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Comparison of Outer Membrane Protein Subtypes of *Haemophilus influenzae* Type b Isolates from Healthy Children in the General Population and from Diseased Patients

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Over a 12-month period we obtained throat cultures from 1,448 children less than 5 years of age attending well-child clinics and identified 24 carriers of *Haemophilus influenzae* type b (1.7%). The outer membrane protein subtypes of the strains from the carriers were compared to the subtypes of isolates from 50 patients with *Haemophilus* type b disease hospitalized in St. Louis, Mo., during the same period (1981 to 1982), and the latter were compared to the subtypes of isolates from 51 patients hospitalized between 1977 and 1980. There were no significant differences in the frequencies of the five most common subtypes (1L, 1H, 2L, 2H, and 3L), comparing isolates from the carriers to those from the patients. However, 5 of the 24 throat isolates had the unusual 13L subtype compared with only 1 of the 50 invasive isolates ($P = 0.02$). The lower frequency of 13L strains among the invasive isolates suggests that type b isolates with this subtype may be less pathogenic than type b isolates with other subtypes. Subtype 2L strains accounted for only 2% of recent cerebrospinal fluid or blood isolates, compared with 22% of those from 1977 to 1980 ($P = 0.02$). Subtype 1H and 3L strains together accounted for 73%, compared with 47% of the earlier ones ($P = 0.02$). Thus, temporal shifts may also occur in the subtype distribution of *Haemophilus* type b strains causing invasive disease in a community.

Haemophilus influenzae type b isolates can be subtyped based upon differences in the sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of their outer membrane proteins (3). To date, 21 different outer membrane protein subtypes have been identified, but 5 subtypes account for more than 90% of the isolates from patients hospitalized with invasive diseases in 22 states (9). This subtyping system has been proven useful for epidemiological studies of *Haemophilus* type b disease (2, 5a, 7, 9).

The outer membrane protein subtyping system was originally defined on isolates from patients with invasive (bacteremic) disease (3). Before the present study, no information was available on the subtypes of type b isolates from healthy, nasopharyngeal carriers in the community who have no known exposure to patients. This group of carriers represents a natural reservoir of *Haemophilus* type b, and information on the subtypes of the strains could add to our understanding of the epidemiology of *Haemophilus* type b infection. The purpose of the present investigation, therefore, was to compare the outer membrane protein subtypes of *H. influenzae* type b isolates from carriers in the community to the subtypes of invasive isolates from patients with disease. We also compared the subtype distribution of blood and cerebrospinal fluid (CSF) isolates from patients hospitalized in 1981 and 1982 with the previously reported subtype distribution of strains isolated between 1977 and 1980 (3).

MATERIALS AND METHODS

Study population and source of isolates. The study population consisted of 1,448 healthy children less than 5 years of age attending 11 immunization clinics in the St. Louis, Mo., metropolitan area. During the 1-year period from 1 August 1981 to 31 July 1982, one of the authors (C.M.H.) periodically visited each of the clinics and obtained throat specimens for culture from healthy children. Children were excluded from the study if they had received antibiotics within the previous week, if a sibling had been previously cultured as part of the study, if they had ever attended a day care center or nursery school, or if they had known contact with a patient with *Haemophilus* type b disease. No child had a culture obtained on more than one occasion. Written informed consent was obtained from parents before the child was enrolled in the study.

Haemophilus type b strains from the blood or CSF of 50 representative children hospitalized in St. Louis with invasive disease over the same period as the

TABLE 1. Frequency of *H. influenzae* type b pharyngeal colonization[a]

Age (mo)	No. of children tested	No. of carriers (%)	
0–11	624	11 (1.8)	18/1341 (1.3)[b]
12–23	494	4 (0.8)	
24–35	131	2 (1.5)	
36–47	92	1 (1.1)	
48–59	104	6 (5.8)[b]	
Unknown	3	0 (0.0)	
Total	1,448	24 (1.7)	

[a] Throat cultures were obtained over a 12-month period in 1981 to 1982 from 1,448 healthy children <5 years of age who were attending immunization clinics in the St. Louis metropolitan area. Exact ages of three of the children were inadvertently lost and are shown as unknown.

[b] $\chi^2 = 9.0$; df = 1; $P < 0.001$.

study were used as a comparison group. Of the 50 children, 29 were cared for at Cardinal Glennon Hospital for Children, and 21 at St. Louis Children's Hospital. These two hospitals together provide care for the majority of children with serious bacterial infections in the St. Louis metropolitan area (a total of ca. 150 cases of invasive *Haemophilus* disease per year). The subtyping results on the 50 strains from this group were also compared with previously published results on invasive isolates from 51 children hospitalized in St. Louis between 1977 and 1980 (3).

Bacteria. Isolation of *H. influenzae* type b from throat swabs was accomplished by methods previously described (15). In brief, specimens were plated onto brain heart infusion agar supplemented with Levinthal base, NAD, bacitracin, and *Haemophilus* type b antiserum prepared in burros. Plates were incubated at 37°C in a 5% CO_2 environment. Colonies showing iridescence or forming halos of immune precipitation after 24 to 48 h of incubation were subcultured on chocolate agar and identified as *H. influenzae* by standard methods (11). Capsular typing was performed by latex particle agglutination (19) or countercurrent immunoelectrophoresis (17) with type b-specific rabbit antiserum (Burroughs Wellcome Co., Research Triangle Park, N.C.). *Haemophilus* type b strains isolated from the blood or CSF were identified similarly. Biotypes were assigned to isolates by assessing their ability to produce urease, indole, and ornithine decarboxylase in assays modified from those described by Kilian (5, 10).

Subtyping method. Isolates were subtyped on the basis of differences in the outer membrane protein patterns observed on sodium dodecyl sulfate-polyacrylamide gels (3). In brief, detergent-insoluble outer membrane derivatives were examined on a 4 to 24% gradient gel system and an 11% modified Laemmli gel (12). On the gradient gel, type b isolates were assigned to 1 of 18 previously defined categories (9). Isolates were further classified as being of either the H or L type based upon the apparent molecular weight of a heat-modifiable protein of 50,000 or 49,000, respectively, observed on the 11% acrylamide gels (3). Subtype designations were then assigned to each isolate by combining the classification from each of the gels (e.g., 1H, 1L).

Statistical methods. The statistical significance of differences between groups was determined by the 2 × 2 contingency tables, employing the chi-square test with Yates correction for small sample size (18).

RESULTS

Table 1 shows the frequency of *H. influenzae* type b pharyngeal colonization. Among the 1,448 children, 24 asymptomatic nasopharyngeal carriers of *H. influenzae* type b were identified. The overall colonization rate was 1.7% and was increased among the group of children between 48 and 59 months of age. Colonization rates were not affected by the sex or race of the individuals nor by the season of the year when the culture specimens were obtained (data not shown).

Table 2 compares the distribution of the outer membrane protein subtypes of the 24 throat isolates from the healthy carriers with that of 50 blood or CSF isolates obtained from patients hospitalized during the comparable 1981 to 1982 study period. Also shown are the previously reported subtyping results from 51 patients hospitalized in St. Louis between 1977 and 1980 (3). There were no significant differences in the frequencies of the five most common subtypes (1L, 1H, 2L, 2H, and 3L), comparing the throat isolates from the carriers with the invasive isolates from the patients hospitalized in the same 1981 to 1982 period. However, 5 of the 24 throat isolates were of the unusual 13L subtype, compared with only 1 of the 50 blood or CSF isolates ($P = 0.02$). With one exception, 13L strains were isolated at different times of the year from children of different ages attending different immunization clinics.

Examples of the three most common outer membrane protein patterns (1, 2, and 3) and patterns of three representative 13L isolates as

TABLE 2. Outer membrane protein subtypes of *H. influenzae* type b isolates

Subtype	No. of throat isolates from healthy asymptomatic carriers in 1981–1982 (%)	Blood or CSF isolates from patients with invasive disease	
		No. in 1981–1982 (%)	No. in 1977–1980 (%)
1L	2 (8)	6 (12)	7 (14)
1H	7 (29)	22 (44)	16 (32)
2L	2 (8)	2 (4)[a]	11 (22)[a]
2H	0 (0)	3 (6)	5 (10)
3L	7 (29)	14 (28)	8 (16)
13L	5 (21)[b]	1 (2)[b]	1 (2)
Other	1 (4)	2 (4)	3 (6)

[a] $\chi^2 = 5.47$; df = 1; $P = 0.02$.

[b] $\chi^2 = 5.40$; df = 1; $P = 0.02$.

resolved on a gradient gel are shown in Fig. 1. The 13L pattern appears to be similar to the 3L pattern commonly associated with invasive isolates, with the exception of a single major peptide migrating at 28 kilodaltons (kd) in the 13L strain instead of 26.5 kd in the 3L strain.

H. influenzae strains can also be subdivided by biochemical markers by the method of Kilian (10, 11). We compared the biotype distribution of the 24 throat isolates from the healthy carriers to that of the invasive isolates obtained during the same period. Among both groups of isolates, the predominant biotype was biotype I (71% of throat isolates and 94% of invasive isolates). However, 29% of the throat isolates were biotype II, compared to only 4% of the invasive isolates ($\chi^2 = 7.40$; df = 1; $P < 0.007$).

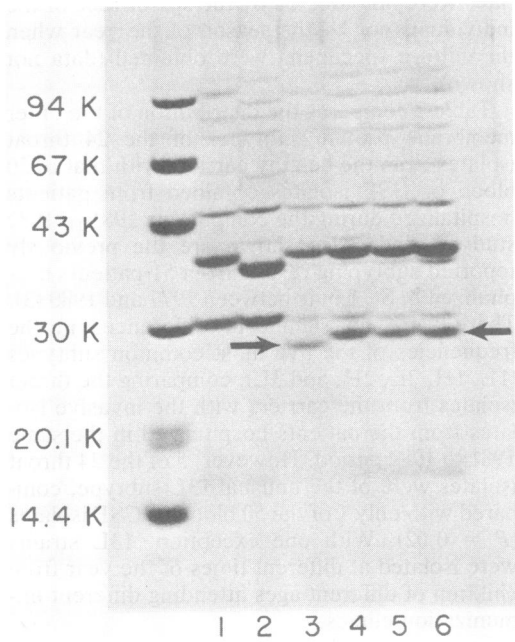

FIG. 1. Sodium dodecyl sulfate–4 to 24% polyacrylamide gradient gel electrophoresis of detergent-insoluble outer membrane preparations of *H. influenzae* type b isolates. Lanes 1 to 3, Profiles of isolates with outer membrane protein patterns 1, 2, and 3, respectively; lanes 4 to 6, profiles of representative isolates with outer membrane protein pattern 13. The isolate in lane 4 was from the CSF of a patient with invasive disease, whereas the isolates in lanes 5 and 6 were from throat cultures of healthy asymptomatic carriers. Isolates were subclassified for the presence of the H or L protein by another gel system (3). On this second gel, all pattern 13 isolates showed the presence of an "L" band (data not shown), and therefore the isolates were designated subtype 13L. The 13L pattern appears to be similar to the 3L pattern with the exception of a single major peptide migrating at 28 kd in the 13L strain (arrow on right) instead of 26.5 kd as in the 3L strain (arrow on left). Molecular weight standards are α-lactalbumin (14.4×10^3), soybean trypsin inhibitor (20.1×10^3), carbonic anhydrase (30×10^3), ovalbumin (43×10^3), bovine serum albumin (67×10^3), and phosphorylase *b* (94×10^3).

Beta-lactamase production, as measured by the rapid acidometric method (8), was detected in 6 of 24 throat isolates (25%), compared with 13 of 50 invasive isolates (26%) obtained during the same period (P = not significant).

Significant differences were apparent in the outer membrane protein subtype distribution of invasive isolates from 1981 to 1982 compared with that of invasive isolates from 1977 to 1980 (Table 2). Subtype 2L strains, which accounted for 22% of the isolates from the earlier period, comprised only 4% of the isolates from 1981 to 1982 ($P = 0.02$). Additionally, subtype 1H and 3L strains, combined, comprised a larger percentage of isolates from the 1981 to 1982 period than from the earlier period, (72 versus 47%; $P = 0.02$).

DISCUSSION

The carriage of *H. influenzae* type b was detected in 1.7% of the healthy children. This rate is lower than previously reported carriage rates for children of similar ages (13, 14). However, a large proportion of the children in our study were less than 2 years old (Table 1), a group that may have lower carriage rates than older children (14, 16). Also, we excluded siblings, avoiding the potential problem of including multiple carriers from the same family in our statistics.

Subtype 13L accounted for 5 of the 24 throat isolates but was rarely found among invasive isolates (1 of 50 isolates from 1981 to 1982 and 1 of 51 from 1977 to 1980). Thus, based on the higher frequency of 13L strains among asymptomatic carriers in the community compared to patients with disease, it would appear that isolates with the 13L subtype are less pathogenic than type b strains with other subtypes. The biological basis for this observation is unclear and is not necessarily directly related to the outer membrane proteins themselves. Of note is that subtype 13L strains appeared to be as fully encapsulated as strains with other subtypes as assessed by production of iridescent colonies on Levinthal agar or by formation of precipitin halos on agar containing *H. influenzae* type b antiserum (data not shown).

The frequency of biotype II strains among the isolates from the carriers was significantly greater than among the isolates from patients with disease (29% compared with 4%). Several investigators have previously reported that *H. influenzae* strains from the respiratory tract are often biotype II, but most of the isolates examined were nontypable (1, 10). In the present study,

four of five of the 13L throat strains and both of the 13L invasive isolates (Table 2) were biotype II. The increased frequency of biotype II strains among the type b throat isolates resulted primarily from the presence of the 13L strains in this group. In contrast, in a previous study of 21 invasive biotype II strains (4), 13L strains were observed only once. Thus, although the majority of the biotype II throat isolates in the present study were subtype 13L, this subtype has been observed infrequently among biotype II invasive isolates. Among 13L strains, the concordance of the outer membrane protein pattern and the general concordance of the biotype (II) suggest that isolates with this subtype may be descendants of a distinctive *Haemophilus* type b clone. Furthermore, the epidemiological evidence suggests that descendants of the clone have successful colonizing properties but relatively low pathogenicity. Interestingly, we recently found that a *Haemophilus* type b throat strain isolated in 1949 from a patient with conjunctivitis in Allen, Tex., was also subtype 13L and biotype II (isolate kindly provided by Margaret Pittman, U.S. Bureau of Biologics). Thus, subtype 13L strains are not new and are not limited to the St. Louis area but presumably have been present for some time and have a wider geographical distribution.

Finally, we detected a recent temporal shift in the subtype distribution of isolates causing invasive disease in St. Louis (Table 2). It is true that the isolates obtained in 1981 to 1982 were from patients hospitalized at either Cardinal Glennon or St. Louis Children's Hospital, whereas the isolates from 1977 to 1980 were primarily from patients from the latter. However, the respective subtype distributions of the isolates from the two hospitals in 1981 to 1982 were virtually identical. Therefore, it is unlikely that differences in sample selection in the two periods accounted for the shift observed in the frequencies of the different subtypes.

Temporal shifts in the frequencies of outer membrane protein subtypes have been observed previously with other bacterial pathogens such as *Neisseria meningitidis* (6). For *H. influenzae* type b, such temporal shifts may have important implications for vaccine development, particularly with regard to the use of outer membrane proteins as immunogens. Data from our laboratory suggest that passive protection against experimental *Haemophilus* disease in infant rats is subtype specific when monoclonal antibodies directed against outer membrane proteins or antisera prepared against purified outer membrane proteins are tested (R. S. Munson, Jr., J. L. Shenep, S. J. Barenkamp, and D. M. Granoff, J. Clin. Invest., in press). Therefore, in the future it will be important to continue to monitor the subtype distribution of isolates responsible for disease in the community. By doing so, additional temporal shifts in subtype distribution may be identified, and strains with new subtypes which possess different antigenic and pathogenic properties may be recognized.

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ADDENDUM IN PROOF

The statuses of 20 of the 24 carriers have been determined at a minimum follow-up period of 12 months. None had developed invasive *Haemophilus* disease. However, 1 of the 24 carriers (aged 18 months, with a biotype I, OMP subtype 2L strain) was determined to have had *Haemophilus* meningitis 6 weeks before his throat culture and therefore should have been excluded from the study. This change does not affect our conclusions (omitting this subject strengthens the observed statistical associations related to age [Table 1], 13L OMP subtype [Table 2], and biotype II [text]).

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