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Human Immune Response against Outer Membrane Proteins of *Moraxella* (*Branhamella*) *catarrhalis* Determined by Immunoblotting and Enzyme Immunoassay

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The role of *Moraxella* (*Branhamella*) *catarrhalis* as a respiratory tract pathogen is increasingly recognized. We looked at the human immune response against individual outer membrane proteins of *M. catarrhalis* and against the 81-kDa CopB protein, which has previously been shown to be a target for protective antibodies. Paired serum samples from six elderly patients with pneumonia were tested by Western blot (immunoblot) analysis by using outer membrane vesicles of *M. catarrhalis* 035E as antigen. All of the six convalescent-phase serum samples reacted with a protein which migrated at the position of the CopB protein and with a high-molecular-weight protein of *M. catarrhalis*; three serum samples also reacted with a 34-kDa outer membrane protein. Paired serum samples from 18 patients, 10 of which had *M. catarrhalis* infection on the basis of previous serology results, were tested by enzyme immunoassay (EIA) with the CopB protein and whole cells of *M. catarrhalis* 035E as antigens. Nine patients showed a significant rise in EIA titer between acute- and convalescent-phase sera when whole bacterial cells were used as antigens. Six (67%) patient samples that were positive by the EIA with the whole-cell antigen were also positive by the EIA with the CopB antigen, and six of nine patient samples negative by the EIA with the whole-cell antigen were also negative by the EIA with the CopB antigen. These results suggest that both the CopB and a high-molecular-weight protein are major targets of the immune response against *M. catarrhalis*, and further studies with greater amounts of patient materials are needed to elucidate the usefulness of CopB as an antigen in etiologic studies.

Moraxella (*Branhamella*) *catarrhalis* is an increasingly important cause of respiratory tract infections (8). It is the third most common etiologic agent of otitis media and sinusitis in children and an important pathogen in bronchopulmonary infections (8, 9, 19, 21, 33, 35, 36, 39). *M. catarrhalis* can also cause systemic infections such as septicemia, endocarditis, meningitis, and arthritis mainly in immunocompromised patients (5). The rapid acquisition of β-lactamase by *M. catarrhalis* during the 1980s has complicated the treatment of diseases caused by these bacteria (35, 36). Respiratory tract infections are an important cause of morbidity and mortality in industrialized countries and in developing nations, respectively, and therefore, the development of vaccines against the major pathogens *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *M. catarrhalis* has become an important target of research.

The etiology of infection is easily obtained in patients with otitis media and sinusitis in which the causative organism is cultivated from normally sterile body compartments. In bronchopulmonary infections the interpretation of bacteriological findings is more difficult since *M. catarrhalis* is known to exist as a harmless inhabitant of the upper respiratory tract and the expectorated sputum commonly used in the diagnosis of pulmonary infections may be contaminated by the normal flora (11, 18, 40). Because of these diagnostic difficulties in pulmonary infections, a method for measuring the serologic response in patients with *M. catarrhalis* infections would be useful.

Previous studies have shown that *M. catarrhalis* is highly homogeneous both in its outer membrane and in its lipooligosaccharide compositions. The outer membrane of *M. catarrhalis* is composed of six to eight major proteins, and the lipooligosaccharide consists of two major antigenic groups (1, 10, 29, 38). The role of individual outer membrane proteins (OMPs) in the host's immune response against this bacterium has not been elucidated yet. However, the outer membrane of another gram-negative upper respiratory tract pathogen, *H. influenzae*, is an important target for the host's defense mechanisms (7, 42). For the development of better diagnostic methods, it is important to know more about the antigenic conservation of these proteins in *M. catarrhalis*. Recently, genes coding for two OMPs of *M. catarrhalis*, the 81-kDa CopB protein and the heat-modifiable CD protein, have been sequenced, which greatly facilitates research on these individual proteins (16, 30). The purpose of the study described here was to determine the serologic responses of patients with *M. catarrhalis* pulmonary infection against individual OMPs and against the 81-kDa CopB protein.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *M. catarrhalis* 035E is a clinical isolate from the middle ear fluid of a patient with otitis media treated at Children's Medical Center, Dallas, Tex. This strain has been well characterized and was the source of the CopB protein (27, 37). The cloning of the gene coding for the CopB protein and the sequence of this protein have been described previously (16). *M. catarrhalis* 035E was grown in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, Mich.) at 37°C or on BHI agar at 37°C in a 5% CO_2 atmosphere. *Escherichia coli* HB101 and *E. coli* BL21 (DE3) (Novagen, Madison, Wis.) were grown on LB medium supplemented with the appropriate antibiotics when needed.

Serum specimens. Paired serum samples taken about 14 days apart came from 18 mostly elderly patients with X-ray-positive acute pneumonia requiring hospitalization. The sera were collected in connection with different studies on the etiology of pneumonia in adults (4, 19, 22). Ten of these patients (patients 1 to

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10 in Table 1) had shown at least a threefold serum immunoglobulin G response to *M. catarrhalis* in an enzyme immunoassay (EIA) with a mixture of whole bacterial cells of 10 different *M. catarrhalis* strains as antigens. For three of the patients (patient 1, 2, and 6) sputum culture was positive for *M. catarrhalis*, while for the other patients sputum cultures were not done.

Preparation of antigens. Outer membrane vesicles from *M. catarrhalis* 035E were prepared for Western blot (immunoblot) experiments by the EDTA and heat induction method described by Murphy and Loeb (31). Whole cells of *M. catarrhalis* 035E and *E. coli* HB101 were prepared for EIA as follows. The bacteria were grown on BHI agar overnight at 37°C in a 5% CO_2 atmosphere. The next morning the bacteria were harvested in cold phosphate-buffered saline (PBS; pH 7.2) and washed twice. The whole cells were resuspended in PBS (pH 7.2) to an optical density of 0.150 at 650 nm. This suspension was used as antigen in the EIA and was stored at −70°C until use. To obtain sufficient amounts of the CopB protein for the EIA a protein expression system from Novagen was used. The gene coding for the CopB protein was ligated with the expression vector pET-21+ (Novagen). The plasmid obtained, designated pRAB100, was transformed into protein-expressing *E. coli* BL21 (DE3) (Novagen), and overexpression was performed with isopropyl-β-D-thiogalactopyranoside (IPTG) according to the manufacturer's instructions. Briefly, 50 ml of *E. coli* BL21 (DE3) cultures containing pRAB100 or appropriate control plasmids was grown to a Klett reading of 100. IPTG was then added to a final concentration of 1 mM. After 3 h 1-ml samples were harvested and CopB induction was analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). Frozen pellets were stored at −70°C following a wash with 50 mM Tris-HCl (pH 8.0)–2 mM EDTA. The isolation of the soluble and insoluble (inclusion bodies) protein fractions was performed by resuspending the thawed cell pellets into 5 ml of 50 mM Tris-HCl (pH 8.0)–2 mM EDTA. To this suspension lysozyme (100 μg/ml) and Triton X-100 (0.1%) were added. After incubation at room temperature for 30 min, the suspension was sonicated and then centrifuged at 12,000 × *g* for 15 min. The pellet, which contained the protein, was resuspended in 1 ml of PBS buffer and was used as antigen in the EIA.

Monoclonal antibody. Monoclonal antibody 10F3 to the CopB protein was produced by standard methods as described previously (34).

SDS-PAGE and Western blot analysis. SDS-PAGE analysis was performed by standard methods, and the proteins were stained with Coomassie blue when needed (23). Western blot (immunoblot) analysis was carried out as follows. The proteins were separated after boiling in a reducing buffer by using an SDS–12% polyacrylamide gel and were transferred to Immobilon-P transfer membranes (Millipore Corporation, Bedford, Mass.). A low-molecular-weight standard (Pharmacia LKB Biotechnology, Uppsala, Sweden) was included in each experiment. The transfer membranes were blocked with 0.04% skim milk in PBS (pH 7.2) for an hour, after which they were allowed to incubate with the test sera diluted 1:8 to 1:256 in PBS–Tween 20 (1:2,000) overnight. A separate strip was incubated with monoclonal antibody 10F3. The next morning, after three washes a peroxidase-conjugated rabbit anti-human immunoglobulin G (Dako A/S, Glostrup, Denmark) diluted 1:1,000 in PBS–Tween 20 was added. When using monoclonal antibody 10F3, a peroxidase-conjugated rabbit anti-mouse gamma chain (Dako A/S) diluted 1:1,000 in PBS–Tween 20 and a second goat anti-rabbit antibody (Bio-Rad Laboratories, Richmond, Calif.) diluted 1:3,000 were used. After washes the substrate was added and the color was allowed to develop for 3 min.

EIA. Flat-bottom, 96-well, Maxisorp plates (Nunc, Roskilde, Denmark) were used in the EIA. The wells were coated with 100 μl of the CopB protein pellet (10 μg/ml), with *M. catarrhalis* 035E whole-cell antigen (1:400), or with *E. coli* HB101 whole-cell antigen (1:400), all of which were diluted in PBS (pH 7.2), and were allowed to incubate overnight at 37°C. After four washes with PBS–Tween 20 (0.5%) the wells were blocked with 5% sheep serum in PBS (pH 7.2) for an hour, the serum samples, diluted 1:100 to 1:10,000 in 10% fetal calf serum, were added in duplicate, and the mixtures were allowed to incubate for 2 h. After four washes the alkaline phosphatase-conjugated polyvalent goat anti-human immunoglobulins (Caltac Laboratories, San Francisco, Calif.), diluted 1:1,000 in 10% fetal calf serum, were added, the mixtures were incubated for 2 h, and the plates were again washed. Finally, the substrate solution (1 mg of *p*-nitrophenyl phosphate [Sigma Chemical Co., St. Louis, Mo.] in 1 M diethanolamine buffer containing 2 mmol of $MgCl_2$; pH 9.8) was allowed to incubate for 30 min at 37°C, and the optical density was read at 405 nm with a multiscan photometer (Labsystems Multiscan, MCC/340; Labsystems, Helsinki, Finland). When whole bacterial cells were used as antigen a threefold or greater increase in titer was regarded as positive, and when CopB protein was used as antigen a rise of twofold or more in titer was considered positive. These criteria are based on previous studies (25, 26).

RESULTS

***M. catarrhalis* CopB OMP.** The CopB protein was produced with the protein expression vector pET-21+ (Novagen). The inclusion bodies obtained were used as antigen in the EIA. Figure 1 shows the CopB inclusion bodies running at the same level as the original protein when compared with the level at which the outer membrane vesicles of *M. catarrhalis* 035E ran. The inclusion bodies were also recognized by monoclonal antibody 10F3 both in EIA and in Western blot analyses (data not shown), supporting the study of the protein as antigen in the EIA.

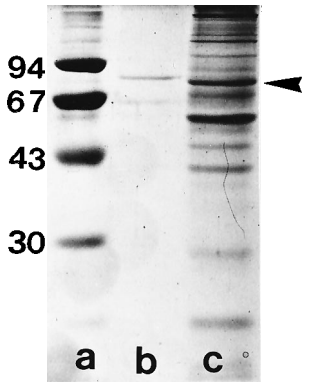

FIG. 1. Comparison of the CopB protein with the 81-kDa OMP of *M. catarrhalis* when electrophoresed on an SDS-polyacrylamide gel. Coomassie blue-stained gel shows molecular weight markers in lane a, the CopB protein (inclusion bodies) in lane b, and outer membrane vesicles of *M. catarrhalis* in lane c. The arrowhead indicates the 81-kDa CopB protein.

Comparison of whole *M. catarrhalis* cells and CopB protein as antigen in EIA. Eighteen paired serum samples were studied in the EIA with whole bacterial cells and the CopB protein as antigens. The results are presented in Table 1. On the basis of previous studies, 10 of the patient serum samples were immunoglobulin G positive when a mixture of whole *M. catarrhalis* cells from 10 strains was used as the antigen and eight were negative. With *M. catarrhalis* 035E whole cells as antigen, nine of the samples were positive and nine were negative. Of these nine positive samples, six were also positive when the CopB protein was used as the antigen and six of the nine samples that were negative by the whole-cell EIA were negative when the CopB protein was used as the antigen. Because the CopB protein (inclusion bodies) preparation also contained some *E. coli* antigens, we simultaneously tested the *E. coli* response of the serum samples when using whole *E. coli* HB101 cells as the antigen in the EIA to see if there was any correlation with the CopB antibody response. There was none (data not shown).

Western blot identification of immunodominant antigens. Six paired serum specimens were chosen for Western blot analysis on the basis of EIA results when whole cells of *M. catarrhalis* 035E were used as the antigen. Five of the serum samples (from patients 1, 4, 6, 7, and 9) were considered positive by the whole-cell EIA and one (from patient 18) was negative. Figure 2 shows the bands obtained after immunoblotting with the test sera. By diluting out the serum samples, differences between the acute- and the convalescent-phase sera were obtained in Western blot reactions, indicating an immune response against the protein(s) during infection. The most prominent reaction in each experiment was with a protein running at the approximate position of the 81-kDa CopB protein identified by its reaction with monoclonal antibody 10F3 when OMPs of *M. catarrhalis* 035E were used as antigens. Patients 1, 4, 6, and 7 showed an antibody response against the protein during infection, whereas patient 9 had constantly high levels. In five of the six blots the test sera also light up a high-molecular-weight protein(s) detected at the very top of the blot. In the sixth blot there was a very faint reaction at this position. There were also several minor reactions

TABLE 1. Titers of serum IgG antibody to two whole bacterial cell antigens and the CopB protein of *M. catarrhalis* 035E in paired serum specimens from patients with pneumonia

Patient no.	Serum specimen no.	EIA titer (RF) to[a]:		
		WBC1	WBC2	CopB
1	1	800	6,580	800
	2	5,300 (6.6)	>100,000 (>15)	2,650 (3.3)
2	1	450	1,440	400
	2	4,600 (10.2)	12,300 (8.6)	740 (1.9)
3	1	310	2,790	240
	2	3,200 (10.1)	10,730 (3.8)	370 (1.5)
4	1	2,980	5,560	810
	2	>10,000 (>3.4)	>100,000 (>18)	1,900 (2.3)
5	1	250	210	100
	2	3,825 (15.3)	4,020 (19.0)	70 (0.7)
6	1	420	120	740
	2	4,650 (11.7)	>100,000 (>800)	5,670 (7.7)
7	1	2,250	2,475	320
	2	8,010 (3.6)	22,580 (9.1)	1,270 (4.0)
8	1	<100	355	620
	2	230 (>2.3)	1,070 (3.0)	1,570 (2.5)
9	1	750	4,900	920
	2	7,570 (10.1)	38,140 (7.8)	2,800 (3.0)
10	1	470	8,950	340
	2	3,620 (7.7)	21,970 (2.5)	2,130 (6.3)
11	1	5,000	12,470	270
	2	>10,000 (>2)	19,670 (1.6)	1,410 (5.3)
12	1	500	3,430	570
	2	1,370 (2.7)	9,210 (2.7)	760 (1.3)
13	1	594	3,660	590
	2	676 (1.1)	5,930 (1.6)	680 (1.1)
14	1	2,340	3,380	1,540
	2	4,250 (1.8)	4,740 (1.4)	2,110 (1.4)
15	1	1,680	8,115	980
	2	2,030 (1.2)	7,640 (0.9)	1,260 (1.3)
16	1	510	6,380	620
	2	510 (1.0)	10,750 (1.7)	2,460 (4.0)
17	1	1,940	6,350	2,360
	2	2,510 (1.3)	15,445 (2.4)	2,160 (0.9)
18	1	<100	760	320
	2	160 (>1.6)	1,640 (2.2)	490 (1.5)

[a] RF, fold rise in titer; WBC1, mixture of whole bacterial cells of 10 strains; WBC2, whole bacterial cells of strain 035E.

with the test sera. Three serum samples (from patients 1, 6, and 9) detected a protein band(s) running at the level of approximately 34 kDa, and two of these test sera (from patients 6 and 9) also reacted with a 21-kDa protein(s). With sera from patient 4 there was a reaction with a protein running at approximately 40 kDa. With antibody dilutions a difference was detected between the acute- and convalescent-phase serum reactions against the 34-kDa protein(s) (patients 1 and 6) and against the 21-kDa protein(s) (patient 6).

DISCUSSION

We have previously cloned and sequenced the gene encoding an 81-kDa OMP of *M. catarrhalis* called CopB (16). This protein can be widely detected in different *M. catarrhalis* strains, and the monoclonal antibody recognizing the protein protects mice against *M. catarrhalis* infection. To further clarify the nature of this protein we have constructed a mutant lacking the CopB protein and have shown it to be less virulent than the parent strain and also susceptible to serum, whereas the original strain was resistant to serum (17). On the basis of those results the CopB protein is an interesting target for vaccine research and may also be useful in *M. catarrhalis* diagnostics. In the present study we showed that the CopB protein and several other OMPs of *M. catarrhalis* are important targets for the host immune response, further emphasizing the need to study more thoroughly the OMPs from the point of view of vaccine research and to obtain better diagnostic tools.

There are several reports of the host immune response against *M. catarrhalis*, but the role of individual OMPs is still unresolved. All healthy adults seem to have specific *M. catarrhalis* antibodies as demonstrated by EIA by Goldblatt et al. (13). They also showed by Western blot analysis preliminary evidence that an OMP of 82 kDa seems to be the major target of the host immune response (14). Various methods have been used to detect specific *M. catarrhalis* antibodies in diseased states, making comparison of the results difficult. Antibodies have been detected by complement fixation, immunodiffusion, EIA, bactericidal assays, and immunofluorescence (2, 3, 6, 13, 26, 32). Leinonen et al. (26) have shown by EIA that 9 of 19 children with culture-confirmed *M. catarrhalis* middle ear infection had an increase in serum anti-*M. catarrhalis* whole-cell titer between their acute- and convalescent-phase sera. Similar results were obtained when Faden et al. (12) used OMPs as the antigen in EIA, in which 57% of children with otitis media showed an antibody response. Chapman et al. (6) showed partly strain-specific bactericidal antibodies in the convalescent-phase sera of patients with respiratory tract infections. Another indication of antigenic differences between strains comes from the study of Jonsson et al. (20), who demonstrated differences in immunofluorescence titers with different strains and sera.

There is no widely accepted and uniformly reliable diagnostic method for evaluation of the role of bacteria in lower respiratory tract infections. We compared EIA titers between acute- and convalescent-phase sera when using either *M. catarrhalis* whole cells or the CopB protein as antigen. On the basis of previous studies a threefold or greater increase in antibody titer was considered diagnostic when whole cells of *M. catarrhalis* were used as the antigen and a twofold or greater increase in titer was considered diagnostic when CopB protein was used as the antigen (25, 26). In the present study the CopB protein recognized two-thirds of the samples positive by EIA and two-thirds of the samples negative by EIA when compared with the results obtained by the whole-cell assay. There are several explanations for these discrepancies. In the three patients (patients 10, 11, and 16) with negative EIA results with the whole-cell antigen and positive EIA results with the CopB protein, the EIA titers with the whole-cell antigen were already high in the acute-phase serum sample, indicating that the immune response had probably started before the acute-phase sample was obtained. It is common for *M. catarrhalis* infections to manifest only in patients with underlying chronic lung disease, and then with relatively subtle symptoms that are sometimes difficult to separate from the underlying condition and viral infections, which makes it difficult to detect the beginning of the infection (41). When using whole bacterial cells as antigen, the immune response consists of antibody rises against several different antigens, including the CopB protein. Thus, in our study, the timing for the acute-phase serum sample might have been too late for the titer change in the EIA with the whole-cell antigen to be positive but accurate enough when the CopB protein was used. Asymptomatic nasopharyngeal carriage of *M. catarrhalis* is also possible, a situation which, with other bacteria, has been shown to induce specific antibodies, which might also explain the high titers in the acute-phase sera (15). Since CopB is only one of the many antigens present on the bacteria, the immune response against CopB may be hidden under very high titers in serum, explain-

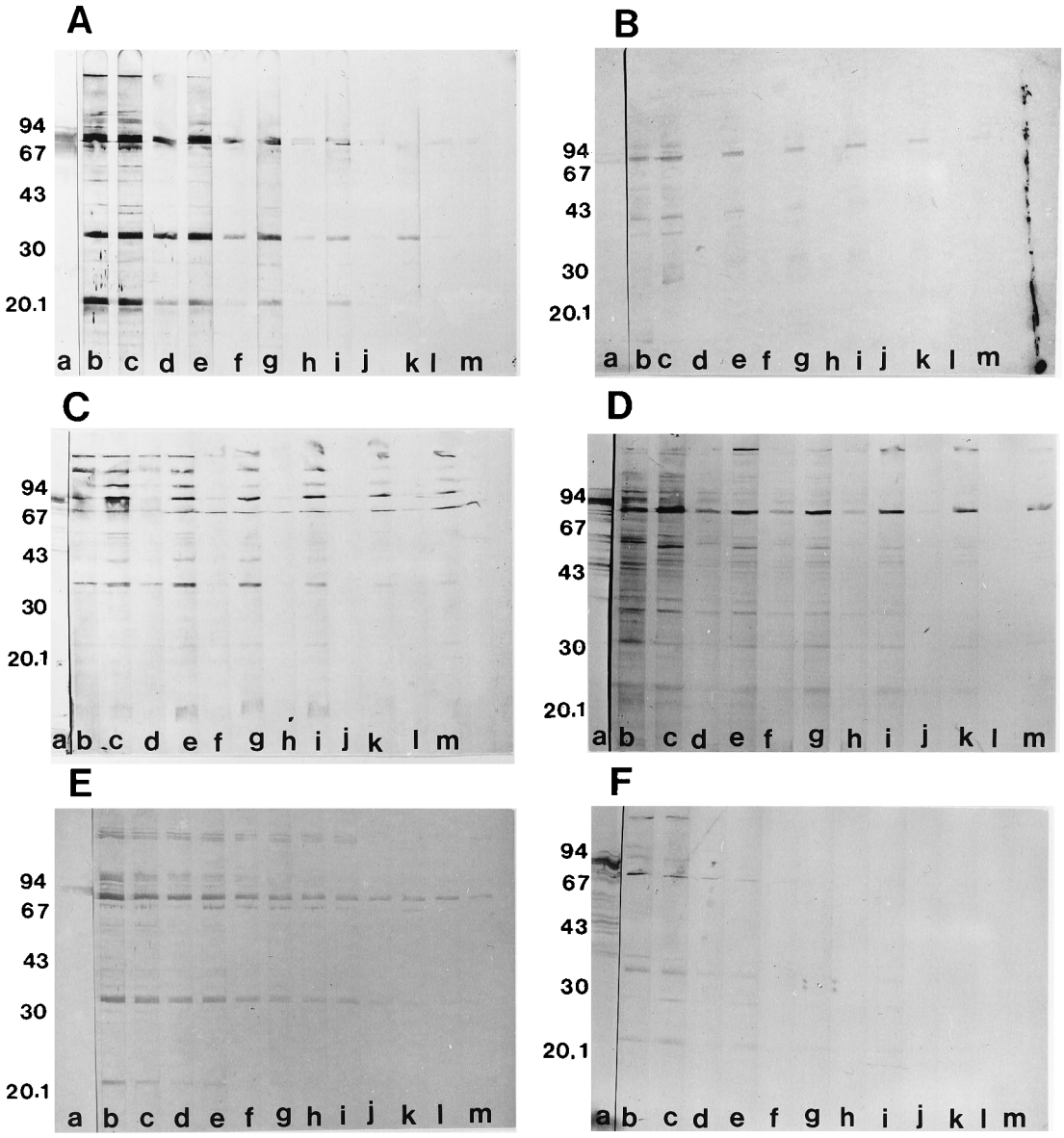

FIG. 2. Western blot analysis of OMPs of *M. catarrhalis* 035E with six paired human serum samples. Outer membrane vesicles of strain 035E were allowed to react with acute-phase (lanes b, d, f, h, j, and l) and convalescent-phase (lanes c, e, g, i, k, and m) test sera diluted sequentially from $1:2^3$ to $1:2^8$ (lanes b and c to l and m) or with the monoclonal antibody 10F3 (lane a). (A) EIA-reactive patient 6; (B) EIA-reactive patient 4; (C) EIA-reactive patient 1; (D) EIA-reactive patient 7; (E) EIA-reactive patient 9; (F) EIA-nonreactive patient 18.

ing the negative EIA result when whole cells are used as the antigen. On the other hand, three paired serum samples (from patients 2, 3, and 5) showed a negative result when CopB was the antigen, although there was a significant rise in the titer in serum when whole cells were used as the antigen. Since we did not have the original causative *M. catarrhalis* isolates, we could not test whether they possessed the CopB protein. However, there was a small but not significant enough rise in titer with the CopB antigen in these negative samples, suggesting that the strains did possess the antigen. The only exception could be patient 5, whose sera had such low titers that they may have been caused by background reaction to *E. coli* antigens. It has also been shown previously that the epitope recognized by monoclonal antibody 10F3 is not present on all strains, although they do have the CopB protein, emphasizing the importance of other epitopes as targets of the immune response (16).

The importance of the CopB protein was further confirmed by Western blot analysis, in which the CopB protein reacted with all six paired serum samples. Another protein of interest is the high-molecular-weight protein of *M. catarrhalis*, which reacted clearly with five of the paired serum samples and faintly with the sixth sample in Western blot analysis. This high-molecular-weight protein was not included in the original OMP classification of *M. catarrhalis* by Murphy (28). However, Klingman and Murphy (24) later described a high-molecular-weight protein of *M. catarrhalis*. The molecular mass of this protein varies between 350 and 720 kDa among different *M. catarrhalis* strains, and it may consist of smaller subunits on the basis of the results obtained with formic acid treatment.

Another protein of *M. catarrhalis* also missing from the original OMP studies on the basis of the SDS-PAGE results is the 34-kDa protein, which in our study could be detected with at least three serum samples. Murphy and Bartos (29) later studied the antigenic conservation of *M. catarrhalis* OMPs and described a protein with an approximate molecular size of 34 kDa, which corresponds to the size of the protein detected in our study. The results presented here are only preliminary, and greater numbers of specimens are needed to evaluate thoroughly the human immune response against the 81-kDa CopB protein and against other OMPs of *M. catarrhalis*. However, on the basis of our results both the CopB and the high-molecular-weight proteins are potential candidates for use as diagnostic antigens, and further research on these proteins is warranted. When purified proteins are available, the diagnosis of *M. catarrhalis* infections becomes more accurate, and new information regarding the importance of individual proteins as targets of immune response can be obtained.

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