Virulence mechanisms of *Moraxella* in the pathogenesis of infection

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Current Opinion in Infectious Diseases 2009, 22:279–285

Purpose of review

Moraxella catarrhalis is an emerging human-specific pathogen responsible for upper and lower respiratory tract infections. Understanding the events in the complex pathogenesis and underlying mechanisms during *M. catarrhalis* infection is a key to the development of novel therapeutics and vaccines.

Recent findings

Several novel findings have been reported on *Moraxella* pathogenesis and, in parts, explain how the species stands as a commensal in preschool children and survives in the host. Molecular structures for different adhesins in addition to target ligands with respect to signalling and invasion have been defined. Evasion of the complement system allows *Moraxella* to survive in the mucosa and by neutralizing α1-antichymotrypsin the protease activity is increased, resulting in tissue destruction and thus promotion of bacterial attachment. *Moraxella*-dependent cell activation via immunoglobulin D in addition to toll-like receptors and specific epithelial cell inhibition by cross-linking of carcinoembryonic antigen-related cell adhesion molecule-1 in the early innate immune response and, finally, the ability of *M. catarrhalis* to form biofilms are other specific research areas of interest.

Summary

Recent advances have allowed a more detailed picture of the processes involved in bacteria–host cell interactions, the cause of inflammatory processes and specific host defense responses against the intriguing species *Moraxella*.

Keywords

innate immune response, microbial pathogenesis, *Moraxella catarrhalis*

Curr Opin Infect Dis 22:279–285

0951-7375

Introduction

Moraxella catarrhalis is a Gram-negative aerobic diplococcus and an exclusive human respiratory pathogen. After *Streptococcus pneumoniae* and nontypeable *Haemophilus influenzae*, *M. catarrhalis* is the third most common cause of bacterial respiratory tract infections, including acute otitis media (AOM), sinusitis, laryngitis and finally bronchitis or pneumonia. The relationship of *M. catarrhalis* with the human host occurs early with nasopharyngeal colonization being common in early childhood. The highest incidence of AOM appears to occur before the second year of life, and 50–85% of children have had at least one episode of AOM by the age of 3 years and nearly half had three or more episodes [1,2]. *Moraxella* is responsible for approximately 20% of these cases [3].

The plethora of bacterial species found in AOM may change after the introduction and widespread use of multivalent pneumococcal conjugate vaccines [4–6]. This replacement phenomenon in which pathogens not targeted in a vaccine take the place of eradicated vaccine strains is potentially serious and has been increasingly recognized [7–9]. Furthermore, the potential future usage of vaccines against nontypeable *H. influenzae* [10,11] combined with pneumococcal conjugate vaccines could locate *M. catarrhalis* in a privileged position compared with the other childhood respiratory pathogens. Almost all strains of *M. catarrhalis* produce β-lactamases [12], and hence another aspect of an increased *Moraxella* incidence is that the use of antibacterial agents other than beta-lactam antibiotics may increase and drive the resistance rates also in other pathogens.

During the last years, increased evidence underlines the importance of *M. catarrhalis* as an emerging pathogen responsible not only for AOM in children but also for recurrent and persistent respiratory tract infections in patients suffering from chronic obstructive pulmonary disease (COPD), which is one of the leading causes of morbidity and mortality worldwide [13–15]. *M. catarrhalis* likely causes approximately 10% of the exacerbations and also significantly contributes to the chronic airway inflammation which is a hallmark of the disease [15].

Interestingly, isolation of new strains of *M. catarrhalis* in COPD patients is associated with exacerbation episodes [16]. Elucidating the dynamics of bacterial chronic colonization and characterizing the role of bacterial pathogens in causing exacerbations have important implications in the design of innovative therapies and intervention strategies.

It is generally accepted that several virulence factors involved in adherence to the respiratory tract and complement resistance allow *M. catarrhalis* to evade the host innate immune defense and thus establish infection, though the exact pathogenic mechanisms are not well understood. Recently, it has also been suggested that *Moraxella* carriage in the community might be underestimated due to its ability to invade respiratory epithelial cells and form biofilm [17,18,19••]. Available data clearly show that *M. catarrhalis* is an important pathogen, and its role in causing human infections will certainly be greater in the near future. Thus, a future need for a vaccine also against *M. catarrhalis* is emphasized, and the understanding of its virulence mechanisms is the obvious starting point for the rational design of an effective vaccine. This review summarizes recent progress over the key stages of *Moraxella* pathogenesis including adhesin-mediated attachment to the target tissue, specific interactions with the innate immune system and finally persistence in the host.

Bacterial attachment

Bacterial colonization is initiated by attachment of multifunctional surface adhesins that may additionally act in defensive roles to evade immune recognition. A number of *Moraxella* adhesins and its corresponding receptors have been identified and characterized [20,21•]. The family of ubiquitous surface proteins (Usps), which consists of at least three proteins, UspA1, UspA2 and the hybrid protein designated UspA2H, are the most extensively studied outer membrane proteins (OMPs) of *M. catarrhalis*. UspA are oligomeric coiled-coil adhesins with multifunctional binding sites. These include domains attaching to the extracellular matrix proteins fibronectin [22], laminin [23] and vitronectin [24]. Approximately 20% of *M. catarrhalis* strains express UspA2H in replacement of UspA2 [25]. UspA2H possesses properties of both UspA1 and UspA2 as the UspA2H molecule participates both in adhesion to epithelial cells and serum resistance [25,26].

An interesting finding is that UspA1 has been reported to include a critical binding site for carcinoembryonic antigen-related cell adhesion molecule-1 (CEACAM-1), which is widely distributed on epithelial cells including those of the oropharynx and lower respiratory tract [27]. Electron microscopy studies have shown that UspA1 and A2 form a dense forest of lollipop-like structures at the bacterial surface projecting between 600 and 700 Å from the cell membrane [28], with a tripartite structure consisting of a head group, extended stalk region and membrane anchor domain. In contrast to the laminin and fibronectin-binding domains that are located in the N-terminal region of UspAs, the most protruding part of the UspA1 molecule, the CEACAM-1-binding domain, was found in the extended stalk region [29]. Using X-ray crystallography, Conners *et al.* [30••] demonstrated that the CEACAM-1 receptor-binding region of UspA1 unusually consists of an extended, rod-like left-handed trimeric coiled-coil. That study also supported a model in which the stalk of UspA1 is capable of changing conformation after CEACAM-1 binding to allow a closer approach between *Moraxella* and the host cell membrane and explain how UspA1 can engage CEACAM-1 at a site far distant from its head group, permitting closer proximity of the respective cell surfaces during infection.

The great variety of functions attributed to the UspA family is remarkable. However, a diversity among UspAs exists at the amino acid level as well as there being large potential to exchange variable functional motifs [31•,32]. It is proposed that the structural and functional determinants of each UspA are phylogenetically fluid, allowing the acquisition of various combinations of binding functions. These potential combinations of binding activities could define the attachment ability and virulence of each strain.

The *Moraxella* immunoglobulin D-binding protein (MID, also named Hag; hemagglutinin) is another extensively studied and highly conserved OMP that has a unique ability to bind immunoglobulin D (IgD) in a nonimmune manner [33]. In addition, MID functions as a hemagglutinin and adhesin that binds to type II alveolar epithelial cells [34–36]. Analysis of the MID sequence revealed that it resembles members of a family of autotransporter proteins designated oligomeric coiled-coil adhesins (Oca) in analogy with the *Yersinia enterocolitica* OMP YadA and the *H. influenzae* adhesin (Hia) [28,36,37]. Recently, Hallstrom *et al.* [38•] showed that MID forms a multimeric complex of at least three MID monomers, whereas the last 210 aa of the C-terminal region (translocator domain) of each one contributes to form a β-barrel structure composed of 10 transmembrane β-sheets. The rest of the MID protein forms an approximately 120 nm long, fibrillar structure in which the individual monomers fold back on themselves to expose a globular distal domain at their tips composed of both the IgD binding and adhesive domains [38•].

Interactions with the innate immune system

The innate and adaptive (acquired) immune systems protect our body against invading microbes and comprise

a complex network consisting of tissues, cells and molecules working together to eliminate intruders. In contrast to the adaptive immune response, the innate counterpart is present at birth and can be induced within a few minutes. The innate immune system comprises phagocytes in addition to an array of soluble and membrane-bound molecules including complement and lysozymes.

Evasion of complement system

The complement system plays an important part of the innate immunity in the first line of defense against invading microbes. Complement resistance is considered as a major virulence factor of pathogenic bacteria. The majority of *M. catarrhalis* strains are resistant to serum killing, and several proteins have been reported to contribute to its survival in serum. The most important OMPs involved in serum resistance are the UspAs, capable of interfering both with the classical complement pathway by binding the complement inhibitor C4 binding protein (C4BP) and the alternative complement pathway by neutralizing C3 [39,40]. *M. catarrhalis* has also been shown to inhibit the direct formation of the membrane attack complex by binding vitronectin to UspA2 [24].

M. catarrhalis releases outer membrane vesicles (OMVs), which carry some of the underlying periplasm, together with OMPs, and possess porins, receptors, pores and lipopolysaccharide from the outer membrane layer. *M. catarrhalis* OMVs carry UspA1 and A2 that also has been proven in clinical nasopharyngeal specimens from a 9-year-old child [41]. UspA-expressing OMVs specifically bind C3 and hence counteract the complement cascade. Most importantly, *Moraxella* OMVs also increase the survival of the extraordinary serum susceptible nontypeable *H. influenzae* when exposed to human serum. As *Moraxella* often is isolated as a copathogen with *H. influenzae*, this phenomenon could thus explain how these two respiratory pathogens collaborate.

Airway inflammation

In COPD patients, airway inflammation is characterized by increased infiltration of neutrophils, lymphocytes and macrophages with the concomitant prevalence of bioactive substances and inflammatory mediators [42,43]. It was recently demonstrated that UspA1 and A2 can bind and neutralize α1-antichymotrypsin [44••], which is a serine protease inhibitor considered as a key player in several inflammatory processes protecting host tissues from proteolytic and oxidative damage. The UspA-dependent α1-antichymotrypsin neutralization is a novel microbial virulence mechanism unique for *Moraxella*. The increased protease activity may lead to excessive inflammation, resulting in more exposed extracellular matrix that is beneficial for bacterial colonization, and also might explain how *M. catarrhalis* contributes to the chronic inflammation seen in patients with COPD.

Among all the inflammatory mediators, the cytokine interleukin (IL)-8 has a critical role in regulating neutrophil and monocyte chemotaxis toward sites of infection. It was recently demonstrated that *M. catarrhalis* infection activates protein kinase C through nuclear factor (NF-κB) signalling pathways which in turn induce the IL-8 production [45]. The activation of the protein kinase C (PKC)/NF-κB signalling pathway was found to be UspA2 dependent. Another interesting observation is that UspA1-dependent binding to CEACAM-1 induces apoptosis in epithelial cells [46]. Pulmonary epithelial cells exposed to *Moraxella* showed an increased activity of caspases 3, 6 and 9 in addition to a reduced expression of Bcl-2, translocation of Bax into the mitochondria and cytosolic increase of apoptosis-inducing factor, suggesting a direct involvement of mitochondrial death pathways. Thus, *Moraxella* promotes airway inflammation not only by increasing the protease activity and proinflammatory cytokine production, but also through a direct apoptosis induction of infected cells. It remains, however, for these findings to be examined in detail in a suitable in-vivo model.

Toll-like receptor activation

Together with the complement system, toll-like receptors (TLRs) are important components of the innate immune system and sense microbial molecules designated pathogen-associated molecular patterns (PAMPs). The specific inflammatory responses are mediated upon activation of these pathogen recognition receptors. In particular, TLR2 is upregulated during various pulmonary disorders such as COPD and seems to be especially important for the modulation of airway inflammation [47,48]. *M. catarrhalis* induces an inflammatory immune response in respiratory epithelial cells that is mainly dependent on TLR2 [19••,49]. More interestingly, a recent report demonstrated that the TLR2-mediated epithelial IL-8 release caused by *M. catarrhalis* was inhibited upon UspA1-dependent interaction with CEACAM-1 [50••]. CEACAM-1, which is a member of the immunoglobulin superfamily proteins, has a cytoplasmic domain containing an immunoreceptor tyrosine-based inhibitory motif (ITIM) [51]. Phosphorylation of this domain results in recruitment of the Src homology 2 domain-containing cytoplasmic protein tyrosine phosphatase 1 (SPH-1), which initiates negative feedback pathways by limiting phosphorylation of the p85α regulatory subunit of the class A phosphoinositide 3-OH kinase [PI(3)K] [52]. Recruitment of the p85α subunit of PI(3)K to TLR2 has also been shown to be important for TLR2-induced activation of NF-κB [53]. Slevogt *et al.* [50••] found that CEACAM-1 colocalized with TLR2 on the epithelial surface and that engagement of CEACAM-1

by UspA1 induced recruitment of the SPH-1 to CEACAM-1 and to TLR2. However, subsequent formation of the signaling complex composed of TLR2 and PI(3)K was abrogated as a consequence of UspA1–CEACAM-1 interactions, which lead to inhibition of the PI(3)K–NF-κB signaling pathways. This is a previously unknown strategy of pathogen evasion of the human immune response and, more notably, the TLR2-regulatory effect was not restricted to *M. catarrhalis* alone, as *Neisseria meningitidis* Opa proteins elicited the same response [50••].

Similar to other Gram-negative bacteria, *M. catarrhalis* also possesses lipooligosaccharide (LOS) in its outer membrane which consists of an oligosaccharide core and lipid A without repeated O-antigen polysaccharide side chains. A recent study showed that *M. catarrhalis* LOS stimulates pro-inflammatory cytokine production and selectively induces intercellular adhesion molecule (ICAM)-1 expression on human monocytes via TLR4-dependent and CD14-dependent pathways [54]. LOS-activated monocytes also influence adjacent naive monocytes to produce pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α, which is partially mediated through enhanced surface ICAM-1 expression and IL-8 secretion. This study also demonstrated that the LOS-induced surface ICAM-1 expression involved a TNF-α-dependent autocrine mechanism and could be further augmented by lipopolysaccharide-binding protein (LBP) in serum. Despite enhanced cell–cell interactions via ICAM-1 and cytokines playing a critical role in leukocyte recruitment and bacterial clearance, these activities may also lead to a prolonged and excessive airway inflammation resulting in an increased bacterial adhesion to the respiratory tract. Taking into account all the different PAMPs and their receptors resulting in inflammation, a complicated view of *M. catarrhalis* pathogenesis is revealed. In particular, *Moraxella* possesses very sophisticated ways of increasing the survival as exemplified by inhibition of the TLR2-induced activation via CEACAM-1 [50••].

Bacterial persistence in the host

Persistence is the consequence of a continuous struggle between host and microbial interests in which a successful pathogen has several strategies to thwart host responses and gain a niche. Many bacterial mechanisms have been recognized to mediate persistence in the host, for example, complement resistance as discussed above, intracellular localization, variation of surface-exposed antigen, inhibition of phagocytosis and resistance to phagocytosis killing, adaptative immune response evasion and finally biofilm formation. New studies indicate that *M. catarrhalis*, a species that in some age groups can be considered as a commensal, possesses some of these abilities which, at least partially, can help to explain the carriage of this pathogen in humans.

Invasion of respiratory epithelial cells

Entry into epithelial cells may protect bacteria against host clearance and also plays an important role in overcoming the epithelial barrier for subsequent spreading. *M. catarrhalis* is capable of invading respiratory epithelial cells including bronchial epithelial cells lines and primary small airway epithelial cells *in vitro* [19••]. By using a combination of cytoskeleton inhibition and tissue culture invasion assays, it was demonstrated that *M. catarrhalis* actively induces its own uptake into epithelial cells by a microfilament-dependent trigger-like mechanism. Internalization of *M. catarrhalis* was shown to be dependent on bacterial viability, which suggests that some *Moraxella* secretory products might be actively involved in the induction of macropinocytosis. It was also demonstrated that both TLR2 and NOD1 participated in the IL-8 respiratory epithelial cell response to *M. catarrhalis*. More recently, Spaniol *et al.* [55•] showed that UspA1 and also the LOS has a role in invasion of epithelial cells using Chang conjunctival cells. Furthermore, the inhibition of UspA1-mediated binding to cell-associated fibronectin and α5β1 integrin showed a decreased invasion of *M. catarrhalis* and indicated that these receptors could be involved in the UspA1-promoted invasion [55•]. Importantly, the ability of *M. catarrhalis* to invade non-phagocytic cells has also been proven *in vivo* by Heiniger *et al.* [56••]. The traditional concept of *Moraxella* as an 'obligate' extracellular respiratory pathogen is now changing in the light of these new reports. Although more studies are needed to determine the mechanisms involved in this process, it is most likely that *M. catarrhalis* uses an intracellular strategy to persist in the host.

Thymus independent B-cell activation and nonspecific antibodies

It has been suggested that *Moraxella* carriage might be underestimated due to its ability to hide in the pharyngeal lymphoid tissue, in which naive B cells reside [56••,57]. In contrast to conventional surface cultures of tonsils and adenoids, modern molecular biology techniques revealed *M. catarrhalis* in more than 85% of preschool children undergoing tonsillectomy. This clinical observation is important, and the reservoir of *Moraxella* in the pharyngeal lymphoid tissues may explain the bacterial preference for B cells. With regard to *Moraxella*-dependent B-cell interactions, the large adhesin MID induces B-cell activation independently of physical T-cell help resulting in a nonspecific IgM production [58]. The activation is initiated through a nonimmune cross-linking of the IgD constant heavy chain region 1 and leads to upregulation of an array of different B-cell surface molecules [59•]. Importantly, MID-expressing *M. catarrhalis* does not induce apoptosis in tonsillar B

cells isolated from children. IgD binding alone is not enough, however, to induce a strong proliferative response, and we have found that particularly activation via TLR9, which is essential for the recognition of CpG motifs present in bacterial DNA, is required in synergy with IgD signaling for an efficient *Moraxella*-dependent B-cell activation [60]. Available data thus suggest that the bacterial interaction with B cells serves to redirect and elude the early adaptive immune response in order to increase the possibilities for bacterial survival. However, it cannot be excluded that also the host responds by taking advantage of the bacterial IgD interaction as clones of IgD^+ IgM^- B cells exist, albeit at low numbers, and may represent unique B lineage subsets generated in response to superantigens such as MID [61].

Biofilm development

It is most likely that *M. catarrhalis* exists in biofilms together with commensals on the mucosal surfaces in the nasopharynx. In fact, detection of *M. catarrhalis* biofilms in the middle ear mucosa specimens obtained from children with recurrent otitis media supports the hypothesis that the bacterial biofilms play a key role in chronic middle ear infections [18]. To date, there have been only a few studies of biofilm formation by *M. catarrhalis in vitro* [17,34,62,63]. Two of these studies exploit crystal violet-based assays to demonstrate that UspA1, UspA2H and MID proteins are involved in biofilm formation [17,63]. More recently, Luke *et al.* [64•] showed that *M. catarrhalis* forms a mature biofilm in continuous-flow chambers and that biofilm formation is enhanced by type IV pili expression. Type IV pili are cell surface organelles found on many Gram-negative bacteria and mediate a variety of functions including adhesion, twitching motility and competence for DNA uptake, and thus are recognized as important virulence determinants. The same group previously identified the genes that encode proteins involved in the biosynthesis of type IV pili and demonstrated that the pili are highly conserved and essential for natural transformation of *M. catarrhalis* [65]. The potential role of type IV pili in *M. catarrhalis* colonization was also proved both *in vitro* and *in vivo* by using epithelial cell attachment assays and the chinchilla model system [64•]. Interestingly, the biofilms produced by *M. catarrhalis* under flow cell conditions resemble the complex structures composed of microcolony pillars interspersed with water channels produced by *Pseudomonas aeruginosa* [64•].

Conclusion

Studies completed during the last year enhance our understanding of the complex virulence mechanisms underlying the development of *M. catarrhallis* infection. *M. catarrhalis* has an important number of multifunctional virulence factors and presents, in some cases, previously unknown strategies to circumvent the immune defense. The ability of UspA to neutralize α1-antichymotrypsin, reduce TLR2-dependent pro-inflammatory responses of primary pulmonary epithelial cells via its interaction with CEACAM-1 and also interfere with the complement system in delicate ways illustrates the potential of this pathogen to interplay with the human host. The superantigen MID mediates adhesion to respiratory epithelial cells but is also capable of inducing proliferation and unspecific IgM production in tonsillar B cells. It is now proven that *M. catarrhalis* is able to invade epithelial cells and form biofilms both *in vitro* and *in vivo*. Taken together, available data explain the persistance of *Moraxella* as a commensal, and it is fascinating how this species can conquer in particular the innate immune system. Future research should be directed to further investigate the role of the multifaceted *Moraxella* virulence factors in bacterial pathogenesis in order to increase the understanding of bacterial colonization and infection in the large groups of patients suffering from AOM or COPD.

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