Immunization with the Truncated Adhesin Moraxella catarrhalis Immunoglobulin D-Binding Protein (MID764-913) Is Protective against M. catarrhalis in a Mouse Model of Pulmonary Clearance.

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Published in:
Journal of Infectious Diseases

2004

Immunization with the Truncated Adhesin *Moraxella catarrhalis* Immunoglobulin D–Binding Protein ($MID^{764–913}$) Is Protective against *M. catarrhalis* in a Mouse Model of Pulmonary Clearance

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Most *Moraxella catarrhalis* isolates express the outer membrane protein MID. In addition to its specific affinity for immunoglobulin D, MID functions as an adhesin and binds to human epithelium. The adhesive part is localized within $MID^{764–913}$. Two *mid*-deficient *M. catarrhalis* isolates were constructed and examined in a mouse model of pulmonary clearance. *M. catarrhalis* devoid of MID was cleared more efficiently, compared with the wild-type counterparts. Furthermore, mice immunized with $MID^{764–913}$ cleared *M. catarrhalis* much more efficiently, compared with mice immunized with bovine serum albumin. $MID^{764–913}$ is suggested as a promising candidate in a future *M. catarrhalis* vaccine.

After *Haemophilus influenzae* and *Streptococcus pneumoniae, Moraxella catarrhalis* is the third most common bacterial agent in acute otitis media in children. *M. catarrhalis* is often implicated as a cause of sinusitis in both children and adults. In adults and the elderly, *M. catarrhalis* is a common cause of lower respiratory tract infections, particularly in those with predisposing conditions, such as chronic obstructive pulmonary disease. The emergence of *M. catarrhalis* as a significant cause of human disease and the increase of antibiotic resistance in the species have generated much interest in the identification of potential vaccine antigens [1].

Recently, a novel surface protein of *M. catarrhalis* that displays a high affinity for IgD (MID) was solubilized in Empigen (Calbiochem) and isolated by use of ion-exchange chromatography and gel filtration [2]. The apparent molecular mass of monomeric MID was estimated to be ~200 kDa by use of SDS-PAGE. The *mid* gene was cloned and expressed in *Escherichia coli.* The complete *mid* nucleotide gene sequence was determined, and the deduced amino acid sequence consists of 2123 or 2139 residues, depending on 2 alternative translation starts. MID is most likely identical to the 200-kDa protein described by Fitzgerald et al. [3] and the Hag protein described by Pearson et al. [4]. The sequence of MID has no similarity to other immunoglobulin-binding proteins and was found to exhibit unique immunoglobulin-binding properties [2]. Thus, MID bound 2 purified IgD myeloma proteins, 4 IgD myeloma sera, and, finally, 1 IgD standard serum. No binding of MID was detected to IgG, IgM, IgA, or IgE myeloma proteins. MID was also attracted to the surface-expressed B cell receptor IgD, but not to other membrane molecules, on human peripheral blood lymphocytes [5]. The smallest fragment of MID with essentially preserved IgD binding comprised 238 aa residues ($MID^{962–1200}$) [6]. Ultracentrifugation experiments and gel electrophoresis revealed that native $MID^{962–1200}$ is a tetramer. Interestingly, tetrameric $MID^{962–1200}$ attracted IgD >20-fold more efficiently than did the monomeric form. Thus, a tetrameric structure of $MID^{962–1200}$ was crucial for optimal IgD-binding capacity.

The *mid* gene was detected in all 98 strains studied, as revealed by homologic analysis of the signal peptide sequence and a conserved area in the 3′ end of the gene [7]. When the *mid* genes from 5 different strains were compared, an identity of 65.3%–85.0% and a homology of 71.2%–89.1% was detected. Gene analyses showed several amino-acid repeat motifs in the open-reading frames. Eighty-four percent of the *Moraxella* strains expressed MID-dependent IgD binding, as revealed by flow cytometric analysis. It was shown that bacteria reduced their expression of MID by removing a guanosine (G) in their poly(G) tracts downstream of the start codons. *Moraxella* strains isolated from the nasopharynx, blood, and sputum expressed MID at approximately the same frequency. In addition, no variation was observed between strains from different geographical origins. MID and the *mid* gene were found solely in *M. catarrhalis;* related *Neisseria* and *Moraxella* species did not express MID.

Our previous work shows a strict correlation between hem-

Received 24 October 2003; accepted 10 February 2004; electronically published 9 June 2004.

Financial support: Alfred Osterlund Foundation; Anna and Edwin Berger Foundation; Scandinavian Society of Antimicrobial Chemotherapy; Greta and Johan Kock Foundation; Ake Wiberg Foundation; Swedish Medical Research Council; Cancer Foundation at Malmo University Hospital.

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The Journal of Infectious Diseases 2004;190:352–5

0022-1899/2004/19002-0020$15.00

agglutination and expression of MID in *M. catarrhalis* [8]. Isolated MID and a 150-aa recombinant MID–derived protein ($MID^{764–913}$) bound to erythrocytes and type II alveolar epithelial cells. Antibodies to MID, $MID^{764–913}$, or the consensus sequence $MID^{775–804}$ effectively inhibited adherence to the alveolar epithelial cells. Since *M. catarrhalis* isolates expressing MID at high concentrations bound considerably more effectively to epithelial cells, compared with isolates expressing MID at low concentrations and 2 MID-deficient mutants, the MID protein and, in particular, the fragment $MID^{764–913}$ are suggested to be attractive vaccine candidates.

In the present report, we demonstrate that MID is important for bacterial survival, in a mouse pulmonary clearance model, as revealed by MID-deficient *M. catarrhalis* mutants. In addition, mice immunized with the adhesive part of MID ($MID^{764–913}$) more strongly cleared *M. catarrhalis* wild-type *(wt)* bacteria, compared with control mice immunized with bovine serum albumin.

Materials and methods. Female BALB/c mice (6–8 weeks old; Taconic) were used in all experiments. Animal-experimentation guidelines were followed, in accordance with the Swedish National Board for Laboratory Animals at Lund University, Lund, Sweden. Mice were immunized intraperitoneally with 50 μg of purified recombinant $MID^{764–913}$ [6] emulsified in complete Freund's adjuvant (Difco; Becton Dickinson) and received booster doses on days 14 and 48, with the same dose of protein in incomplete Freund's adjuvant. Ten days after the last booster dose, mice were challenged with *M. catarrhalis*.

M. catarrhalis strains are described in table 1. All bacterial strains were grown on solid blood agar medium or in brain-heart infusion liquid medium (Oxoid). MID-deficient *M. catarrhalis* isolates were constructed by use of a standard protocol [8].

For the pulmonary clearance model, the procedure described by Unhanand et al. was followed [9]. Mice were sedated with enfluran in combination with oxygen. A bolus inoculum of 10^8 cfu in PBS containing 0.15% gelatin (final volume, 20 μL) was deposited into the bronchial tubes by use of an intratracheal (int) cannula. Lungs were removed under sterile conditions, weighed, and homogenized by use of a DI 18 dispenser (IKA yellow line; Staufen). The homogenates were serially diluted and plated on blood agar plates, followed by incubation overnight at 37°C. Measurements of antibodies to $MID^{764–913}$ and tests of the bactericidal activity were performed according to standard protocols [10].

Results. To study the influence of MID on pulmonary clearance in mice, MID-deficient mutants of 2 *M. catarrhalis* clinical isolates were constructed. Resulting mutants were deficient in MID, as revealed by Western blotting using polyclonal antibodies (PAbs) against the sequence $MID^{962–1200}$ [8]. Furthermore, the 2 mutants were deficient in expression of MID, as shown by flow cytometric analysis using the same PAbs. In addition, no significant difference in cell density and generation time was found between the *wt* and mutant strains (figure 1), nor could any difference in susceptibility to normal human and mouse serum be detected between MID-deficient mutants and *wt* bacteria.

In initial experiments, pulmonary clearance of 8 strains of *M. catarrhalis* from diverse clinical sources was investigated in the murine model system (table 1). After a bolus inoculum of 10^8 cfu, recovery of bacteria from the lungs was followed by viable count, by use of disintegrated lungs. No correlation was detected between isolates from patients with sinusitis, bronchiolitis, otitis media, or septicemia. Surviving *M. catarrhalis* could not regularly be detected at $\geq$6 h after bacterial inoculation. Consequently, the importance of MID for pulmonary clearance was studied 3 h after bacterial inoculation. As shown in figure 2, the *M. catarrhalis wt* strain RH4 displayed slight net growth over the course of the 3-h experiment. In contrast, the derived mutant was cleared by 80% from the lungs. Another *wt* strain, BBH18, was cleared such that only 30% of the initial inoculum remained in the lungs at 3 h after inoculation. However, only 15% of the derived mutant bacteria could be detected

Table 1. *Moraxella catarrhalis* strains used in the present study.

Strain[a]	Site of isolation	Diagnosis (age of person derived from)	Reference
SÖ-1914	Tympanic cavity	Otitis media (9 months)[b]	
S7-1340	Bronchoalveoli	Myeloma (56 years)	
S6-4568	Nasopharynx	Otitis media (1 year)	
S6-688	Nasopharynx	Leukemia, fever (29 years)	
S6-177	Nasopharynx	Upper respiratory tract infection (1 year)	
Bc5	Nasopharynx		[2]
BBH18	Sputum	Exacerbation, COPD[c]	[7]
BBH18Δ*mid*			
RH4	Blood	Septis	[7]
RH4Δ*mid*			

[a] Most *M. catarrhalis* strains were from Sweden, except for BBH18 and RH4, which were from Denmark.
[b] *Streptococcus pneumoniae* was also isolated.
[c] COPD, chronic obstructive pulmonary disease.

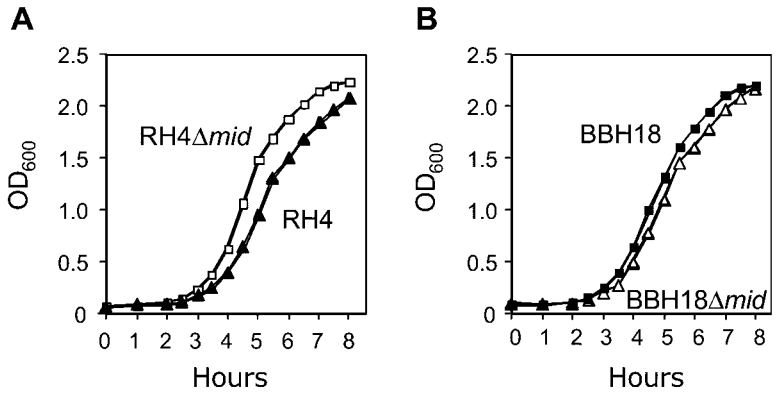

Figure 1. Comparison of the growth rates of the wild-type *Moraxella catarrhalis* parent strains and the MID-deficient mutants. *A, M. catarrhalis* RH4 and RH4Δ*mid*. *B*, BBH18 and BBH18Δ*mid*. Bacteria were grown in brain-heart infusion liquid medium at 37°C, with agitation at 200 rpm. Samples were obtained every 30 min, and the optical density at 600 nm (OD_{600}) was determined. Data are from 1 representative experiment of 3 experiments performed.

at the same time. Thus, the findings suggest that MID is involved in the survival of *M. catarrhalis* in the respiratory tract.

The importance of MID was also studied after immunization of mice with $MID^{764–913}$, a truncated fragment of MID that previously has been shown to be critical for adherence to epithelial cells [8]. Serum samples from mice immunized with $MID^{764–913}$ showed a >10,000-fold increase in antibody titer but no significant increase in bactericidal titer. However, immunized mice had a significantly improved efficiency to clear 2 clinical isolates of *M. catarrhalis* from the lungs (figure 2*B*).

Discussion. The molecular interactions that occur between the pathogen and the host cell are among the earliest events in bacterial infections. These interactions are required for extracellular colonization and internalization to occur and are usually mediated by adhesins on the surface of the microbe. The pathogenesis of *M. catarrhalis* is not completely understood, but, in recent years, an increasing number of virulence factors involved in adhesion and colonization have been determined. We have shown that MID is a highly conserved outer membrane protein (OMP) and have suggested that it plays an important role in the pathogenesis of *M. catarrhalis* [7, 8]. Recently, we demonstrated that MID is an adhesin, by promoting attachment to alveolar epithelial cells, and that the adhesive part of MID is localized within the 150-aa fragment $MID^{764–913}$.

Our observations have been confirmed by Lafontaine et al., who have shown that the Hag protein, which is most likely

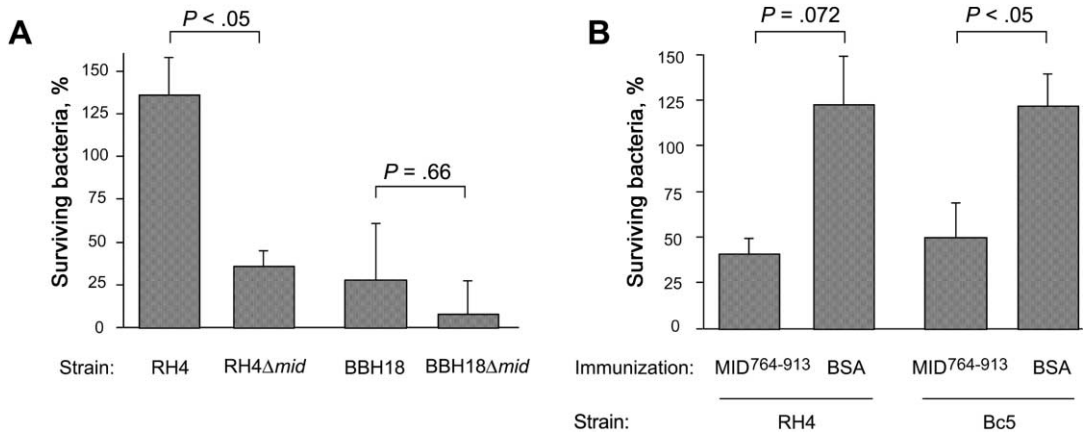

Figure 2. Effect of active immunization with $MID^{764–913}$ on the pulmonary clearance of wild-type *(wt) Moraxella catarrhalis*. *A*, Increased pulmonary clearance occurs with the MID-deficient *M. catarrhalis* RH4 and BBH18 mutants, compared with the *wt* counterparts. *B*, The clinical *Moraxella wt* isolates RH4 and Bc5 have an increased clearance in mice immunized with $MID^{764–913}$, compared with immunization with bovine serum albumin (BSA). Mice were immunized intraperitoneally with either $MID^{764–913}$ or BSA. Ten days after the second booster dose, 5 mice/group were challenged with 10^8 bacteria. Mice were killed after 3 h, and lungs were homogenized and plated on blood agar plates. Surviving bacteria (percentage) were related to the no. of bacteria detected in lungs at the initiation of the experiment. Data are representative of 2 independent experiments. Error bars indicate SEM of the mean. *P* values were calculated using Student's *t* test (paired data).

identical to MID, is an adhesin for cells derived from human lungs and the middle ear [11]. However, Hag-deficient mutants adhered at *wt* levels to Chang conjunctival epithelial cells. Partly in contrast with the our results and those of Holm et al. [11], Pearson et al. [4] tested 1 isogenic *hag* mutant only and showed that it was attached at *wt* levels to several cell lines, including Chang, Hep-2, 16HBE14 bronchial-epithelial cells, and, finally, NCI-H292 epithelial cells derived from a lungmucoepidermoid carcinoma. Other studies with *M. catarrhalis* have identified ubiquitous surface protein (Usp) A1, UspA2, UspA2H, OMP CD, OMP *M. catarrhalis* adherence protein (McaP), and *Moraxella* lipooligosaccharide as potential adhesins [1, 4, 11–14]. However, of these, only UspA1, UspA2H, and OMP McaP have been directly shown to mediate adherence to human cells.

The low virulence of *M. catarrhalis* in laboratory animals has hampered protection experiments and pathogenicity studies. The most frequently used animal model for the study of pulmonary clearance of *M. catarrhalis* is a mouse model [9]. This model consists of transoral inoculation of bacteria into the lung and enumeration of surviving bacteria after removal and homogenization of lungs. It has been shown that antibodies against the OMP Cop B of *M. catarrhalis,* immunization with detoxified lipooligosaccaride from *M. catarrhalis* (as well as vaccine regimens involving intra Peyer's patch), and intranasal and int routes of immunization with killed *M. catarrhalis* enhance pulmonary clearance of the pathogen. Furthermore, in an aerosol-challenge model, passive immunization with antisera to the bacterial strain used for challenge was shown to enhance clearance [15]. In light of hitherto discovered *M. catarrhalis* OMPs [1, 4, 11–14] and the increased understanding of the pathogenesis of *M. catarrhalis,* MID and its adhesive domain $MID^{764–913}$ [2, 6–8] are very promising components to be included in a future vaccine against *M. catarrhalis.*

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