Pulmonary Clearance of *Moraxella catarrhalis* in an Animal Model

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The virulence mechanisms of *Moraxella catarrhalis* that are involved in producing pulmonary infection are unknown. A well-characterized murine model was used to study the pulmonary clearance of *M. catarrhalis* and analyze the histopathologic changes and the role of phagocytic cells in the infected lungs. Ten strains of *M. catarrhalis* from various isolation sites were evaluated for their ability to resist pulmonary clearance. The rates of clearance of these strains, based on the percentage of the original inoculum remaining at 6 h after challenge, varied considerably. Histopathologic examination of lungs infected with 2 strains that exhibited very different clearance rates revealed similar pathologic responses. Analysis of the phagocytic cell response to these 2 strains revealed significant alveolar recruitment of granulocytes at 3, 6, and 24 h after bacterial challenge. However, granulocyte recruitment in response to strain B22, which was cleared readily, was significantly greater than to strain 035E, which resisted pulmonary clearance. This model system should facilitate investigation of the molecular basis of the interaction between *M. catarrhalis* and the lower respiratory tract.

Moraxella catarrhalis, formerly named *Branhamella catarrhalis* [1], until recently was considered to be a nonpathogenic, commensal inhabitant of the upper respiratory tract. It is now known to be a cause of sinusitis and acute otitis media in children [1–5]. It has also become increasingly recognized as an important pathogen in the lower respiratory tract, producing acute bronchitis and exacerbation of chronic bronchitis and pneumonia in adults, especially in patients with compromised respiratory function [6–15]. Fatal *M. catarrhalis* pneumonia without bloodstream or central nervous system invasion has been described in immunocompromised patients [16], and a nosocomial outbreak of lower respiratory tract infections caused by *M. catarrhalis* has been reported [17].

Essentially nothing is known about the virulence mechanisms used by *M. catarrhalis* in the production of respiratory tract disease. There is a similar paucity of information concerning the immune response to this pathogen, although recent studies have indicated that there are a few outer membrane proteins that are exposed on the surface of this organism and that could be targets for protective antibodies [18, 19]. Studies of both virulence and immunity have been hindered by the lack of an inexpensive animal model for studying the interaction of *M. catarrhalis* with the respiratory tract. Animal models using chinchillas or gerbils to study experimental otitis media resulting from direct middle ear inoculation of *M. catarrhalis* have been described [20] but are either very expensive (the chinchilla model) or technically difficult (both models).

We describe here the use of a well-characterized murine model for studying the clearance of *M. catarrhalis* from the lower respiratory tract after direct bolus inoculation of the lungs. Ten different strains of *M. catarrhalis* were evaluated for their ability to resist pulmonary clearance. The effect of growth of *M. catarrhalis* in the murine lung was examined histopathologically, and the phagocytic cell response to this bacterial challenge was assessed. The outer membrane protein (OMP) profile and lipooligosaccharide (LOS) characteristics for each strain were determined to provide a basis for identification of possible phenotypic differences that could be correlated with findings obtained from the animal model.

Materials and Methods

Animals. Female BALB/c VAF/Plus mice (Charles River Breeding Laboratories, Wilmington, MA), 8–10 weeks old and weighing 20–25 g, were used in these experiments. All animals were housed in the specific pathogen–free barrier room facility in the Animal Resources Center at this institution.

Bacterial strains and culture conditions. M. catarrhalis strains B21 and B22 were clinical isolates from nasopharyngeal swabs (from E. Juni, University of Michigan, Ann Arbor). 035E and 046E were recovered from the middle ear fluid of patients with otitis media treated at Children's Medical Center, Dallas. FR2682 and FR2213 were recovered from tracheal aspirates of clinical pneumonia cases (from R. Wallace, University of Texas Health Center, Tyler). TTA24 and P48 were isolated from tracheal aspirates of patients with confirmed *M. catarrhalis* pneumonia (from S. Berk, East Tennessee State University [ETSU], Johnson City). W1 and BC1 were isolated from blood cultures

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(from S. Berk, ETSU, and G. Doern, University of Massachusetts Medical Center, Worcester, respectively).

All *M. catarrhalis* strains were cultured in brain-heart infusion (BHI) broth (Difco Laboratories, Detroit). Before each experiment, a few colonies from a BHI agar plate were used to inoculate 10 ml of BHI broth. This culture was then incubated at 37°C in a rotary water-bath shaker until the bacteria were in the midlogarithmic phase of growth. Cells were then harvested by centrifugation at 7600 *g* at 4°C for 10 min. The cell pellet was suspended in 5 ml of pyrogen-free PBS, pH 7.2, and diluted with this same buffer to the desired bacterial cell density immediately before inoculation into the animals.

Characterization of OMPs and LOSs. Outer membrane vesicles were extracted from whole cells of *M. catarrhalis* using the EDTA-based method [21]. Proteins present in these vesicles were resolved by SDS-PAGE and stained with Coomassie blue [22, 23]. For characterization of LOS, whole-cell lysates of *M. catarrhalis* cells were prepared and treated with proteinase K (Boehringer-Mannheim, Indianapolis) as described [24]. The LOS present in these lysates was resolved by SDS–polyacrylamide gradient gel electrophoresis (PAGGE) [25] and stained with silver by the method of Tsai and Frasch [26].

Method of bacterial inoculation in the pulmonary clearance model. The method of bacterial inoculation was described previously [27]. Briefly, mice were anesthetized by intramuscular injection of 2 mg of ketamine HCl (Fort Dodge Laboratory, Fort Dodge, IA) and 0.2 mg of acepromazine maleate (Fort Dodge Laboratory). After tracheal exposure, mice were intubated transorally, and a 20-gauge intravenous catheter was advanced until it could be seen through the translucent tracheal wall. A PE-10 polyethylene tube (Clay Adams, Division of Becton Dickinson, Parsippany, NJ) containing 5 μl of bacterial suspension was passed through the catheter into the lung. The inoculum was then expressed from the tube by using 150 μl of air. This technique delivered the inoculum to a localized, peripheral segment of the lung.

Pulmonary clearance. In each experiment, four or five mice were killed by intraperitoneal injection of 0.75 mg of pentobarbital sodium (Abbott Laboratories, North Chicago) immediately after inoculation (0 h) to determine bacterial deposition in the lung and at 6 or 24 h after inoculation. The lungs were removed aseptically and homogenized in 2 ml of sterile BHI in a tissue homogenizer (Omni-mixer; Omni International, Waterbury, CT), then were ground in a tissue grinder as described previously [28, 29]. The homogenate was serially diluted in BHI broth, plated on BHI agar, and incubated at 37°C in an air incubator with a 5% Co_2 atmosphere for 24 h. Clearance of *M. catarrhalis* from the lungs was expressed as the percentage of colony-forming units (cfu) remaining in the lung at each time point compared with the mean cfu of bacteria present at 0 h in the same experiment. Each pulmonary clearance experiment was repeated at least once.

Bronchoalveolar lavage. Bronchoalveolar lavage (BAL) was done on groups of five mice each at 0 h and 3, 6, and 24 h after inoculation of $1–3 \times 10^5$ cfu of *M. catarrhalis* into the lung. Control mice were challenged with 5 μl of pyrogen-free PBS, pH 7.2. All mice were sacrificed by intraperitoneal injection of pentobarbital immediately before BAL. Mice were intubated transorally with a 20-gauge catheter as described above, and the lungs were irrigated with 3 ml of heparinized saline introduced into the lungs in 0.6-ml aliquots. Fluid recovered from the lungs was collected on ice; total cells were counted in a hemocytometer, and differential cell counts were done on cytocentrifuged Wright-stained preparations.

Lung histology. At 0 h and at 3, 6, and 24 h after challenge with 10^5 cfu of *M. catarrhalis*, equivalent numbers of mice were killed and their lungs were aseptically removed. The lungs were fixed in 10% neutral formalin; vertical midline sections were stained with hematoxylin-eosin for histologic examination. Tissue from all lobes was present in these stained sections. Pneumonia was diagnosed when an inflammatory infiltrate was identified. The extent of involvement was estimated from the percentage of the tissue that contained an infiltrate. The intensity of the interstitial infiltrate and the amount of intraalveolar exudate was estimated using a 0 to 3+ scale: 0, normal, unperturbed lung; 1+, light polymorphonuclear leukocyte (PMNL) infiltrate; 2+, moderate interstitial infiltrate composed of PMNL and mononuclear cells, with some evidence of an intraalveolar exudate composed of proteinaceous fluid and macrophages; 3+, interstitial infiltrate composed of PMNL and mononuclear cells, together with capillary congestion, focal hemorrhage, and an intraalveolar exudate composed of proteinaceous fluid, macrophages, and sloughed pneumocytes. Control and infected lung sections were coded to conceal the identity of each sample before examination by pathologists.

Statistical analysis. To analyze phagocytic cell response to *M. catarrhalis*, we applied a distribution-free test for ordered alternatives based on the Mann-Whitney-Wilcoxon two-sample test modified by Jonckheere [30] for a one-way layout design with randomized trials. For comparison of the increase in the number of PMNL over time between animals inoculated with different strains of *M. catarrhalis*, regression analysis techniques [31] were used to define indicator variables to compare the slopes of two regression lines.

Results

Pulmonary clearance of M. catarrhalis. Pulmonary clearance of 10 strains of *M. catarrhalis* from diverse clinical sources was investigated in the murine model system. The number of viable bacteria remaining in the lungs 6 h after bolus deposition of $1.04–4.73 \times 10^5$ cfu was determined and expressed as a percentage of the initial inoculum (table 1). The 10 strains differed markedly in terms of their ability to resist clearance from the lung. Two strains, 035E and TTA24, displayed slight net growth over the 6-h experimental period. All of the other strains were cleared with various degrees of efficiency such that from 6% (strain FR2213) to 66% (strain BC1) of the initial inoculum remained in the lungs at 6 h after inoculation.

There appeared to be no correlation between the original site of isolation of a particular strain and the ability of that strain to persist in the lung; strains isolated from the same anatomic site exhibited a wide range of clearance rates that

Table 1. Pulmonary clearance of *Moraxella catarrhalis* at 6 h after challenge.

Isolation sites of strains, *M. catarrhalis* strain	Deposition at 0 h, mean cfu ± SD*	% bacteria remaining at 6 h, mean ± SD
Nasopharynx		
B21	$4.65 \times 10^5 \pm 2.24$	47 ± 51
B22	$2.41 \times 10^5 \pm 1.63$	11 ± 13
Blood		
W1	$3.79 \times 10^5 \pm 1.16$	12 ± 11
BC1	$1.25 \times 10^5 \pm 0.88$	66 ± 43
Middle ear		
035E	$1.04 \times 10^5 \pm 0.96$	109 ± 27
046E	$4.73 \times 10^5 \pm 0.64$	22 ± 9
Tracheal aspirate (pneumonia)		
P48	$2.20 \times 10^5 \pm 0.49$	41 ± 17
TTA24	$1.20 \times 10^5 \pm 0.11$	108 ± 43
FR2682	$3.43 \times 10^5 \pm 0.14$	40 ± 29
FR2213	$3.38 \times 10^5 \pm 1.58$	6 ± 4

* Each value represents mean of 8–10 animals from two independent experiments.

overlapped those obtained with strains from other isolation sites (table 1). In all instances, bacteria were completely cleared from the lungs by 24 h after challenge, as occurred with other pathogens used previously in this murine model [27, 28]. The use of smaller challenge inocula (i.e., $<10^5$ cfu) resulted in complete clearance of *M. catarrhalis* by 6 h after challenge. When larger challenge inocula (i.e., 10^6 cfu) were used, the clearance patterns were similar or identical to those obtained by using the 10^5 cfu inoculum (data not shown).

Phagocytic cell response to bacterial challenge. The observed differences in the rates of clearance of these 10 *M. catarrhalis* strains from the lung suggested that there may have been quantitative or qualitative differences in phagocytic cell recruitment to the alveoli in response to challenge with these different strains. Two *M. catarrhalis* strains were selected to test this hypothesis. Strain 035E was cleared relatively poorly, with 109% of the inoculum still viable in the lung at 6 h after challenge, while strain B22 was cleared rapidly from the lung such that only 11% of the initial inoculum remained at 6 h after inoculation (table 1). A bolus of 10^5 cfu of these two *M. catarrhalis* strains was deposited in the lungs of 20 mice each. BAL was done on groups of 5 mice each after they were sacrificed at 0 h and at 3, 6, and 24 h after inoculation to determine the numbers and types of phagocytic cells in the alveoli at each time point (table 2).

Similar numbers of alveolar macrophages and PMNL were present in the BAL obtained from control (PBS-challenged) and *M. catarrhalis*–challenged mice immediately after intrabronchial challenge. The difference between the numbers of alveolar macrophages present in the BAL at 3, 6, and 24 h after challenge with strain B22 and the number of cells of this type present at 0 h was small; however, statistical analysis provided strong evidence of an increase in cell numbers over time ($P = .02$). In contrast, the number of PMNL present in the BAL increased significantly over time, with a 10-fold increase at 3 h, a 135-fold increase at 6 h, and a 436-fold increase at 24 h after B22 deposition ($P \leq .001$).

In animals challenged with strain 035E, the numbers of alveolar macrophages in the BAL increased 1.7-fold at 3 and 6 h and 1.9-fold at 24 h after inoculation ($P = .001$). PMNL also showed significant increases in number in response to 035E challenge, increasing 9-, 23-, and 24-fold at 3, 6, and 24 h after deposition, respectively ($P = .001$). The increase in numbers of PMNL recruited to the alveoli of animals challenged with strains B22 and 035E was analyzed by using a comparison of the two regression lines. The values at 0 h were not statistically different ($P = .63$), but there was a significant slope for each line ($P = .001$) and there were significantly different rates of increase for each strain ($P = .001$). In control animals, there were no statistically significant changes in the numbers of alveolar macrophages ($P = .20$) or PMNL ($P = .26$) at 3, 6, or 24 h after challenge compared with 0 h values.

Lung histology. Histologic examination was done on formalin-fixed lungs obtained from PBS- (control), 035E-, and B22-challenged mice at 0, 3, 6, and 24 h after inoculation. Interstitial infiltrates comprised both macrophages and PMNL and were associated with widened alveolar septa, capillary congestion, and focal hemorrhages of varying intensity. There was intraalveolar exudate, in some animals, that comprised proteinaceous fluid, PMNL, macrophages, and scarce, sloughed pneumocytes. The infiltrates were found in all lobes, although the right lung was generally more involved than the left. In some lungs, pneumonia surrounded the large bronchi at the hilum, but in most lungs the peripheral alveoli were principally involved. No significant bronchitis was seen in any animal.

In control (PBS-challenged) animals, the inflammatory indices at various times were similar, being graded 0 (normal) to 1+ at all time points (figure 1A). A slight degree of inflammation (1+) present in a few members of this control group may represent an inflammatory response to mechanical injury resulting from the deposition of the PBS itself. In animals inoculated with *M. catarrhalis* strains B22 and 035E, inflammatory indices at 0 h were similar to those of the control group. By 3–6 h after challenge, B22-infected animals had an inflammatory infiltrate of principally 2+ intensity (figure 1B). By 24 h after challenge, the interstitial infiltrates were uniformly 2+ to 3+ (figure 1C), and an intraalveolar exudate was present in all mice (figure 1D). In animals challenged with 035E, the inflammatory infiltrates ranged from 1+ to 2+, with indices of 2+ predominating at 3 h, similar to the B22-challenged group at 3 h and 6 h. In addition, there was an intraalveolar exudate at 3 h in these 035E-challenged animals. At 6 and 24 h after challenge, inflammatory indices

Table 2. Phagocytic cell response in the lungs after challenge with *Moraxella catarrhalis* strains B22 and 035E.

	Time (h) after inoculation				
	0	3	6	24	*P*
Strain B22					
Macrophages × 10^5	1.78 ± 0.07	1.52 ± 0.33	1.61 ± 0.48	2.71 ± 0.80	≤.02
Polymorphonuclear leukocytes (PMNL) × 10^{4*}	0.04 ± 0.09	0.48 ± 0.39	6.20 ± 2.30	20.00 ± 3.33	≤.001
Strain 035E					
Macrophages × 10^5	1.84 ± 0.17	3.13 ± 1.31	3.12 ± 0.80	3.58 ± 0.60	≤.001
PMNL × 10^{4*}	0.06 ± 0.11	0.59 ± 0.61	1.49 ± 1.77	1.54 ± 0.99	≤.001
Control (pyrogen-free PBS)					
Macrophages × 10^5	1.81 ± 0.13	2.38 ± 0.90	1.82 ± 0.47	2.65 ± 1.42	≤.20
PMNL × 10^4	0.04 ± 0.09	0.45 ± 0.21	0.42 ± 0.61	0.07 ± 0.11	≤.26

NOTE. Each value represents mean ± SD of 8–10 animals at each time point.
* $P = .003$ when increase in number of PMNL over time between 035E- and B22-derived results was compared using regression lines analysis.

were uniformly 2+ with intraalveolar exudate present in all mice challenged with 035E.

OMP and LOS profiles. OMP profiles for each of the 10 *M. catarrhalis* strains used in the pulmonary clearance model are shown in figure 2. Two of these 10 strains, FR2213 (figure 2, lane 5) and W1 (figure 2, lane 7) had OMP profiles that differed significantly from those of the other eight isolates used in this study. These two strains were confirmed to be *M. catarrhalis* by standard clinical microbiology laboratory procedures [32] and by a *M. catarrhalis*–specific genetic transformation assay (Juni E, University of Michigan, personal communication). The remaining eight isolates, including strains 035E (figure 2, lane 1) and B22 (figure 2, lane 4), were similar to each other in OMP profile, having all major bands previously reported for *M. catarrhalis* [13, 33, 34], with only minor differences among these strains. All 10 strains exhibited LOS profiles in SDS-PAGGE typical of *M. catarrhalis* and other LOS-expressing bacteria. The LOS profiles of these 10 strains were all different from each other (data not shown). These results confirm that all organisms used in the study were *M. catarrhalis* and that each represented a different strain.

Discussion

M. catarrhalis is a well-known member of the normal flora of the oropharynx [5, 7]. The pathogenic potential of this organism becomes evident when this bacterium gains access to the middle ear or the lower respiratory tract [4–7]. The method of bolus inoculation used in the animal model described here resembles the mechanism presumably by which these bacteria enter the human lung (i.e., aspiration of oropharyngeal fluids [35]). Once the organisms are deposited in the lung, the extent of net growth that can be achieved in vivo depends on both the ability of the organism to grow in this environment (i.e., to use nutrient sources) and its ability to resist host defense mechanisms (i.e., phagocytosis, antibody-mediated killing, etc.).

The rates of pulmonary clearance of oropharyngeal bacteria in this and similar murine models vary greatly, with differences being dependent on both the bacterial pathogen and mouse strain (e.g., C5-sufficient vs. C5-deficient mice). Some organisms (e.g., *Streptococcus sanguis, Streptococcus salivarius*) are readily cleared from the lungs such that only a tiny fraction of the original inoculum remains viable by 6 h after inoculation [36]. Other oropharyngeal bacteria (e.g., *Haemophilus influenzae*) are apparently better equipped to survive and grow in the lower respiratory tract in this model [27]. After bolus inoculation of nontypeable *H. influenzae* into mouse lungs, a biphasic pattern of growth and clearance was observed. During the first 6 h after challenge, bacterial multiplication exceeded killing such that the total number of viable organisms in the lung increased to a level about fourfold greater than that of the original inoculum. During the second phase, killing exceeded multiplication, and the bacteria were eradicated by 24 h after challenge. These results have been interpreted to indicate that resident host defenses in the mouse lung are unable to effect eradication of certain oropharyngeal bacteria and must be augmented somehow for clearance to occur [27]. The recruitment of PMNL to the lungs has been shown to correlate with enhanced clearance of these same bacteria [28].

A previous study of the interaction of a number of members of the normal flora of the human oropharynx with the lower respiratory tract of mice revealed that *M. catarrhalis* differed from the other organisms in at least two ways [36]. First, after aerosol challenge of the mice, *M. catarrhalis* persisted in the lungs significantly longer than did the other bacterial species, even though there was no net increase in viable numbers of *M. catarrhalis* over the 4-h experimental

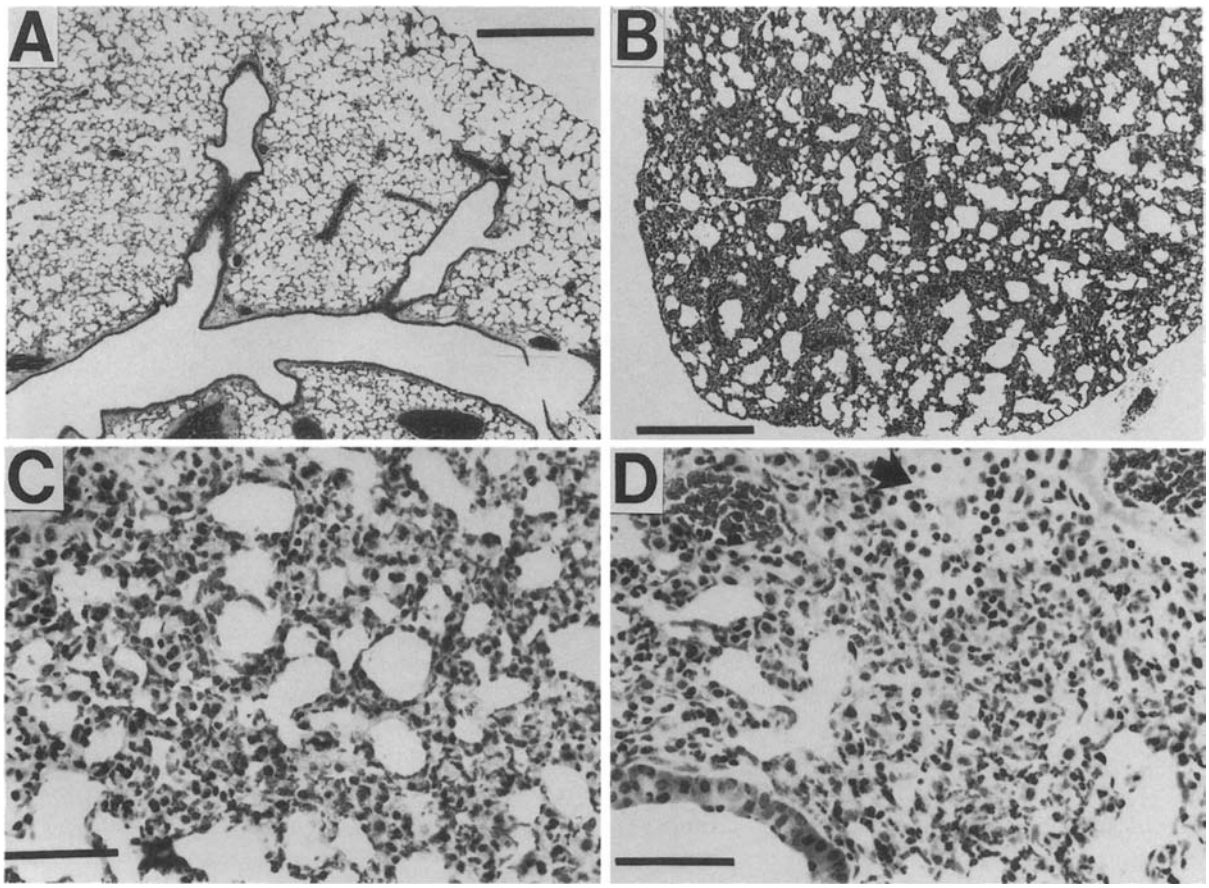

Figure 1. **A**, Normal mouse lung immediately after inoculation (0 h) of pyrogen-free PBS. Mild, focal interstitial response (1+) was present in lungs of some control animals. Bar = 500 μm. **B**, Pulmonary lobe containing 2+ inflammatory response characterized by diffuse widening of alveolar septa, present in B22-challenged animals at 3 and 6 h after inoculation and in 035E-challenged animals at 3, 6, and 24 h after inoculation. Bar = 500 μm. **C**, Interstitial infiltrate associated with mild intraalveolar proteinaceous exudate seen in some 2+ interstitial responses present in both B22- and 035E-challenged animals. Bar = 80 μm. **D**, Prominent intraalveolar inflammatory exudate with the presence of polymorphonuclear leukocytes in alveolar spaces (arrow) seen in B22-challenged animals at 24 h and 035E-challenged animals at 3, 6, and 24 h after inoculation. Bar = 80 μm.

period. Second, challenge of the lungs with *M. catarrhalis* resulted in much greater numbers of PMNL eventually appearing in the alveoli than did challenge with the other organisms [36].

In the present study, we evaluated 10 strains of *M. catarrhalis* for their ability to resist clearance from the mouse lung after bolus inoculation of 10^5 cfu. The rates of clearance of these strains varied considerably, with 2 strains (035E and TTA24) persisting in the lung to a greater extent than did the other 8 strains (table 1). The ability of these 2 strains to survive better in the lungs is apparently not related to growth rate because all 10 strains exhibited similar rates of growth in vitro (data not shown). All of these strains appeared to be different from each other on the basis of comparison of their OMP and LOS profiles (figure 2). The relative abilities of these strains to resist pulmonary clearance also appeared to be unrelated to the original sites of isolation of these strains.

Histopathologic evaluation of the damage produced by these strains during growth in the lungs revealed that both a strain that persisted well in the lungs (e.g., 035E) and a strain that was readily cleared from the lungs (e.g., B22) produced identical types of pathologic reactions in the alveoli (figure 1). However, there was a significant difference in granulocyte cell recruitment to the alveoli in response to challenge with these two strains. Strain B22 recruited PMNL to the alveoli in greater numbers than did strain 035E (table 2). Whether this greater degree of recruitment of phagocytes by strain B22 was directly responsible for the more rapid clearance of this organism cannot be determined at this time, although it has been shown that impairment of PMNL recruitment to the alveoli will enhance the persistence of nontypeable *H. influenzae* in the mouse lung [28]. A previous study involving one strain of *M. catarrhalis* suggested that the ability of this organism to recruit PMNL to the alveoli might contribute to protease-mediated lung destruction [37].

This model system provides a mechanism for distinguish-

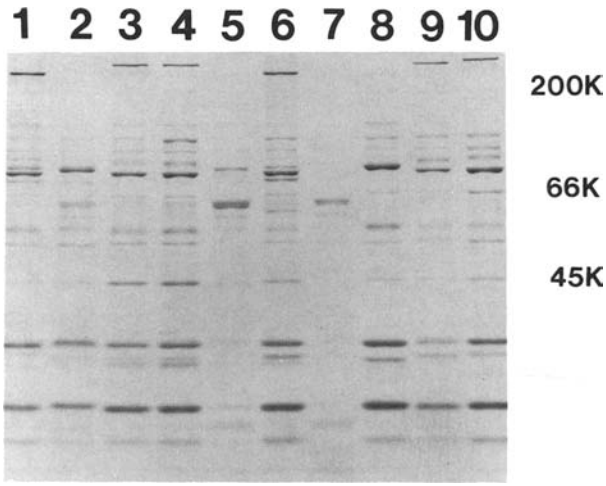

Figure 2. Outer membrane protein profiles of 10 *Moraxella catarrhalis* strains. Outer membrane vesicles of each strain were resolved by SDS-PAGE and stained with Coomassie blue. Molecular weight position markers are shown at right in thousands. Lane 1, 035E; lane 2, 046E; lane 3, B21; lane 4, B22; lane 5, FR2213; lane 6, FR2682; lane 7, W1; lane 8, BC1; lane 9, TTA24; lane 10, P48.

ing strains of *M. catarrhalis* on the basis of their ability to resist pulmonary clearance. In addition, it should be possible to use this model system to study the interaction of this organism with the immune defense system in the lower respiratory tract, as has been accomplished with nontypeable *H. influenzae* [29, 38], another member of the normal oropharyngeal flora that can cause both upper and lower respiratory tract disease [39, 40]. The existence of strains of *M. catarrhalis* that readily persist in the lungs during the 6-h experimental time period will allow experimental determination of the ability of both humoral and cellular immune defenses to augment the pulmonary clearance process. This in turn may permit the identification of potential vaccine candidates among the surface antigens of *M. catarrhalis.*

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