteria (10^6 CFU) were incubated with the anti-UspA1/A2 pAb and washed as described above. FITC-conjugated goat anti-rabbit pAb, diluted according to the manufacturer’s instructions, was used for detection. To assure that EDTA did not disrupt the outer membrane proteins, UsA1 and UsA2, M. catarrhalis was incubated with or without EDTA, followed by detection of UsA1/A2 expression. EDTA, at the concentrations used in the NHS-EDTA experiments, did not change the density of UsA1/A2.

**Serum and serum bactericidal assay**

NHS was obtained from five healthy volunteers. The blood was allowed to clot for 30 min at room temperature and thereafter was incubated on ice for 60 min. After centrifugation, sera were pooled, aliquoted, and stored at −70°C. To inactivate both the classical and alternative pathways, 10 mM EDTA was added. In contrast, Mg-EGTA was included to inactivate the classical pathway by absorbing divalent cations (Mg2+). To inhibit the classical and alternative pathways, 25 mM EDTA was added to NHS (not shown). Thus, M. catarrhalis survived in NHS without any chelators, but not as long as the wild-type bacteria. The survival of UspA1 and UspA2, 10% serum was preincubated with 100 nM recombinant UspA150–770 and/or UspA250–539 proteins for 30 min at 37°C and thereafter added to the erythrocytes at 0–4°C. EDTA did not disrupt the outer membrane proteins, UspA1 and UspA2, M. catarrhalis was incubated with or without EDTA, followed by detection of UsA1/A2 expression. EDTA, at the concentrations used in the NHS-EDTA experiments, did not change the density of UsA1/A2.

**Serum and serum bactericidal assay**

Human serum was isolated from five healthy volunteers. The blood was allowed to clot for 30 min at room temperature and thereafter was incubated on ice for 60 min. After centrifugation, sera were pooled, aliquoted, and stored at −70°C. To inactivate both the classical and alternative pathways, 10 mM EDTA was added. In contrast, Mg-EGTA was included to inactivate the classical pathway by absorbing divalent cations (Mg2+). To inhibit the classical and alternative pathways, 25 mM EDTA was added to NHS (not shown). Thus, M. catarrhalis survived in NHS without any chelators, but not as long as the wild-type bacteria. The survival of UspA1 and UspA2, 10% serum was preincubated with 100 nM recombinant UspA150–770 and/or UspA250–539 proteins for 30 min at 37°C and thereafter added to the erythrocytes at 0–4°C.

**Helminocytic assay**

Rabbit erythrocytes were washed three times with ice-cold 2.5 mM veronal buffer, pH 7.3, containing 0.1% (w/v) gelatin, 7 mM MgCl2, 10 mM EGTA, and 25% dextrose (Mg2+EGTA) and were resuspended at a concentration of 0.5 × 10^6 cells/ml. Erythrocytes were incubated with various concentrations of serum diluted in Mg2+EGTA. After 1 h at 37°C, erythrocytes were centrifuged, and the amount of lysed erythrocytes was determined by spectrophotometric measurement of released hemoglobin at 405 nm. For inhibition with UspA1 and UspA2, 10% serum was preincubated with 100 nM recombinant UspA150–770 and/or UspA250–539 proteins for 30 min at 37°C and thereafter added to the erythrocytes at 0–4°C.

**Isolation of polymorphonuclear leukocytes (PMN) and phagocytosis**

Human PMN were isolated from fresh blood of healthy volunteers using Macrodex (Pharmalink). PMN were centrifuged for 10 min at 300 x g, washed in PBS, and resuspended in RPMI (160 mmol/L in Invitrogen Life Technologies). The bacterial suspension (0.5 × 10^9) was opsonized with 3% of either NHS or NHS-EDTA or 20 μg of purified C3mert for 15 min at 37°C. After washes, bacteria were mixed with PMN (1 × 10^5 cells/ml) at a bacteria to PMN ratio of 10:1, followed by incubation at 37°C with end-over-end rotation. The number of surviving bacteria after 0, 30, 60, and 120 min of incubation was determined by viable counts. The number of engulfed NHS-treated bacteria was compared with bacteria phagocytosed in the absence of NHS. S. aureus opsonized with NHS was used as a positive control.

**Results**

M. catarrhalis outer membrane proteins UspA1 and UspA2 inhibit both classical and the alternative pathways of the complement cascade

UspA2 surface expression is crucial for M. catarrhalis survival in NHS (10, 18), i.e., Moraxella UspA2-deficient mutants are rapidly killed when exposed to NHS. We have recently shown that both UsA1 and UsA2 bind C4BP and thus might inhibit the classical pathway of complement activation (18). To shed light on M. catarrhalis interactions with the complement system, the survival of UsA1/A2 double mutants was studied in serum treated with either EGTA with addition of MgCl2, (Mg-EGTA) or EDTA. Mg-EGTA inhibits the classical and lectin pathways and thus allows separate analysis of the alternative pathway. In contrast, EDTA inhibits all complement pathways by absorbing divalent cations (Mg2+ and Ca2+). The M. catarrhalis RH4 wild type survived after 30 min of incubation, whereas the RH4usA1/A2 double mutant was killed by intact NHS after 10 min (Fig. 2). When the classical pathway was inhibited (NHS plus Mg-EGTA), the RH4usA1/A2 mutant survived for a significantly longer period of time compared with NHS without any chelators, but not as long as the wild-type bacterium. Furthermore, when both classical and alternative pathways were blocked with EDTA, M. catarrhalis RH4usA1/A2 survived. A similar pattern was obtained with the M. catarrhalis BBH18 isolate and the corresponding BBH18ΔusA1/A2 mutants (not shown). In parallel, experiments with C1q- and factor D/properdin-deficient sera demonstrated that both classical and alternative pathways were inhibited by M. catarrhalis UsA1 and UsA2 (not shown). Thus, M. catarrhalis, a pathogen that frequently colonizes the human respiratory tract, counteracts not only the classical pathway, but also the alternative pathway, of the complement system by the outer membrane proteins UsA1 and UsA2.

M. catarrhalis absorbs C3 from EDTA-inactivated serum

C3b covalently binds to the surface of a microbe and hence induces the alternative pathway (Fig. 1B). To analyze whether
**FIGURE 2.** *M. catarrhalis* RH4 counteracts the classical and alternative pathways of the complement system by the outer membrane proteins UspA1 and UspA2. A. *M. catarrhalis* RH4 wild-type (wt) and ΔuspA1, ΔuspA2, or ΔuspA1/ΔuspA2 mutant were incubated in the presence of 10% NHS. B. The ΔuspA1/ΔuspA2 mutant was incubated with 10% NHS supplemented with either EDTA or Mg-EGTA. Bacteria were collected at the indicated time points. After overnight incubation, CFU were counted. The number of bacteria at the initiation of the experiments was defined as 100%. Mean values of three separate experiments are shown, and error bars indicate the SD.

**FIGURE 3.** *M. catarrhalis* binds C3 in serum independently of complement activation. Flow cytometry profiles showing C3 binding to *M. catarrhalis* RH4 (A) or *S. pneumoniae* (B). Bacteria were incubated with NHS or NHS treated with EDTA. Thereafter, a rabbit anti-human C3d pAb and, as a secondary layer, an FITC-conjugated goat anti-rabbit pAb were added, followed by flow cytometric analysis. Bacteria in the absence of NHS, but in the presence of both pAbs, were defined as background fluorescence. One representative experiment of three is shown.

**FIGURE 4.** *M. catarrhalis* noncovalently binds purified methylamine-treated C3 in a dose-dependent manner, and the binding is based on ionic interactions. A. Flow cytometry profiles showing binding with increasing concentrations of C3met. B. The mfi of each profile in A is shown. C, C3met binding of RH4 decreases with increasing concentrations of NaCl. Bacteria were incubated with C3met with or without NaCl as indicated. C3met binding was measured by flow cytometry as described in Fig. 3. Error bars indicate the SD. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001.
**M. catarrhalis is a unique C3- and C3met-binding bacterium**

To extend our analysis of bacterial C3 absorption from NHS, related *Moraxella* subspecies (*n* = 13) as well as common human pathogens (*n* = 13) were incubated in the presence of NHS-EDTA. Interestingly, among all bacterial species tested, *M. catarrhalis* was the only bacterium binding C3 in complement-inactivated serum (Table I). All related *Moraxella* strains as well as the other human pathogens were also analyzed for binding of C3met. In parallel with C3 binding, *M. catarrhalis* was the only species that bound C3met. Taken together, *M. catarrhalis* has a unique feature to strongly bind C3 and C3met in a noncovalent manner.

**M. catarrhalis binds C3met via outer membrane proteins UspA1 and UspA2**

To determine the *M. catarrhalis* protein responsible for C3 binding, we tested a series of bacterial mutants devoid of the outer membrane proteins MID, UspA1, and/or UspA2 (7, 18). Interestingly, the binding of C3met was significantly correlated with Usp expression (Fig. 5). *M. catarrhalis* RH4Δmid bound C3met to the same degree as the wild-type counterpart (Fig. 5, A and B). The RH4Δuspa1 mutant showed only a slightly decreased binding, whereas RH4Δuspa2 was a weaker binder than the wild-type counterpart (Fig. 5, C and D). In parallel, C3met binding to the double RH4Δuspa1/Δuspa2 mutant was completely abolished (Fig. 5E). Furthermore, when the same experiments were performed using NHS-EDTA, the same pattern was seen (Fig. 5, F–J). When NHS was used, all mutants showed similar amounts of C3 on their surfaces, because it was a mixture of covalent deposition and binding of C3 (Fig. 5, K–O). Similar results were obtained with the *M. catarrhalis* BBH18 isolate and the corresponding BBH18 mutants.

To further analyze the interaction between C3 and UspA1/A2, UspA150–770 and UspA230–539 were produced in *E. coli* and purified. The recombinant proteins were dot-blotted onto a nitrocellulose membrane, followed by incubation with iodine-labeled C3met. Recombinant MID962–1200, which is derived from the *M. catarrhalis* outer membrane protein MID (31), was included as a negative control. A weak binding to UspA150–770 was detected, whereas 125I-labeled C3met strongly bound to UspA230–539 (Fig. 6A). These findings were strengthened using surface plasmon resonance (i.e., Biacore). UspA150–770 and UspA230–539 were immobilized on the surface of a CM5 chip using amino coupling, and C3met was injected until saturation was reached. The *K*<sub>d</sub> values for the interactions between C3met and UspA230–539 and between C3met and UspA150–770 were 3 and 14 μM, respectively. In conclusion, we found that UspA2 was the major C3-binding protein of *M. catarrhalis*, whereas UspA1 contributed to binding to a lesser degree.

**A C3-binding domain is located between aa residues 200 and 458 of UspA2**

To define the C3-binding domain of UspA2, recombinant proteins spanning the entire UspA230–539 molecule were manufactured. C3met was incubated with the immobilized full-length UspA150–770, whereas only a weak C3met binding to UspA150–770 is observed. Furthermore, the C3met-binding region of UspA2 was determined to be located between aa residues 200 and 458. A, The rUspA150–770 and rUspA230–539 were immobilized on a nitrocellulose membrane. The membrane was incubated with 125I-labeled C3met overnight, and bound protein was visualized with a Personal FX (Bio-Rad) using intensifying screens. The recombinant protein MID962–1200 was also included as a negative control. B, UspA150–770, UspA230–539, and a series of truncated UspA2 proteins were coated on microtiter plates and incubated with C3met, followed by incubation with goat anti-human C3 pAb and HRP-conjugated anti-goat pAb. The mean values of three experiments are shown. The background binding was subtracted from all samples. Error bars correspond to the SD. *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001.

![Flow cytometry profiles of M. catarrhalis RH4 wild type and UspA1/A2-deficient mutants show UspA1/UspA2-dependent C3met/C3 binding. The profiles of a wild-type clinical isolate (A, F, and K) and corresponding mutants devoid of protein MID (B, G, and L), UspA1 (C, H, and M), UspA2 (D, I, and N), or both UspA1 and UspA2 (E, J, and O) are shown. Bacteria were incubated with C3met (A–E), NHS-EDTA (F–J), or NHS (K–O) and detected as outlined in Fig. 3. One typical experiment of three performed, with the mfi for each profile, is shown.](image-url)

**FIGURE 5.** Flow cytometry profiles of *M. catarrhalis* RH4 wild type and UspA1/A2-deficient mutants show UspA1/UspA2-dependent C3met/C3 binding. The profiles of a wild-type clinical isolate (A, F, and K) and corresponding mutants devoid of protein MID (B, G, and L), UspA1 (C, H, and M), UspA2 (D, I, and N), or both UspA1 and UspA2 (E, J, and O) are shown. Bacteria were incubated with C3met (A–E), NHS-EDTA (F–J), or NHS (K–O) and detected as outlined in Fig. 3. One typical experiment of three performed, with the mfi for each profile, is shown.

**FIGURE 6.** C3met binds to purified rUspA230–539, whereas only a weak C3met binding to UspA150–770 is observed. Furthermore, the C3met-binding region of UspA2 was determined to be located between aa residues 200 and 458. A, The rUspA150–770 and rUspA230–539 were immobilized on a nitrocellulose membrane. The membrane was incubated with 125I-labeled C3met overnight, and bound protein was visualized with a Personal FX (Bio-Rad) using intensifying screens. The recombinant protein MID962–1200 was also included as a negative control. B, UspA150–770, UspA230–539, and a series of truncated UspA2 proteins were coated on microtiter plates and incubated with C3met, followed by incubation with goat anti-human C3 pAb and HRP-conjugated anti-goat pAb. The mean values of three experiments are shown. The background binding was subtracted from all samples. Error bars correspond to the SD. *, *p* ≤ 0.05; **, *p* ≤ 0.01; ***, *p* ≤ 0.001.
UspA2\textsuperscript{30–539}, and a series of truncated Usp2 proteins. Thereafter, the interactions were quantified by ELISA. In agreement with the dot-blot experiments (Fig. 6A), UspA1\textsuperscript{50–770} bound C3met to a much lesser extent compared with UspA2\textsuperscript{30–539} in the ELISA (Fig. 6B). Among the truncated protein fragments, UspA2\textsuperscript{165–318}, UspA2\textsuperscript{280–359}, and UspA2\textsuperscript{302–438} efficiently bound C3met, suggesting that a binding domain was within aa residues 200 and 458.

**Recombinant UspA1/A2 neutralizes C3 activity**

To examine in detail the role of UspA1/A2-dependent inhibition of the alternative pathway, a series of flow cytometry experiments was performed with bacteria incubated with 10% NHS or serum that had been preincubated with 100 nM UspA1\textsuperscript{50–770} and UspA2\textsuperscript{30–539}. Interestingly, significantly decreased C3 deposition/binding at the surface of *M. catarrhalis* RH4\_UspA1/A2 was observed when NHS was pretreated with UspA1\textsuperscript{50–770} and UspA2\textsuperscript{30–539} (Fig. 7A). When the classical pathway was shut down with Mg-EGTA, similar results were obtained (Fig. 7B). Thus, the recombinant proteins UspA1\textsuperscript{50–770} and UspA2\textsuperscript{30–539} absorbed C3 from NHS and inhibited deposition/binding of C3.

To determine whether absorption of C3 by UspA1\textsuperscript{50–770} and UspA2\textsuperscript{30–539} increased bacterial survival, the double-mutant *M. catarrhalis* RH4\_UspA1/A2 was incubated with serum supplemented with UspA1\textsuperscript{50–770} and UspA2\textsuperscript{30–539}, followed by determination of the number of surviving bacteria. Mg-EGTA was included in the reactions to inhibit the classical pathway. Interestingly, addition of UspA1\textsuperscript{50–770} and UspA2\textsuperscript{30–539} to NHS prevented killing of the UspA1/A2-deficient *M. catarrhalis* (Fig. 7C). UspA2\textsuperscript{30–539} was more efficient in inhibiting bacterial killing compared with UspA1\textsuperscript{50–770}. When both recombinant proteins were supplemented together, no additional inhibition of the alternative pathway was detected. Ten percent NHS corresponds to ~600 nM C3. To investigate whether more UspA1 molecules could neutralize C3 activity, UspA1\textsuperscript{50–770} and/or UspA2\textsuperscript{30–539} up to 600 nM were added. However, higher concentrations of the recombinant proteins did not further increase the inhibition (not shown).

We also included an alternative pathway hemolytic assay consisting of rabbit erythrocytes and NHS to establish the roles of UspA1 and UspA2 as inhibitors of the alternative pathway. NHS was preincubated with recombinant UspA1\textsuperscript{50–770}, UspA2\textsuperscript{30–539}, or both proteins together, followed by addition to the erythrocytes. After 1-h incubation, the amount of erythrocyte lysis was determined. Interestingly, significantly decreased hemolysis was observed when NHS was preincubated with UspA1\textsuperscript{50–770} or UspA2\textsuperscript{30–539} compared with untreated NHS (Fig. 8). In parallel with the increased survival of bacteria in the presence of UspA2\textsuperscript{30–539} or UspA1\textsuperscript{50–770} (Fig. 7C), preincubation with UspA2\textsuperscript{30–539} alone resulted in a more efficient inhibition of the alternative pathway compared with that when NHS was preincubated with UspA1\textsuperscript{50–770}. In conclusion, UspA1\textsuperscript{50–770} or UspA2\textsuperscript{30–539} interfered with the activity of the alternative pathway due to its ability to capture C3.

In addition to being a key molecule in the complement cascade, deposited C3b and iC3b (inactivated C3b) target microbes for removal in the process of opsonophagocytosis. To investigate whether C3 or C3met that was noncovalently bound at the surface of *M. catarrhalis* could still function as an opsonin, a series of phagocytosis experiments was performed. *M. catarrhalis* was preincubated with C3met, NHS, or NHS treated with EDTA, followed by addition of PMNs. Interestingly, *M. catarrhalis* was not engulfed in the presence of C3met, whereas NHS strongly promoted phagocytosis (data not shown). However, when NHS was pretreated with EDTA, *M. catarrhalis* was not phagocytosed by PMNs. Thus, C3/C3met was inactive at the *M. catarrhalis* cell surface and did not function as an opsonin.

**Discussion**

Complement resistance is one of the most important bacterial virulence factors (32). The majority (89%) of *M. catarrhalis* isolates...
from patients with lower respiratory tract infections are resistant to complement-mediated killing (33). *M. catarrhalis* UspA1 and A2 are crucial for bacterial survival in human serum in vivo (11, 16), and we have shown that these two outer membrane proteins bind to the complement fluid phase regulator of the classical pathway, C4BP (18). In the present study we demonstrate that *M. catarrhalis* can inhibit the alternative pathway by noncovalently binding C3 (Figs. 7 and 8). The binding of C3 most likely also inhibits the classical pathway. This could not be analyzed in detail, however, because *M. catarrhalis* also binds C4BP. Interestingly, the *M. catarrhalis*-dependent C3-binding is unique, because several related *Moraxella* subspecies as well as common human pathogenic bacteria do not bind C3 (Table I). The interactions with C3 and methylyamine-treated C3 are mediated mainly by UspA2, whereas UspA1 has a minor role (Figs. 5 and 6). The C3-binding region of UspA2 was localized between aa residues 200 and 458. This region contains a stretch of 140 aa residues that is 93% identical with a region in UspA1 (12). However, despite this sequence similarity, UspA1 binds C3 to a much lesser extent. This might be due to a specific difference in conformation between the proteins. The discrepancy in the C3 binding of UspA1 and UspA2 stands in contrast to the UspA1/A2 interaction with C4BP (18).

*M. catarrhalis* is equally resistant to both the classical and alternative pathways (Fig. 2B). The bacterium binds C4BP, which inhibits the classical pathway (18); in this paper we demonstrate an interaction with the alternative pathway through binding of C3. To determine which of these mechanisms is of most importance for the *M. catarrhalis* serum resistance in various in vivo situations is difficult. For example, the importance of the classical pathway will strongly depend on the history of infections with *M. catarrhalis* and the ability to generate complement-activating Abs. However, every mechanism providing protection from the complement is certainly beneficial for a pathogen. Because C3 is a key molecule in the complement system, the binding of C3 most likely results in the regulation of all three activation pathways and may contribute the most to serum resistance.

The importance of the complement system as a primary defense mechanism is mirrored by the fact that microbes have developed various strategies to interfere with and/or neutralize components of the complement system (34–36). In addition to *M. catarrhalis*, *S. pyogenes*, *Bordetella pertussis*, *E. coli K1*, *Candida albicans*, and *N. gonorrhoeae* express specific surface molecules that bind C4BP and, as a consequence, protect the bacteria against the classical complement pathway (18, 37–42). In addition to inhibition of the classical pathway, several bacteria (e.g., *C. albicans*, *Neisseria meningitidis*, *S. pyogenes*, and *S. pneumoniae*) (for reviews, see Refs. 43 and 44) bind factor H and factor H-like molecules and hence are partially protected against the alternative complement pathway.

UspA1 and A2 absorb C3 from serum and thus most likely inhibit the complement activation. Similarly, the pneumococcal surface protein A (PspA) appears to inhibit the alternative pathway both in vitro and in vivo. PspA is an important virulence factor for *S. pneumoniae*. PspA-deficient pneumococcal strains are readily cleared from the blood, whereas PspA-expressing strains survive (45). Furthermore, in a murine model of bacteremia, PspA-deficient pneumococci have a significantly reduced virulence compared with pneumococci that express PspA (46). It has been demonstrated that more C3b is deposited on PspA-negative pneumococci than on PspA-positive pneumococci (45, 47). Thus, the expression of PspA reduces the complement-mediated clearance and phagocytosis of *S. pneumoniae* by limiting opsonization by C3b (47, 48). PspA-deficient pneumococci that are not virulent in normal mice become virulent in C3-deficient and factor B-deficient mice (45).

To our knowledge, there are only two examples of bacterial proteins that noncovalently bind C3 and thereby interfere with complement function. The first is the extracellular fibrinogen-binding protein (Efb) of *S. aureus*, which was found to bind C3b (49). Efb inhibits both classical and alternative pathways independently of thiorester conformation, i.e., binding to C3b is noncovalent. The second example is the pneumococcal choline-binding protein (CbpA), which has been shown to bind methylamine-treated C3, suggesting a noncovalent interaction that is not dependent on complement activation (50). CbpA is a component of the pneumococcal cell wall, but may only bind C3 when CbpA is secreted. To test this hypothesis, which is not firmly established in the literature, we analyzed 11 different pneumococcal isolates for C3 binding (methylamine-treated C3 or NHS-EDTA) by flow cytometry (Fig. 2B and Table I). No bound C3 could be detected on the surface of *S. pneumoniae*. When lysates of *S. pneumoniae* and culture supernatants were analyzed on Western blots using methylamine-treated C3, followed by an anti-human C3 pAb, we confirmed the results reported by Cheng et al. (50) (not shown). In the light of Efb and CbpA, which both are C3BPs secreted by two Gram-positive bacteria, the Gram-negative *M. catarrhalis* is a unique species with membrane-anchored proteins that bind C3 and inhibit the alternative pathway at the surface of the bacterium.

The yeast *C. albicans* has been shown to bind C3b, iC3b, and C3d. However, C3b is bound at a considerably lower affinity than iC3b and C3d (51). We found a large difference between C3 binding to *M. catarrhalis* and *C. albicans* (not shown); despite the fact that *Candida* bound C3met (56% positive cells), the mfi was <2.0 compared with the mfi of 36.9 for *M. catarrhalis*. Furthermore, no detectable binding was seen when *C. albicans* was incubated with EDTA-treated serum. Two C3BPs have been isolated from *C. albicans*, and the most-characterized protein is a 60-kDa mannosaccharide that was initially recognized by an Ab directed against human complement receptor 2 (CD21) (52). However, *M. catarrhalis* UspA1 and UspA2 were not recognized by a pAb directed against CD21 (not shown). In parallel with staphylococci and pneumococci (41, 42), a secreted C3BP from *C. albicans* exists (53). Finally, a *C. albicans* iC3b receptor has been isolated and is structurally similar to human CR3 (CD11b) (54). The mechanisms by which these receptors participate in pathogenesis are not fully known.

The above examples of C3-binding pathogens are notably different from *M. catarrhalis*, in that these species often are bloodstream isolates. *M. catarrhalis* is a mucosal pathogen with rare instances of bacteremic infections. Hence, the binding and inactivating C3 most likely occur at the mucosal surface. This is supported by the fact that there is strong ongoing complement activation and consequent inflammation in disease states such as acute otitis media (55). The complement proteins are believed to be transported to the mucosal surface due to exudation of plasma (56, 57). In middle ear effusions from children, for example, highly elevated concentrations of C3 products can also be found (58). In addition, complement factors in middle ear effusions fluid have been shown to be important in bactericidal activity against other mucosal agents, such as nontypable *Haemophilus influenzae* (59).

*M. catarrhalis* is a strict human pathogen. It does not cause diseases, such as otitis media or pneumonia, in animals. A mouse pulmonary clearance model and an otitis media model with chinchilla have been used on several occasions. However, neither otitis media nor pneumonia develops, and bacteria are rapidly cleared (60, 61). It is thus difficult to test the biological significance of
bacterial C3 binding in vivo. Because UspA1 and UspA2 are multifunctional proteins (11, 13, 15, 17, 18, 27), it would be impossible to relate any differences in the clearance of M. catarrhalis to C3 binding. In particular, the fact that UspA1 is an important adhesin of M. catarrhalis and binds both carcinoembryonic Ag-related cell adhesion molecule 1 and fibronectin (17, 27) would most likely affect the clearance. Nevertheless, due to the strong complement activation in disease states such as otitis media, Moraxella-dependent binding of C3 may represent an important way of combating the mucosal defense.

In addition to the interaction with C3 and C4BP, M. catarrhalis has a strong affinity for soluble and membrane-bound IgD (6, 62). Moraxella-dependent IgD binding to B lymphocytes results in polyclonal Ig synthesis (63), which may produce the inhibition of specific anti-Moraxella mAbs. The fact that M. catarrhalis hampers the human immune system in several ways might explain why M. catarrhalis is such a common inhabitant of the respiratory tract (4). In conclusion, M. catarrhalis has developed sophisticated ways of combating both humoral and innate immune systems. The present data show that M. catarrhalis has a unique C3-binding capacity at the bacterial cell surface that cannot be found in other bacterial species.

Disclosures
The authors have no financial conflict of interest.

References


