Immune Response to *Haemophilus parainfluenzae* in Patients with Chronic Obstructive Lung Disease

JOANNE L. MITCHELL† AND SUSAN L. HILL*

Respiratory Research Laboratory, Division of Medical Sciences, University of Birmingham, Birmingham, United Kingdom

Received 3 May 1999/Returned for modification 13 July 1999/Accepted 28 September 1999

*Haemophilus parainfluenzae* is often isolated from the sputa of patients with chronic obstructive lung disease. We have investigated the immune response to this organism in patients with chronic bronchitis (n = 3) and bronchiectasis (n = 10) and in healthy controls (n = 9). Outer membrane proteins (OMPs) of *H. parainfluenzae* were purified for use in enzyme-linked immunosorbent and immunoblot assays. Whole-cell *H. parainfluenzae* preparations were used to adsorb antibodies from serum samples, which were subsequently immunoblot assayed to investigate the antibody response to surface-exposed epitopes. Levels of *H. parainfluenzae*-specific immunoglobulin G (IgG), but not IgA or IgM, were increased in the sera of patients with chronic obstructive lung disease compared to levels in control subjects. The species specificity of the antibody response was confirmed, although a degree of cross-reactivity with *H. influenzae* antigens was observed. IgA and IgG specific for OMPs of *H. parainfluenzae* were demonstrated to be present in the sputa and sera of five patients with chronic obstructive lung disease. Variation in the pattern and intensity of antigen recognition was observed among patients and among immunoglobulin classes. OMPs of approximately 36, 22, and 15 kDa were confirmed to possess epitopes exposed on the surface of intact *H. parainfluenzae*. We have demonstrated the presence of a species-specific systemic immune response to *H. parainfluenzae* in colonized patients. A specific antibody response was also observed in sputum, and the antigen specificity of these responses in patients with chronic obstructive lung disease was investigated for the first time. The presence of a specific immune response suggests that *H. parainfluenzae* may have a pathogenic role in patients with chronic obstructive lung disease.

*H. parainfluenzae* is a common commensal organism of the normal oropharynx, is becoming recognized as an opportunistic pathogen causing systemic diseases with a spectrum similar to that of the closely related nontypeable *Haemophilus influenzae* (NTHI), including endocarditis, meningitis, and bacteremia (1, 4). This species of *Haemophilus* has also been isolated from the sputa of patients with chronic obstructive lung disease (20), but whereas the role of NTHI as a respiratory pathogen has become established, the role of *H. parainfluenzae* in both acute and chronic lung infections remains to be elucidated.

The presence of a specific antibody response, over and above that observed in healthy individuals, is often used as a marker of current or previous infection by a variety of infectious agents. Studies of the immune response to NTHI in patients with chronic obstructive lung disease have been important in establishing a pathogenic role for the organism (5, 7, 10, 13). In addition, research has focused on the antigenicity of outer membrane proteins (OMPs) of NTHI (6, 8, 12, 16–18), since naturally produced or vaccine-stimulated antibodies specific for surface-exposed epitopes of these proteins are important in immune-mediated bacterial clearance mechanisms. In contrast, few studies on either the presence or specificity of the immune response to *H. parainfluenzae*, particularly in patients with chronic obstructive lung disease, have been performed.

The outer membrane composition of *H. parainfluenzae* is similar to that of other gram-negative bacteria and includes a major heat-modifiable protein of approximately 37 kDa, peptidoglycan-associated proteins (15, 27), and lipopolysaccharides (21). *H. parainfluenzae* also appears to exhibit diversity in OMP profiles similar to that of NTHI (21); however, in contrast to the case of NTHI, little work has been published regarding the antigenic characteristics of the major OMPs of *H. parainfluenzae*. Work on *H. influenzae* has established that this species has OMPs which include a heat-modifiable protein, P5; a porin, P2 (26, 27); and a peptidoglycan-associated lipoprotein, P6 (2). Suzuki et al. (24) have shown that the outer membrane of *H. parainfluenzae* also contains proteins which display homology to P2, P5, and the P6 precursor of *H. influenzae*. These proteins have all been considered as vaccine candidates for protection against *H. influenzae* infection; however, their importance as targets for antibodies in patients with chronic lung disease who are infected with *H. parainfluenzae* has not yet been investigated.

In order to provide evidence for or against a role for *H. parainfluenzae* as a pathogen in chronic lung disease, we performed a pilot study of patients with chronic bronchitis or bronchiectasis, who are frequently infected with or colonized by this species. We investigated the systemic antibody response in 13 of these patients (3 with chronic bronchitis and 10 with bronchiectasis) using an enzyme-linked immunosorbent assay (ELISA) and compared their levels of specific antibody to those in healthy controls (n = 9). The species specificity of the response has been confirmed through adsorption of serum samples with either *H. parainfluenzae* or *H. influenzae*. We also investigated the patterns of OMP recognition of immunoglobulin G (IgG) and IgA in sputa and sera from five patients with chronic obstructive lung disease and the specificity of the systemic antibody response for epitopes exposed on the intact surface of *H. parainfluenzae*. 

---

* Corresponding author. Mailing address: Department of Respiratory Medicine, Queen Elizabeth Hospital, Birmingham, United Kingdom B15 2TH. Phone: 44 121 627 2088. Fax: 44 121 627 2012. E-mail: susan.hill@university-b.wmids.nhs.uk.

† Present address: Micropathology Ltd., University of Warwick Science Park, Coventry, United Kingdom CV4 7EZ.
MATERIALS AND METHODS

Patients and controls. Ten patients with idiopathic bronchiectasis proven by high-resolution computed tomography scanning (seven female and three male; mean age, 69 years; 51 to 83 years) and three patients with Motion Research Council-defined chronic bronchitis (two female and one male; mean age, 67 years; range, 63 to 70 years), all with cough and daily production of sputum from which H. parainfluenzae was isolated regularly, were studied. All patients had evidence of long-standing airflow obstruction (mean FEV₁, as percentage of predicted was 56.9 [standard error, 9.2]). Patients provided sputum from which the sol phase was obtained by centrifugation at 50,000 × g for 90 min at 4°C and venous blood from which serum was obtained by low-speed centrifugation at 300 × g. The sol phase and serum were stored at −20°C. Analysis of sputum from each patient were also subjected to quantitative bacterial culture (19), and H. parainfluenzae was present in the samples studied at a mean of 2 × 10⁵ CFU/ml (range, 2 × 10⁴ to 7 × 10⁶ CFU/ml). The identity of H. parainfluenzae was confirmed by the API NH typing system (bioMerieux, Basingstoke, United Kingdom) and by requirements for NAD (V factor) and hemin (X factor). Isolates were stored in freezing broth (10% [vol/vol] glycerol in brain heart infusion [BHI] broth) at −70°C until required for study.

Nine healthy control subjects (five female and four male; mean age, 68 years; range, 61 to 78 years) provided venous blood samples from which serum was obtained for ELISA, and a further six healthy control subjects (all female; mean age, 45 years; range, 41 to 52 years) provided serum which was pooled for immunoblot assay. Two other healthy control subjects (both male, aged 23 and 26 years) underwent sputum induction. Briefly, subjects inhaled hypertonic saline (30% [wt/vol] sodium chloride) for 30 s at 2.5 l/min, setting 6; Soniprobe; Lucas Dawe Ultrasonics, Hayes, United Kingdom), was modified from the protocols of Loeb (11) and Sethi et al. (22).

ELISA for antibodies to H. parainfluenzae. The capture antigen was prepared as follows. Eight distinct isolates of H. parainfluenzae (distinguished by differences in OMP profiles by sodium dodecyl sulfate-polyacrylamide gel electrophoresis [SDS-PAGE]) were grown overnight in BHI broth (75 ml per isolate). The bacteria were harvested by centrifugation (10,000 × g for 60 min at 4°C) and the bacterial pellet was stored at −20°C until required for study.

Further incubation and washing were followed by the addition of 2.5 M sodium hydroxide (50 ml) in carbonate buffer (0.1 M, pH 9.6). The plates were incubated overnight at 4°C, and nonspecific binding was blocked with PBS-Tw containing 0.5% bovine serum albumin. Serum samples were blocked with PBS-Tw containing 0.1% Tween 20 PBS-Tw). The plates were incubated with 1/1,000 diluted human IgG-specific antibodies (diluted to 1/2,000 in TTBS; The Binding Site, Birmingham, United Kingdom) or for IgG or IgA alone (diluted to 1/2,000; Dako). After 1 h, the nitrocellulose was washed again with TTBS for 5 min at 4°C. The nitrocellulose was washed again and color was developed for 10 min with VIP development solution (Vector Laboratories Ltd., Peterborough, United Kingdom), after which the nitrocellulose was rinsed in distilled water and allowed to dry.

Adsortion and elution assay. Adsorption of serum antibodies by whole H. parainfluenzae was modified from the protocols of Loeb (11) and Sethi et al. (22). Three different isolates of H. parainfluenzae were isolated from the sputa of bronchiectatic patients regularly colonized with this species and were used to adsorb antibodies from sera collected from the same patients. Sputa and sera were collected on the same day. The isolated strains of H. parainfluenzae were grown to stationary phase in 3 ml of BHI broth supplemented with NAD and hemin (10 μg/ml) and inoculated into 75 ml of supplemented BHI broth. These bacteria were incubated for 6 to 8 h at 37°C until the absorbance of the bacterial suspensions reached 0.3 to 0.6. These bacteria were harvested by centrifugation (12,000 × g for 10 min at 4°C), washed at 4°C by resuspension in 10 ml of phosphate-buffered saline (PBS; pH 7.4), and sonicated on ice. The bacterial pellets were stored at −70°C until required for study.

Levels of IgG, IgA, and IgM antibody against whole-cell H. parainfluenzae antigens in the sera of 13 patients with chronic obstructive lung disease (10 with bronchiectasis and 3 with chronic bronchitis) and 9 healthy control subjects are shown in Fig. 1. Antibody recognizing H. parainfluenzae was found to be present in all patients and control subjects studied, with the exception of a single control subject in whom only specific IgM was undetectable. Levels of H. parainfluenzae-reactive IgG were significantly higher in patients than in controls (mean absorbance in patients, 0.51; standard deviation [SD], 0.23; mean absorbance in controls, 0.26; SD, 0.15; P < 0.01). Mean levels of IgA and
IgM recognizing *H. parainfluenzae* were lower than those of IgG in patients and controls, and no significant differences were observed between the two groups with regard to IgA (mean absorbance in patients, 0.22; SD, 0.15; mean absorbance in controls, 0.15; SD, 0.11) or IgM (mean absorbance in patients, 0.07; SD, 0.05; mean absorbance in controls, 0.08; SD, 0.07). There was a significant inverse correlation between bacterial load in sputum and levels of reactive IgG in serum (Spearman’s rank correlation coefficient $r$, $-0.57$; $2P < 0.05$) (Fig. 2). There was no significant correlation between bacterial load and IgA or IgM.

The species specificity of the antibody reacting with a whole-cell preparation of *H. parainfluenzae* was tested by comparing the results of the ELISA after serum samples from all of the patients had been adsorbed with either sonicated whole-cell preparations of *H. parainfluenzae* or similar preparations of *H. influenzae*. Results are shown in Fig. 3, along with the level of reactive antibody measured in unadsorbed serum. A reduction in *H. parainfluenzae*-reactive IgG was observed after adsorption of serum with *H. influenzae* (for unadsorbed sera, the mean absorbance was 0.79 and the SD was 0.33; for sera adsorbed with *H. influenzae*, the mean absorbance was 0.29 and the SD was 0.11 [$P < 0.001$]). However, a far greater reduction was observed when serum samples were adsorbed with *H. parainfluenzae* (mean absorbance, 0.06; SD, 0.03), resulting in levels which were significantly lower than those both in unadsorbed serum ($P < 0.001$) and in serum adsorbed with *H. influenzae* ($P < 0.001$).

**Immunoblot assay of serum and sputum sol phase.** Sera (1/100) and sputum sol phase (1/10) from five patients (two with chronic bronchitis and three with bronchiectasis) were immunoblot assayed for the presence of IgG and IgA specific for OMPs prepared from a single strain (CF3115) of *H. parainfluenzae* (Fig. 4a). Staining by IgG-specific antibodies was
more intense than that by IgA-specific antibodies. Similar patterns of recognition were observed when sputum and serum from the same patient were compared, although patterns did vary among individuals. Samples from two patients displayed low levels of staining of OMPs by sputum IgG (Fig. 4a, lanes B and D). Generally, staining was most intense in IgG-specific assays of serum, whereas for IgA, sputum provided more intense staining than serum. Overall, the specificities of IgG and IgA varied in that an antigen of approximately 22 kDa and several more between 45 and 66 kDa were recognized by IgG only. Two antigens consistently recognized by IgG and IgA had approximate molecular masses of 15 and 37 kDa.

Pooled serum and induced sputum sol phase obtained from healthy controls were also immunoblot assayed for the presence of IgG, IgA, and IgM antibodies recognizing OMP antigens of strain CF3115 (Fig. 4b). Two antigens of approximately
15 and 37 kDa were recognized faintly by antibodies in serum (Fig. 4b, lane C). Staining by sputum antibodies was negligible.

No specific staining of the buffer-only control (Fig. 4b, lane D) was observed in either of the above assays.

**Adsorption and elution assay.** Three adsorption assays were performed; the results of one are shown in Fig. 5. In two of the assays, three *H. parainfluenzae* OMPs (approximate molecular masses of 37, 22, and 15 kDa) were recognized by IgG, IgA, and IgM antibodies in heat-treated homologous serum, and two OMPs (22 and 15 kDa) were recognized by IgG, IgA, and IgM in heat-treated serum from the remaining patient. In all assays, adsorption of sera with the control organism, *E. coli* (Fig. 5, lane B), had no effect on the intensity of antibody recognition of *H. parainfluenzae* OMPs. Adsorption with intact *H. parainfluenzae* (Fig. 5, lane C), however, reduced the recognition of OMPs to negligible levels. After adsorption, antibodies specific for *H. parainfluenzae* OMPs could be eluted from the surface of *H. parainfluenzae* (Fig. 5, lane F) but not from the surface of similarly treated *E. coli* (Fig. 5, lane E).

**DISCUSSION**

*H. parainfluenzae* is isolated from the sputa of patients with chronic lung disease in relatively large numbers and at a frequency similar to that of accepted respiratory pathogens such as NTHI (9). Little work, however, has been performed investigating the contribution of this species to the pathogenesis of chronic lung disease, and since, like other members of the genus including NTHI, *H. parainfluenzae* is frequently isolated from the oropharynx, it has been regarded as a contaminating commensal organism when isolated from sputa. In this pilot study we have therefore begun to investigate the antibody response to *H. parainfluenzae* as part of our studies of the role of this species in chronic lung infection.

Results from the ELISA described here demonstrated that patients who were regularly colonized with *H. parainfluenzae* had higher levels of serum IgG specific for this organism than did healthy control subjects (Fig. 1). Patients with chronic lung disease, however, are likely to have been colonized with NTHI, and *H. parainfluenzae* is known to possess antigens which cross-react with rabbit antibodies to *H. influenzae* (23). The specificity of the systemic immune response observed in our patients was therefore confirmed through adsorption of serum samples with whole-cell preparations of either NTHI or *H. parainfluenzae* prior to measurement of *H. parainfluenzae*-reactive IgG by ELISA. Adsorption with NTHI reduced the amount of IgG specific for *H. parainfluenzae* in serum samples (Fig. 3), though this was to be expected since a sonicated whole-cell preparation was used. This preparation is certain to contain antigens shared with a variety of bacterial species, including *H. parainfluenzae* (23), and will therefore adsorb cross-reactive antibody in serum samples tested. Adsorption of native serum with *H. parainfluenzae*, however, resulted in a reduction of reactive antibody to very low levels (*P < 0.001; 13 times lower than that observed after adsorption with *H. influenzae*). This result, together with the data from the original ELISA, strongly indicates that there is an elevated systemic IgG response to *H. parainfluenzae* in patients from whom this organism is frequently isolated. Interestingly, there was an inverse correlation between bacterial load in the sputa of these patients and levels of *H. parainfluenzae*-reactive IgG in their sera. It is possible that in patients with lower levels of reactive IgG, the larger numbers of colonizing bacteria in their airways are a consequence of lower levels of opsonizing antibody transudating from serum to airway secretions. It is not possible, however, to draw any firm conclusions from the small number of patients studied here.

Immunoblot assays were performed in an effort to identify which OMPs of *H. parainfluenzae* were recognized by specific antibodies in sera and sputa from patients with chronic obstructive lung disease. IgG and IgA antibodies specific for OMPs were demonstrated to be present in both the sera and sputa of the patients studied. Up to 14 different OMPs of the single strain of *H. parainfluenzae* studied, ranging from 15 to 70 kDa, were recognized by serum IgG. Recognition by sputum IgG followed a similar pattern of staining but was generally of lower intensity. Fewer antigens were recognized by IgA in serum or sputum, but two antigens, of 37 and 15 kDa, were most consistently recognized by IgA and IgG. The exact identity of these OMPs is unknown, but it is possible that the 37-kDa OMP is the same as that described by Suzuki et al. (24) and possesses some homology to the porin protein, P2, of NTHI. The consistent antibody recognition of the 15-kDa OMP is similar to that previously observed for P6 (a 16-kDa, surface-exposed, antigenically conserved lipoprotein of NTHI) in patients with bronchiectasis (14). Bogdan and Apicella (3) have shown that of the *Haemophilus* species, *H. parainfluenzae* is the only one that does not contain the protein P6. Suzuki and colleagues (24), however, have identified an OMP which exhibits homology to the OMP P6 precursor of *H. influenzae*, and it is therefore possible that *H. parainfluenzae* possesses a closely related protein.

Since antibody-mediated clearance of infecting organisms can succeed only in the presence of antibodies recognizing epitopes exposed by live bacteria, the potential functional significance of the antibody response to *H. parainfluenzae* in these patients was investigated by using intact bacteria isolated from their sputa to adsorb antibodies from their sera. Antibodies
reacting with surface-exposed epitopes were then eluted from the surface of the bacteria, and the results were visualized by immunoblot assay. The 36- and 15-kDa antigens described above, together with a 22-kDa antigen (recognized by serum IgG in three of the five patients' samples) initially studied by immunoblot, were demonstrated to possess surface-exposed epitopes, which suggests that they may be important in immune recognition and antibody-dependent clearance mechanisms.

These initial studies, therefore, have demonstrated the presence of a specific immune response in the sera and sputa of a group of patients with chronic obstructive lung disease who are colonized by or frequently infected with H. parainfluenzae. We have shown that a variety of OMPs are recognized by antibodies in these patients but that apparently only three of these possess epitopes exposed on the surface of the intact bacteria. Further studies are necessary to confirm the identity of these antigens and to measure the functional capabilities of antibodies recognizing H. parainfluenzae in these patients.

ACKNOWLEDGMENTS

This work was supported by a noncommercial educational grant from the Bayer Corporation.

REFERENCES

1. Albritton, W. L. 1982. Infections due to Haemophilus species other than H. influenzae. Annu. Rev. Microbiol. 11:199–216.
2. Barenkamp, S. J., R. S. Munson, Jr., and D. M. Granoff. 1982. Outer membrane protein and biotype analysis of pathogenic nontypable Haemophilus influenzae. Infect. Immun. 36:535–540.
3. Bogdan, J. A., Jr., and M. A. Apicella. 1995. Mapping of a surface-exposed, conformational epitope of the P6 protein of Haemophilus influenzae. Infect. Immun. 63:4395–4401.
4. Bruun, B., J. J. Christensen, and M. Kilian. 1984. Bacteraemia caused by a beta-lactamase producing H. parainfluenzae strain of a new biotype. Acta Pathol. Microbiol. Immunol. Scand. Sect. B 92:135–138.
5. Burns, M. W., and J. R. May. 1967. Haemophilus influenzae precipitins in the serum of patients with chronic bronchial disorders. Lancet i:354–358.
6. Deich, R. A., A. Anilionis, J. Fulginiti, B. J. Metcalf, S. Quataert, T. Quinn-Dey, G. W. Zlotnick, and B. A. Green. 1990. Antigenic conservation of the 15,000-dalton outer membrane lipoprotein PCP of Haemophilus influenzae and biologic activity of anti-PCP antisera. Infect. Immun. 58:3388–3393.
7. Glynn, A. A. 1959. Antibodies to Haemophilus influenzae in chronic bronchi-
8. Green, B. A., M. E. Vazquez, G. W. Zlotnick, G. Quigley-Reape, J. D. Swarts, I. Green, J. L. Cowell, C. D. Bluestone, and W. J. Doyle. 1993. Evaluation of mixtures of purified Haemophilus influenzae outer membrane proteins in protection against challenge with nontypeable H. influenzae in the chimpanzee otitis media model. Infect. Immun. 61:1950–1957.
9. Hill, S. L., A. Pye, M. M. Johnson, C. Munday, and R. A. Stockley. 1997. A role for Haemophilus parainfluenzae in chronic lung disease. Respir. Crit. Care Med. 155:A105.
10. Korppi, M., M. L. Katila, J. Jaaskelainen, and M. Leinonen. 1992. Role of non-encapsulated Haemophilus influenzae as a respiratory pathogen in children. Acta Paediatr. 81:989–992.
11. Loeb, M. R. 1984. Immunoblot method for identifying surface components, determining cross-reactivity, and investigating cell topology: results with Haemophilus influenzae type b. Infect. Immun. 55:2612–2618.
12. Loeb, M. R. 1987. Protection of infant rats from Haemophilus influenzae type b infection by antiserum to purified outer membrane protein a. Infect. Immun. 55:2612–2618.
13. May, J. R., R. Peto, C. M. Tinker, and C. M. Fletcher. 1973. A study of Haemophilus influenzae precipitins in the serum of working men in relation to smoking habits, bronchial infection, and airway obstruction. Am. Rev. Respir. Dis. 108:460–468.
14. Mitchell, J. L., and S. L. Hill. 1995. Recognition of the P6 outer membrane protein of non-typeable Haemophilus influenzae by antibodies in sputum. Eur. Respir. J. 8(Suppl. 19):2638.
15. Morton, D. J., and P. Williams. 1989. Characterization of the outer mem-
16. Munson, R. S., Jr., and D. M. Granoff. 1985. Purification and partial char-
17. Murphy, T. F., and L. C. Bartos. 1988. Human bactericidal antibody re-
18. Murphy, T. F., L. C. Bartos, P. A. Rice, M. B. Nelson, K. C. Dudas, and M. A. Apicella. 1986. Identification of a 16,600-dalton outer membrane protein on nontypeable Haemophilus influenzae as a target for human serum bactericidal antibody. J. Clin. Invest. 78:1020–1027.
19. Pye, A., R. A. Stockley, and S. L. Hill. 1995. Simple method for quantifying viable bacterial numbers in sputum. J. Clin. Pathol. 48:719–724.
20. Rhind, G. B., G. A. Gould, F. Ahmad, M. J. Coughlan, and M. A. Calder. 1985. Haemophilus influenzae and H. parainfluenzae infections: comparison of clinical features. Br. Med. J. 291:707–708.
21. Roberts, M. C., C. S. Mintz, and S. A. Morse. 1986. Characterisation of Haemophilus parainfluenzae strains with low-Mr or ladder-like LPS. J. Gen. Microbiol. 132:611–616.
22. Sethi, S., S. L. Hill, and T. F. Murphy. 1995. Serum antibodies to outer membrane proteins (OMPs) of Moraxella (Brumallella) catarrhalis in pa-
23. Shoitz, P. O., N. Holby, and J. B. Hertz. 1979. Cross-reactions between Haemophilus influenzae and nineteen other bacterial species. Acta Pathol. Microbiol. Scand. 87:337–344.
24. Suzuki, Y., Nakatomi, S. Odami, H. Sato, F. Geyo, and M. Arakawa. 1996. Circulating IgA, IgG, and IgM class antibody against Haemophilus parain-
25. Suzuki, Y., Nakatomi, and H. Sato. 1994. Haemophilus parainfluenzae antigens and antibody in renal biopsy samples and serum of patients with IgA nephropathy. Clin. Exp. Immunol. 94:306–311.
26. Vachon, V., D. J. Lewy, and J. W. Coulton. 1985. Transmembrane permeability channels across the outer membrane of Haemophilus influenzae type b. J. Bacteriol. 162:918–924.
27. van Alphen, L., T. Riems, J. Poolman, and H. C. Zanen. 1983. Character-
28. Williams, P., and M. R. W. Brown. 1986. Influence of iron-restriction on growth and the expression of outer membrane proteins by Haemophilus influenzae and H. parainfluenzae. FEMS Microbiol. Lett. 33:153–157.