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Lipopolysaccharide Gel Profiles of *Haemophilus influenzae* Type b Are Not Stable Epidemiologic Markers

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Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of lipopolysaccharide (LPS) was performed to assess the usefulness of this technique for the epidemiologic analysis of *Haemophilus influenzae* type b isolates. LPS samples were prepared from isolates which had been passaged either in vitro or in infant rats. Preparations from paired isolates from a number of epidemiologically related clinical situations also were examined. The gel patterns of LPS prepared on different occasions from an individual isolate were stable. However, the LPS gel patterns changed in 5 of 14 (36%) of the passaged isolates, and differences in gel patterns also were observed among epidemiologically related isolates. The variability in LPS electrophoretic patterns of individual isolates indicated that this technique is not useful for the epidemiologic analysis of *H. influenzae* type b disease.

Haemophilus influenzae type b is the most common cause of bacterial meningitis in children in the United States. Outer membrane protein (OMP) profiles (4, 6, 13, 22), serum bactericidal assays with cross-adsorbed antisera (1), and biotype analyses (19) have been used to distinguish among type b isolates in an effort to assess the heterogeneity of these organisms as well as to provide important epidemiologic information on the transmission of this organism. In the United States, more than 94% of the type b isolates from children with bacteremic disease are biotype I (13), and approximately 44% have a common OMP subtype, 1H (13). Therefore, in many situations the application of these techniques provides only limited information to aid in distinguishing among isolates of *H. influenzae* type b.

Recently, Inzana and Pichichero (15, 17) demonstrated that the lipopolysaccharide (LPS) of *H. influenzae* type b also exhibits strain heterogeneity as assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This technique was then applied to evaluate the relatedness of isolates obtained from patients and carriers in an epidemic (17) and to investigate endemic disease in an Apache population (23). More recently, it has been applied to quantitate LPS production by antibody-sensitive and -resistant strains of *H. influenzae* type b (16) and to analyze cell wall alterations in a transformed type b strain (31, 32). One requirement of an epidemiologic tool is that the characteristic being measured be a stable feature of an isolate and not subject to frequent variation. Thus, the present study was undertaken to ascertain the stability of the LPS gel profiles after a variety of experimental manipulations of the organism. We also compared the LPS profiles of isolates obtained from patients with those of isolates from asymptomatic contacts in closely related clinical situations.

MATERIALS AND METHODS

Bacteria. Bacterial isolates were collected, serologically typed for capsular antigen, and stored as described previously (4). The clinical and epidemiologic characteristics of selected bacterial isolates used are summarized in Table 1. Isolates were subcultured from frozen stocks onto chocolate agar and incubated at 37°C in 5% CO_2 overnight. A single, representative colony was selected and streaked onto a fresh chocolate agar plate and incubated at 37°C in 5% CO_2 overnight; colonies were then scraped from the chocolate agar and suspended in Dulbecco phosphate-buffered saline (5) for LPS sample preparation. In some experiments, bacteria were grown to mid-log phase in broth cultures and harvested as described previously (4).

Rapid isolation micromethod, whole-cell lysates, and proteinase K digestion. Two rapid techniques for LPS extraction were developed recently (14, 15). The rapid isolation micromethod of Inzana uses a simplified hot phenol-water extraction followed by ethanol precipitation (avoiding the ultracentrifugation steps in the classic purification procedure [30]) (15), and the other technique uses SDS solubilization followed by proteinase K digestion (14). In previous studies of both *H. influenzae* type b and other bacteria, these extraction procedures yielded preparations with gel profiles similar to those of preparations made by conventional procedures (9, 18, 30) and were more convenient than the conventional methods for examining the large numbers of isolates encountered in epidemiologic studies (14, 17). Recently, Kimura and Hansen (20) showed that *H. influenzae* type b LPS prepared by these two methods yields identical SDS-PAGE profiles. In selected experiments, the LPS was purified by using the hot phenol-water extraction ethanol precipitation procedure described previously (15), with log-phase broth-grown cells as the source of the LPS. However, the proteinase K procedure of Hitchcock and Brown (14) was found to be easier and was used in most of our experiments. There were several minor modifications, and the procedure was as follows. Cells were harvested with sterile cotton swabs and suspended in 10 ml of cold Dulbecco phosphate-buffered saline (pH 7.2) to an optical density of 0.200 ± 0.050 at a wavelength of 600 nm. Portions (either 0.5 or 1.0 ml) of these suspensions were centrifuged for 1.5 min in an Eppendorf Microfuge (model 5412; Brinkmann Instruments, Inc., Westbury, N.Y.), and the supernatants were discarded. The pellets were solubilized in 50 μl of solubilizing buffer containing 2% SDS (BDH, Poole, England), 4% 2-mercaptoethanol (Eastman Kodak Co.,

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TABLE 1. Clinical and epidemiologic characteristics of *H. influenzae* type b and *Salmonella* isolates used

Isolate designation	Site	Diagnosis	Locale	Date	Notes and reference(s)	OMP subtype[a]	Source
Durst	CSF[b]	Meningitis	Cleveland, Ohio	1974	4, 11, 12, 24	2L	
Eagan	CSF	Meningitis	Boston, Mass.	1968	1, 2, 4, 8, 12, 27	1L	Porter Anderson
Minn A	CSF	Meningitis	Minneapolis, Minn.	1979	Isolate from day-care center 2 (3); 4, 24	1H	Janet Gilsdorf
668	Blood	Facial cellulitis	St. Lous, Mo.	1980	4	1H	
851	CSF	Meningitis	St. Louis, Mo.	1980	4	2H	
862	CSF	Meningitis	St. Louis, Mo.	1980	4, 24	3L	
1329	CSF	Meningitis	Madison, Wis.	Unknown	Patient 1 (6); 4	13L	Bruce Edmonson
3215A	CSF	Meningitis	Buffalo, Mo.	12/13/82	Case 1 (10)	1H	Dean Rising
3216A	Blood	Cellulitis	Buffalo, Mo.	2/4/83	Case 2 (10)	1H	Dean Rising
6031B	Throat		Buffalo, Mo.	3/15/83	Community contact (10)	1H	
6113E	Throat		Buffalo, Mo.	6/10/83	Healthy father of a child with meningitis (case 3 [10])	1H	
6125E	Throat		Buffalo, Mo.	6/10/83	Healthy mother of a child with meningitis (case 3 [10])	1H	
6351W	Blood	Cellulitis	Buffalo, Mo.	8/24/83	Case 4 (10)	1H	
3269	Blood	Meningitis	Minneapolis, Minn.	Unknown		1H	Michael Osterholm
G30					Rc mutant of *Salmonella typhimurium* LT2; 26, 27		Mary Jane Osborn
G30A					Re rough mutant of *S. typhimurium* G30; 7		Mary Jane Osborn
ATCC 14028					*Salmonella typhimurium*		

[a] OMP subtypes per Barenkamp et al. (4).
[b] CSF, Cerebrospinal fluid.

Rochester, N.Y.), 10% glycerol, 0.0625 M Tris chloride (pH 6.8), and 0.002% bromphenol blue. After heating at 100°C for 10 min, 25 μg of proteinase K (Sigma Chemical Co., St. Louis, Mo.) in 10 μl of solubilizing buffer was added to each lysate, and the samples were incubated at 60°C for 1 h. Samples were applied immediately to polyacrylamide gels or were frozen for later use. Frozen samples were reheated at 100°C for 5 min before application to the gel. Quantitation of the LPS in the samples added to the wells was not practical because of the small amount of LPS present. Thus, the sample volumes added to the gels were varied to optimize visualization in subsequent gels. Approximately 1 μg of LPS was added to each well, based on comparison with staining intensities of known amounts of purified LPS standards (28).

SDS-PAGE. LPS preparations were subjected to SDS-PAGE by using the system of Laemmli (21) with modifications described by Hitchcock and Brown (14) and Inzana and Pichichero (15, 17). Stacking gels (2.5%) and separating gels (18%) were cast in Laemmli buffers containing 0.1% SDS, 0.002 M EDTA (tetrasodium salt), and 2 M urea. The electrophoresis was performed at room temperature with equipment from Hoefer Scientific Instruments, San Francisco, Calif., at a constant current until the dye front migrated off the gel (11 to 16 h). The running buffer was that of Laemmli (21) with the addition of 0.002 M EDTA (tetrasodium salt). The gels were fixed for at least 4 h in 400 ml of 25% (vol/vol) 2-propanol in 7% (vol/vol) acetic acid before staining. The SDS-PAGE of sarcosyl-insoluble OMP preparations was performed as described before (4).

Silver staining. Gels were stained by a modification of the method of Tsai and Frasch (29) as modified by Hitchcock and Brown (14). After fixation, the gels were oxidized for 5 min in a mixture of 1.05 g of periodic acid–150 ml of distilled water–10 ml of ethanol:acetic acid:distilled water (8:1:11, vol/vol/vol). After four 30-min washes in 300 ml of distilled water, the gels were stained for 10 min in a solution prepared by mixing 5 ml of 20% (wt/vol) $AgNO_3$, 28 ml of 0.1 N NaOH, 1.6 ml of 28 to 30% NH_4OH, and 115 ml of distilled water. After four 15-min washes in 300 ml of distilled water, the gels were developed in 250 ml of distilled water containing 0.125 ml of 36.8% formaldehyde and 0.0125 g of citric acid. The gels were then washed for 1 h in 200 ml of distilled water containing 10 ml of 7% (vol/vol) acetic acid, rinsed, and stored in distilled water.

In vivo and in vitro passage. The procedure for serial in vivo passage of the isolates through infant rats has been described before (4). In addition, 10 clinical isolates were carried on chocolate agar. Isolated colonies from 6 of the 10 isolates were picked and replated 9 times, and the 4 remaining isolates were passaged 20 times.

RESULTS

Stability of LPS mobilities. The LPS was purified by the modified proteinase K method on one or more occasions (mean, 2.7; range 1 to 10) from 38 isolates. These preparations were electrophoresed on multiple SDS gels (mean, 6.2; range 2 to 21). For each isolate, the gel profiles of the LPS samples were reproducible in all cases.

LPS mobilities of clinical isolates. An outbreak of four cases of *H. influenzae* type b disease occurred among

children in a close-knit Amish community in rural Missouri (10). Isolates from the patients and carriers in the community had the same OMP subtypes (Fig. 1, lanes 8, 10, and 12 to 14) and identical alloenzyme patterns (10) characteristic of electrophoretic type 1. (A total of 16 enzymes was tested, and there was an average of three to four variants at each of 15 polymorphic loci [25].) Thus, the isolates were judged to be a single clone. The LPS from the three invasive isolates (from cases 1, 2, and 4) obtained from the Amish outbreak was examined by the proteinase K purification method of Hitchcock and Brown (14). The LPS from cases 1 and 2, separated by 2 months, showed identical mobility (data not shown). The LPS from case 4, which occurred 6 months after case 1, had a different mobility. In several gels, the major fraction of the staining material from isolate 6351W from case 4 (Fig. 2, lane 10) migrated with a slightly lower apparent molecular weight than that of isolate 3216A from case 2 (Fig. 2, lane 8). *H. influenzae* type b throat isolates 6113E and 6125E were available from the parents of another Amish child (case 3) with meningitis. The isolate from the child was not available, so we compared the electrophoretic mobilities of LPS preparations from the two parental throat isolates with those of preparations from the invasive isolates from cases 1, 2, and 4. The LPS from isolate 6113E from one parent (Fig. 2, lane 13) appeared to be identical to the LPS from isolate 3216A from case 2 (Fig. 2, lane 12), whereas the LPS from the other throat isolate (6125E) appeared to have a slightly lower molecular weight (Fig. 2, lane 14).

Six isolates from the outbreak of *H. influenzae* type b disease in an isolated Amish community (10) were passaged 10 times in vitro. Changes in SDS-PAGE mobility were observed in 3216A (Fig. 2, lanes 8 and 9), 6351W (Fig. 2, lanes 10 and 11), and 3215A (data not shown). Compared with the prepassage isolates, isolates 3215A and 3216A both produced LPS with a lower apparent molecular weight after passage, and isolate 6351W produced LPS with a higher apparent molecular weight. Again, the OMP profiles of these three isolates remained unchanged after passage in vitro (3216A, Fig. 1, lanes 8 and 9; 6351W, Fig. 1, lanes 10 and 11; and 3215A, data not shown).

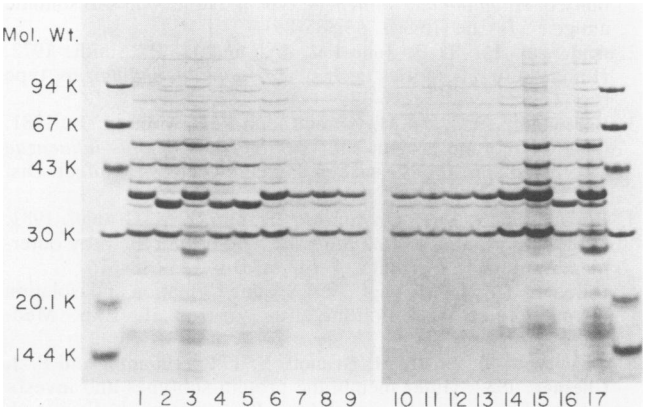

FIG. 1. SDS-polyacrylamide gradient slab gel (4 to 24%) of sarcosyl-insoluble OMP from *H. influenzae* type b isolates. Isolates representing three common subtypes (4) as well as isolates which exhibited changes or differences in electrophoretic mobility of LPS after passage or in epidemiologically related situations are shown. Lanes: 1 and 15, isolate Eagan (OMP subtype 1); 2 and 16, isolate Durst (OMP subtype 2); 3 and 17, isolate 862 (OMP subtype 3); 4 and 5, isolate Durst before and after four rat passages; 6 and 7, isolate 668 before and after four rat passages; 8 and 9, isolate 3216A, a blood isolate from patient 2 (case 2 [10]), before and after 10 in vitro passages; 10 and 11, isolate 6351W, blood isolate of patient 4 (case 4 [10]), before and after 10 in vitro passages; 12, isolate 3216A; 13 and 14, isolates 6113E and 6125E, respectively, throat isolates (taken the same day) from the parents of a child with meningitis (case 3 [10]) in the Amish community (an isolate from the child was unavailable). The method for preparation of sarcosyl-insoluble preparations and gradient gels has been described previously (4). Molecular weight standards, 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); phosphorylase *b* (94×10^3) (13).

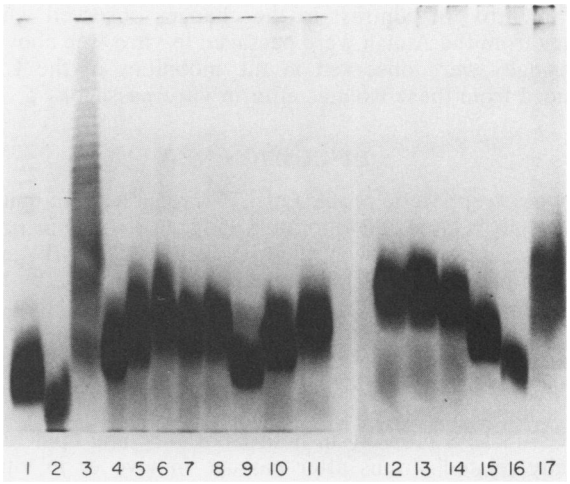

FIG. 2. SDS-polyacrylamide slab gel (18%) of LPS from proteinase K digests of whole-cell lysates of isolates of *H. influenzae* type b and *Salmonella typhimurium*. Profiles of *H. influenzae* type b isolates exhibiting representative changes in the electrophoretic mobilities of LPS are shown in lanes 4 to 14, corresponding to Fig. 1, lanes 4 to 14, in which the OMP profiles of the *H. influenzae* type b strains are shown. The controls are as follows: lanes 1 and 15, strain G30, an Rc mutant of *S. typhimurium* LT2 (26, 27); lanes 2 and 16, strain G30A, an Re rough mutant of *S. typhimurium* G30 (7); lanes 3 and 17, strain ATCC 14028, smooth *S. typhimurium*. Approximately 1 μg of LPS was added to each lane. The preparation of the gels and the LPS samples, as well as the staining of the gels, is described in the text.

LPS mobilities before and after in vivo passage. Four isolates (Durst, Eagan, 668, and 851) were passaged four times each in infant rats. The electrophoretic mobilities of the LPS prepared from proteinase K-digested whole-cell lysates of two of these strains (Eagan and 851) remained unchanged after passage in rats (data not shown). However, the LPS mobilities of the two remaining isolates were different after passage (Durst, Fig. 2, lanes 4 and 5; 668, Fig. 2, lanes 6 and 7). To examine further these changes, LPS was prepared from the isolates obtained after each of the four rat passages. After one passage, isolate Durst produced LPS with a greater apparent molecular weight (slower electrophoretic mobility) than that of the LPS from before passage (data not shown). The mobilities of LPS produced after subsequent passages remained unchanged. In contrast, isolate 668 produced LPS of unchanged mobility over three passages. After passage 4, the LPS had a lower apparent molecular weight (faster electrophoretic mobility; data not shown). The OMP profiles of these isolates before and after passage 4 were unchanged (Durst, Fig. 1, lanes 4 and 5; 668, Fig. 1, lanes 6 and 7).

LPS mobilities before and after in vitro passage. Isolates Durst, Eagan, Minn A, and 1329 were passaged 21 times

each in vitro. In contrast to the changes observed when isolates from the Amish were passaged in vitro (see above), no changes were observed in the mobilities of the LPS produced from these isolates after in vitro passage.

DISCUSSION

The electrophoretic profiles of LPS prepared from epidemiologically related and laboratory isolates of *H. influenzae* type b were compared on silver-stained SDS-PAGE gels. LPS was prepared with log-phase broth-grown cells by the method of Inzana (15) and by the proteinase K method of Hitchcock and Brown (14) with cells scraped from plates. LPS samples prepared from a single isolate yielded reproducible gel profiles, but we observed different mobilities for LPS samples from epidemiologically related isolates and changes in LPS mobility in both laboratory and epidemiologically related strains after passage in vivo or in vitro. Although we were unable to resolve the *H. influenzae* type b LPS patterns into several distinct bands as previously described (15–17, 20, 31), changes in the LPS profiles of individual isolates were apparent in gels of samples prepared by both the methods of Hitchcock and Brown (14) (Fig. 2) and Inzana (15) (data not shown). These changes were obvious and indicative of as yet undefined changes in the LPS from these strains. An increase in the resolution of the gels would only enhance the differences observed and would not alter the conclusions of our study.

Although multiple LPS preparations from a single isolate yielded stable gel patterns, we observed that the electrophoretic mobility patterns of LPS from several isolates were unstable after repeated passage in vivo or in vitro. Four isolates were passaged in infant rats; two produced LPS with altered mobilities. Three of six isolates from a recent outbreak of *H. influenzae* type b disease in an Amish community produced LPS with altered mobilities after passage in vitro. Shifts to lower apparent molecular weights as well as shifts to higher apparent molecular weights were observed. A direct comparison of LPS patterns from isolates from different patients and carriers in the Amish community indicated that variation in LPS structure among isolates also occurred after transmission among humans. The outbreak in the Amish community occurred during a 6-month period. Therefore, it might be argued that more than one *H. influenzae* type b strain was involved. However, the community is located in rural Missouri, and the children have little or no contact with non-Amish children. All of the *H. influenzae* type b isolates from the community had identical antibiotic susceptibility patterns, OMP profiles, and alloenzyme patterns. Furthermore, differences in LPS mobilities were observed in these isolates after passage in vitro. Finally, throat isolates 6113E and 6125E cultured from the parents of patient 3 on the same day differed in LPS mobility (Fig. 2, lanes 13 and 14); it is highly unlikely that these two household contacts, who earlier in the outbreak had had negative throat cultures, became colonized by different *H. influenzae* type b strains.

LPS mobility on SDS-PAGE has been used in previous studies to investigate the transmission of *H. influenzae* type b in an epidemic (17) and to examine the spread of *H. influenzae* type b infection on an Apache reservation (23). In the former study, isolates from two patients with onset of disease 6 weeks apart and who attended the same day-care center differed in LPS subtype but exhibited an identical OMP profile (17). The researchers concluded that LPS subtype is more useful for distinguishing between these two isolates than are OMP profiles, implying that case 2 was caused by the introduction of a new *H. influenzae* type b strain into the day-care center. Similar conclusions were reached concerning isolates from patients and carriers in the latter study (23). However, our results indicate that isolates producing LPS of altered electrophoretic mobility occur frequently after in vivo or in vitro passage in the laboratory and may be seen in epidemiologically related situations. While this manuscript was in preparation, Kimura and Hansen (20) independently reported similar instability in *H. influenzae* type b LPS electrophoretic mobility. Thus, their data and ours indicate that LPS electrophoretic gel mobility is an unstable strain marker and that this technique is not a useful epidemiologic tool to study the transmission of *H. influenzae* type b infection. The chemical basis and the genetic mechanisms responsible for these changes remain to be elucidated.

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