Haemophilus influenzae Survival during Complement-Mediated Attacks Is Promoted by Moraxella catarrhalis Outer Membrane Vesicles

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Moraxella catarrhalis causes respiratory tract infections in children and in adults with chronic obstructive pulmonary disease. It is often isolated as a copathogen with Haemophilus influenzae. The underlying mechanism for this cohabitation is unclear. Here, in clinical specimens from a patient with M. catarrhalis infection, we document that outer membrane vesicles (OMVs) carrying ubiquitous surface protein (Usp) A1 and UspA2 (hereafter, UspA1/A2) were secreted. Further analyses revealed that OMVs isolated in vitro also contained UspA1/A2, which mediate interactions with, among other proteins, the third component of the complement system (C3). OMVs from M. catarrhalis wild-type clinical strains bound to C3 and counteracted the complement cascade to a larger extent than did OMVs without UspA1/A2. In contrast, UspA1/A2-deficient OMVs were significantly weaker inhibitors of complement-dependent killing of H. influenzae. Thus, our results suggest that a novel strategy exists in which pathogens collaborate to conquer innate immunity and that the M. catarrhalis vaccine candidates UspA1/A2 play a major role in this interaction.

Moraxella catarrhalis is an emerging pathogen responsible for acute otitis media in children and for exacerbation of chronic obstructive pulmonary disease in adults. It is frequently found in mixed infections, with pathogens such as Streptococcus pneumoniae or Haemophilus influenzae being reported in up to 50% of cultures [1, 2]. The pathophysiology underlying this coexistence is unknown. M. catarrhalis does, however, release outer membrane vesicles (OMVs), or “blebs.” These blebs are produced during in vitro growth in broth and can also be induced by EDTA-heat treatment [3]. Various reports of OMVs from other gram-negative bacteria (such as Serratia marcescens, Proteus mirabilis, Borrelia burgdorferi, Neisseria gonorrhoeae, Pseudomonas aeruginosa, and Vibrio species) have shown that, in general, OMVs are released from the surface of the cell during growth and occur in various environments, including liquid culture, solid culture, and biofilms. Blebs are thought to carry some of the underlying periplasm, together with outer membrane proteins (OMPs), and to possess porins, receptors, pores, and lipopolysaccharide from the outer membrane layer [4, 5]. The size of OMVs ranges from 50 to 250 nm in diameter, and they are pelletable from a cell-free supernatant [4, 6, 7]. Blebs from M. catarrhalis have not been studied in detail, although EDTA-heat–induced blebs have frequently been used for OMP analysis [3, 8, 9].

The ubiquitous surface protein (Usp) A family consists of UspA1, UspA2, and the hybrid protein UspA2H [10, 11]. These proteins share several identical domains and form stalk-like structures with a lollipop-like head at the tip and cover M. catarrhalis as a dense layer [12, 13]. A wide range of functions have been attributed to

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UspA1 and UspA2 (hereafter, UspA1/A2), including adhesion to epithelial cell–associated fibronectin, carcinoembryonic antigen–related cell-adhesion molecules on epithelial cells, and laminin on the basement membrane [14–16]. We have recently shown that the OMPs UspA1/A2 contribute to the serum resistance of M. catarrhalis by binding to the third component of the complement system (C3) and inactivating it in a noncovalent manner [17]. In addition, UspA1/A2 interact with vitronectin and C4b-binding protein (C4bBP) [9, 18]. Thus, UspA1/A2 are the main virulence factors involved in the complement resistance of M. catarrhalis. In the present study, we show that M. catarrhalis produces blebs natively in broth in vitro as well as during in vivo infections. These OMVs carry UspA1/A2 and interfere with the complement cascade directed against H. influenzae. Hence, OMVs from M. catarrhalis most likely contribute to the indirect pathogenicity of H. influenzae by binding and depleting C3 in their immediate environment.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The clinical isolates M. catarrhalis BBH18 and RH4 as well as their respective UspA1/A2-deficient double mutants used have been described elsewhere [9]. Their ability to interact with the complement factor C3 was shown recently [17]. M. catarrhalis bacteria were grown in brain-heart infusion (BHI) broth for 18 h at 37°C and were harvested for OMV isolation. The capsule-deficient H. influenzae type b strain (RM804) [19] and 4 other non-typeable H. influenzae (NTHi) strains (6-601, 772, 7-120, and 6-6267, all clinical isolates collected at the Malmö University Hospital) were cultured on chocolate agar plates overnight before being suspended in PBS for use in the bactericidal assays.

Antibodies. The anti-UspA1/A2 polyclonal antibodies (PAbs) used have been described elsewhere [9]. The rabbit anti–human C3 PAb was purchased from Serotec, and the horse-radish peroxidase (HRP)–conjugated swine anti-rabbit PAb was purchased from Dakopatts. In some experiments, the anti-UspA1/A2 PAb was labeled with 5-nm colloidal thiocyanate gold [20].

OMVs. OMVs from M. catarrhalis strains BBH18 and RH4 as well as their respective UspA1/A2-deficient mutants were prepared according to the Rosen method [21]. Briefly, 500 mL of BHI cell culture was subjected to centrifugation at 10,000 g for 20 min. The culture supernatant was filtered through a filter with a pore size of 0.2 μm and was concentrated using 100,000-kDa Vivaspin columns (Vivasience). The cell-free concentrates were then centrifuged at 100,000 g for 60 min. The precipitates containing extracellular vesicles were resuspended in PBS.

SDS-PAGE and detection of proteins on membranes (Western blots). To study the OMPs of the UspA1/A2-deficient M. catarrhalis mutants and the wild-type strains, vesicles were analyzed on SDS-PAGE and Western blots. Gels were stained using the BioRad Silver Stain Plus Kit. Electrophoretic transfer of protein bands from the gel to an Immobilon-P membrane (Millipore) was done at 20 V overnight, to transfer the high-
molecular-mass complexes. After transfer, the Immobilon-P membrane was blocked in PBS with 0.1% Tween 20 (PBS-Tween) containing 5% milk powder. After several washings in PBS-Tween, the membrane was incubated with rabbit anti-UspA1/A2 antiserum diluted 1:500 in PBS-Tween containing 2% milk powder for 1 h at room temperature. HRP-conjugated swine anti-rabbit antiserum was added after washings in PBS-Tween. After incubation for 1 h at room temperature and additional washings in PBS-Tween, development was performed with enhanced-chemiluminescence (ECL) Western blotting detection reagents (Amersham Pharmacia Biotech).

**Transmission electron microscopy (TEM).** For TEM, specimens were fixed in 0.15 mol/L sodium cacodylate buffer (pH 7.4) containing 2.5% glutaraldehyde for 1 day at room temperature. They were rinsed in isotonic cacodylate buffer for 30 min and postfixed in cacodylate buffer containing 1% (wt/vol) osmium tetroxide for 2 h. Specimens were then rinsed in isotonic cacodylate buffer, dehydrated in a graded series of ethanol, and embedded in Epon 812 (Agar Scientific). Thin sections were cut on a Leica Ultracut S instrument. Sections were poststained with 5% uranyl acetate for 2 h and then with a saturated lead citrate solution for 7 min. In some experiments, antigen...
Figure 3. Inhibition of the bactericidal effect of normal human serum (NHS) acting against multiple clinical nontypeable Haemophilus influenzae (NTHi) strains by Moraxella catarrhalis wild-type strain RH4 outer membrane vesicles (OMVs) carrying ubiquitous surface protein (Usp) A1 and UspA2 (UspA1/A2). Shown are results for H. influenzae strains NTHi 6-601 (A), 772 (B), 7-120 (C), and 6-6267 (D). All 4 strains were incubated with wild-type or UspA1/A2-deficient OMVs. Improved survival could be seen when incubation was with wild-type blebs compared with UspA1/A2-deficient blebs, with statistical significance being seen at 30 min for NTHi 7-120 and 6-6267. The mean values from 3 separate experiments are shown. Error bars represent SDs. NS, not significant. * P < 0.05; ** P < 0.01.

retrieval was performed by etching the sections on drops of saturated sodium metaperiodate solution for 60 min at room temperature. The sections were then subjected to immunolabeling as described elsewhere [22], with the modification that Aurion BSA-c was used as a blocking agent. Specimens were observed in a JEM-1230 electron microscope (JEOL) operated at 60 kV (accelerating voltage). Images were recorded with a Multiscan 791 CCD camera (Gatan). In addition, fresh nasal discharge from a 9-year-old child with M. catarrhalis sinusitis (pure growth of M. catarrhalis on nasal aspirate culture) was examined. This was prepared by suspending a drop of the purulent nasal discharge in 1 mL of PBS with 4% paraformaldehyde. The cellular fraction was obtained by centrifuging the specimen at 214 g. Aliquots were then examined by TEM as described above.

H. influenzae serum bactericidal inhibition assay. Normal human serum (NHS) was prepared from pooled blood from 5 healthy volunteers by a standard protocol. The serum was aliquoted and stored at −70°C. Serum that was inactivated at 56°C for 30 min was used as a control. The reaction buffer consisted of 2.5 mmol/L veronal buffer (pH 7.3) containing 0.1% (wt/vol) gelatin, 1 mmol/L MgCl2, and 0.15 mmol/L CaCl2 (dextrose gelatin veronal buffer [DGVB]2+) with 5% NHS or heat-inactivated NHS. In inhibition experiments, the serum (5 μL) was preincubated with 5 μg of blebs from either wild-type M. catarrhalis or UspA1/A2-deficient mutants for 30 min at 4°C. Then H. influenzae (1 × 10⁶ cfu) was added in a final volume of 100 μL. This mixture was incubated at 37°C, and 10-μL aliquots were removed at 0, 10, 20, and 30 min and spread onto chocolate agar plates.
Figure 4. Inhibition of the bactericidal effect of normal human serum (NHS) acting against multiple clinical nontypeable Haemophilus influenzae (NTHi) strains by Moraxella catarrhalis wild-type strain BBH18 outer membrane vesicles (OMVs) carrying ubiquitous surface protein (Usp) A1 and UspA2 (UspA1/A2). Shown are results for H. influenzae strains NTHi 6-601 (A), 772 (B), 7-120 (C), and 6-6267 (D). All 4 strains were incubated with wild-type or UspA1/A2-deficient OMVs. Improved survival could be seen when incubation was with wild-type blebs compared with UspA1/A2-deficient blebs, with statistical significance being seen at 30 min for all except NTHi 772. Mean values from 3 separate experiments are shown. Error bars indicate SDs. NS, not significant. *; **.

Dot blot assays. M. catarrhalis BBH18 and RH4 wild-type blebs and their corresponding UspA1/A2-deficient mutant blebs were diluted in 3-fold steps in 100 μL of 0.1 mol/L Tris-HCl (pH 9.0) and then manually applied to nitrocellulose membranes (Schleicher & Schüll Microscience) by use of a dot blot device. After saturation, the membranes were incubated for 2 h with PBS-Tween containing 5% milk powder at room temperature and washed 4 times with PBS-Tween. Thereafter, they were incubated with 10% NHS in PBS overnight at 4°C. Several washes were applied followed by the detection of bound C3 by use of an anti-C3 PAb. Finally, filters were incubated with anti-rabbit HRP-conjugated PAb. Detection was done by means of ECL Western blotting detection reagents.

Total complement hemolytic assays and C3 measurements. The hemolytic activity of serum treated with OMVs was determined using sheep erythrocytes sensitized with rabbit anti-sheep antibodies (amboceptor) by use of a simplified Mayer method [23]. In these reactions, 2% sheep erythrocytes were presensitized with an in-house–produced amboceptor (1:2000) for 1 h at room temperature. Serial 2-fold dilutions of the bleb-treated serum were added to a 50-μL mixture of the sensitized sheep erythrocytes, constituting a 100-μL reaction volume in 96-well round-bottom plates (with the first dilution constituting a 1:100 dilution of NHS). After an incubation of 1 h at 37°C, the plates were centrifuged at 130 g at 4°C. Supernatants were transferred to flat-bottom ELISA plates, and the extent of hemolysis was determined by measuring the absorbance of the supernatant at 540 nm. Total hemolysis was achieved with 2% erythrocytes in 100 μL of deionized water. Background hemolysis consisted of 2% erythrocytes in 100 μL of DGVB²⁴.
The hemolytic activity of 5% NHS not treated with OMVs constituted the control. In addition, the residual amount of C3 in the supernatant of the bleb-treated serum was measured using a human C3 ELISA kit (AssayMax ELISA; Assaypro), in accordance with the manufacturer’s instructions.

Statistics. Student’s t test was used for statistical calculations. Statistical significance was set at \( P < 0.05 \).

RESULTS

Characterization of OMVs from M. catarrhalis. Gram-negative bacteria have the capacity to produce OMVs. To analyze OMV formation by M. catarrhalis, 2 strains (BBH18 and RH4) grown overnight in broth were examined by TEM. These bacteria secreted structures that were consistent with OMVs (figure 1A and 1B); the size was 50–150 nm, which is in line with that described for other species [4].

Blebs from M. catarrhalis wild-type strains (RH4 and BBH18) and their corresponding UspA1/A2-deficient mutants were also analyzed by SDS-PAGE and silver staining (figure 2A). A high-molecular-weight band (>250 kDa) could be seen in blebs from both wild-type M. catarrhalis strains but was not detected in blebs isolated from the UspA1/A2-deficient mutants. Western blots using anti-UspA1/A2 PAb confirmed that these bands were indeed UspA1/A2 (figure 2B). Direct examination of the wild-type M. catarrhalis strains also revealed that they secrete blebs with UspA1/A2, as demonstrated by use of gold-conjugated anti-UspA1/A2 antiserum (figure 2C).

Counteraction of the bactericidal effect of NHS on H. influenzae by M. catarrhalis OMVs containing UspA1/A2. Through the OMPs UspA1/A2, M. catarrhalis has a unique ability to absorb C3 in a noncovalent manner from human serum and thereby mediate serum resistance [17]. Other species, including Moraxella subspecies, do not bind C3 in a similar manner. Given that the OMVs carry UspA1/A2, we hypothesized that this ability might confer a protective effect on copathogens in the immediate environment. To test this hypothesis, we preincubated NHS with M. catarrhalis blebs and followed this with incubation with H. influenzae. Several NTHi clinical isolates (6-601, 772, 7-120, and 6-6267) were incubated with serum that had been pretreated with OMVs originating from M. catarrhalis strain RH4 (figure 3A–3D). All strains showed improved survival when incubated with wild-type OMVs compared with UspA1/A2-deficient OMVs, with statistical significance being seen with NTHi 7-120 and 6-6267 at 30 min (figure 3C and 3D, respectively). A similar survival advantage was seen with the same strains incubated with OMVs from M. catarrhalis wild-type BBH18 compared with the UspA1/A2-deficient OMVs (figure 4A and 4D). In these experiments, all strains showed a significant difference in survival at 30 min, with the exception of NTHi strain 772 (figure 4B).

This phenomenon of a survival advantage being conferred by OMVs carrying UspA1/A2 was also seen with a capsule-deficient H. influenzae type b strain (RM804), although a statistically significant difference was seen only for OMVs from M. catarrhalis BBH18 (figure 5B). Thus, blebs carrying UspA1/A2 protected H. influenzae from complement-mediated killing, suggesting that M. catarrhalis may promote the survival of this species of bacteria during coinfection.

Figure 5. Inhibition of the bactericidal effect of normal human serum (NHS) acting against the capsule-deficient Haemophilus influenzae type b strain (RM804) by Moraxella catarrhalis outer membrane vesicles carrying ubiquitous surface protein (Usp) A1 and UspA2 (UspA1/A2), H. influenzae RM804 was incubated with wild-type RH4 or UspA1/A2-deficient blebs (A) or with BBH18 wild-type or UspA1/A2-deficient blebs (B). Mean values from 3 separate experiments are shown. Error bars indicate SDs. NS, not significant. ** \( P < 0.01 \).
Increased binding of C3 to OMVs from the wild-type M. catarrhalis strains than to OMVs from the UspA1/A2-deficient mutants. Blebs carrying UspA1/A2 were clearly beneficial for H. influenzae survival in NHS (figures 3–5). When expressed at the M. catarrhalis cell surface, UspA1/A2 interact with various complements and serum factors (C4bBP, C3, and vitronectin) [9, 17, 18]. It is most likely the case that similar interactions occur with blebs. However, only UspA1/A2-dependent C3 binding and depletion would allow for better survival of a copathogen. In contrast, depletion of C4bBP and/or vitronectin would actually promote the bactericidal activity of serum against H. influenzae. Hence, we tested the C3 binding of blebs in a direct ligand assay. Wild-type blebs blotted on nitrocellulose filters appeared to bind to C3 to a greater extent than did UspA1/A2-deficient blebs (figure 6A). As expected, blebs from the UspA1/A2-deficient mutants still bound some C3. This could be explained by the fact that blebs contain gram-negative cell wall components such as lipooligosaccharide (LOS), and, therefore, some C3 would be deposited irrespective of UspA1/A2 content.

Reduction of the total complement hemolytic activity of NHS by M. catarrhalis OMVs carrying UspA1/A2, compared with that induced by M. catarrhalis OMVs not carrying UspA1/A2. Because M. catarrhalis blebs carrying UspA1/A2 more strongly counteracted the bactericidal activity of NHS and because more C3 was deposited on blebs with UspA1/A2, we analyzed the total complement hemolytic activity of the bleb-treated NHS. Sensitized sheep erythrocytes were added to NHS preincubated with blebs from M. catarrhalis wild-type strains or their UspA1/A2-deficient mutants. After incubation for 1 h, the extent of erythrocyte lysis was determined. Interestingly, decreased hemolysis was observed when NHS was preincubated with blebs carrying UspA1/A2, compared with that observed for blebs from the UspA1/A2-deficient mutants. This difference was statistically significant for blebs from M. catarrhalis strain RH4 (figure 6B). Unexpectedly, there was no difference in C3 levels between serum treated with wild-type blebs and that treated with UspA1/A2-deficient blebs when directly assayed with a commercial kit (data not shown).

Secretion of OMVs carrying UspA1/A2 by M. catarrhalis in vivo. To confirm that OMVs also exist in vivo and are released during infection, a nasal sample from a child with newly diagnosed M. catarrhalis sinusitis was examined by TEM. Figure 7A and 7B shows M. catarrhalis in close association with a single polymorphic leukocyte, with blebs being secreted. Another example of the secretion of blebs by M. catarrhalis is demonstrated in figure 7C and 7D. In a fashion similar to that for in vitro samples, this in vivo sample was also subjected to probing with gold-conjugated anti-UspA1/A2 antiserum. OMVs
Figure 7. Secretion of outer membrane vesicles (OMVs) by *Moraxella catarrhalis* during infection in a child with sinusitis. Panels A and B show secretion of blebs by *M. catarrhalis* in the vicinity of a polymorph. Panels C and D show another example of a *M. catarrhalis* bacterium with OMVs. Panel E shows *M. catarrhalis* blebs carrying ubiquitous surface protein (Usp) A1 and UspA2 (UspA1/A2; arrows), as demonstrated by gold-labeled anti-UspA1/A2 antibodies around the margin of the bacterium. Black bars represent 0.2 μm at higher magnification (B, D, and E) and 1 μm at lower magnification (A and C).

observed in the vicinity of the bacterium were also found to express UspA1/A2 (figure 7E).

**DISCUSSION**

*M. catarrhalis* blebs have been used extensively in the study of *M. catarrhalis* OMPs, although they have been largely EDTA-heat induced [3, 8, 9]. Here, we provide evidence that OMVs are produced during infection in a pediatric patient. The role these blebs play in the pathophysiological process is, however, unclear. Gram-negative bacteria such as *N. gonorrhoeae* have been known to secrete blebs that act as decoys for serum bactericidal factors against the bacterium itself [24]. *P. aeruginosa* bacteria package hydrolases in blebs, which lyse other species [25]. Several other examples exist and have been reviewed in detail elsewhere [5]. Thus, blebs are virulence factors, and it is likely that blebs produced by *M. catarrhalis* play a pathogenic role. The OMPs on these blebs are, thus, predictably important agents for the virulence mechanism if carried over from the bacterial cell membrane.

The OMPs on *M. catarrhalis* have been closely studied in recent years. Mechanisms of serum resistance via proteins such as UspA1/A2 have been elucidated [9, 17, 18]. Other mechanisms contributing to the pathogenicity of this species have also been intensely characterized [26]. However, the pathophysiology behind the common phenomenon of mixed infections with *M. catarrhalis* and copathogens remains unclear. Here, we describe how the UspA1/A2 OMPs are carried over on the blebs of *M. catarrhalis* and demonstrate that they play a direct role in promoting the survival of *H. influenzae* when exposed to human serum. Such a relationship might also benefit *M. catarrhalis*, given that the promotion of a copathogen such as *H. influenzae* might cause increased inflammation, resulting in the exposure of extracellular matrix proteins and the up-regulation of epithelial cell-surface receptors. These changes in host tissue may, in fact, pave the way for *M. catarrhalis* adherence and survival.

We have recently shown that UspA1/A2 has a unique ability to bind to C3 in a noncovalent manner [17]. C3 is a crucial component in the complement cascade. Not only is it an opsonin, but it is also the common link between the classical, lectin, and alternative pathways of complement activation. In the present study, the majority of experiments showed that NTHi strains appeared to survive better with blebs that carried UspA1/A2. We speculate that these lollipop-like proteins are radially more spread out in blebs than in whole bacteria. Their binding sites for C3, which are located along the stalk region [17], may be more readily available for interactions with C3, because there would be less steric hindrance from each other. In our dot blot experiments, wild-type blebs bound more C3 from NHS than did UspA1/A2-deficient mutant blebs, a finding that supports this hypothesis (figure 6). A hemolytic assay measuring the total complement activity of the OMV-treated serum showed that there was a higher decrease in complement activity when the OMVs carried UspA1/A2. This supported the results of our bactericidal assays, although it is not possible to pinpoint the serum factor affected by UspA1/A2 in such experiments. Because C3 is important as an amplifier, a small decrease may result in a measurable decline in the total hemolytic activity. Thus, the UspA1/A2-dependent C3 noncovalent interaction to-
Bacterial OMVs and C3 consumption.

Although lower C3 levels were expected in serum exposed to OMV carrying UspA1/A2 (compared with those not carrying UspA1/A2), this was not measurable. It is likely that the quantitative competitive sandwich ELISA that we used could not differentiate between UspA1/A2-bound C3 and free C3. Attempts at separating the blebs from the supernatant (containing free C3) by ultracentrifugation were unsuccessful, because it resulted in a marked decrease in C3 levels in all of the samples.

There are several other antigens and OMPs that could contribute to *M. catarrhalis* serum resistance besides UspA1/A2. Mutations leading to alterations in OMPs such as CopB, OMP CD, OMP E, and a carbohydrate epitope of the LOS have all resulted in increased serum sensitivity [27–30]. The underlying mechanisms responsible with respect to these antigens are unclear. Furthermore, a direct interaction of Clq without the participation of antibodies has been previously reported in some strains of *M. catarrhalis*, and this could also possibly alter complement activation [31]. Thus, the possibility that these *Moraxella* surface components could have contributed to the complement-inhibitory effect of the OMVs cannot be excluded. However, because we compared the effect of OMVs carrying UspA1/A2 with the effect of those not carrying UspA1/A2, the differential survival advantage was solely due to UspA1/A2.

UspA1/A2 are multifunctional proteins, and they mitigate several other mechanisms of serum resistance. UspA2 binds to vitronectin and C4bBP [9, 18]. Binding to these proteins confers resistance to the bacteriolytic effect of the complement cascade. Depletion due to UspA1/A2 binding would theoretically result in higher serum sensitivity for *H. influenzae*. Hence, binding to these proteins by *M. catarrhalis* blebs would probably be of lesser significance than the binding of C3 in these 2 strains.

In conclusion, we have provided data showing that blebs are produced in vivo during *M. catarrhalis* infection and that the biologically active molecules UspA1/A2 can be found on blebs. Furthermore, the previously described UspA1/A2-dependent inactivation of C3 [17] contributes to an increased survival of *H. influenzae* in human serum when incubated with blebs isolated from *M. catarrhalis*. Another clinical significance of the present findings may be that a vaccine targeting UspA1/A2 might block the C3 interaction. Although speculative, this may result in lower rates of both *M. catarrhalis* and *H. influenzae* infection.

**References**


