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Decreased Capacity for Type-Specific-Antigen Synthesis Accounts for High Prevalence of Nontypeable Strains of Group B Streptococci In Mexico

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The low incidence of group B streptococcal (GBS) invasive neonatal disease in Mexico has been attributed to the low prevalence of serotype III strains, a major serotype in developed countries. In addition, nontypeable strains account for 12% of the isolates in Mexico and <1% of the isolates in the United States. In this study, 57 GBS isolates (28 nontypeable by the Lancefield procedure) from carrier and infected neonates and women from Mexico were also examined for the presence of type-specific antigen by an enzymatic procedure using *N*-acetylmuramidase digestion of the cell wall to release soluble type-specific antigen. Of the 28 nontypeable strains from Mexico, 23 were typeable by the enzyme extraction procedure, with serotype III being the predominant serotype in invasive disease. These results suggest that nontypeable isolates of GBS should be further examined by the enzymatic extraction procedure to determine the presence of type-specific antigen. Furthermore, these limited results suggest that serotype III is likely a major serotype in invasive disease also in Mexico.

Streptococcus agalactiae (group B streptococcus [GBS]) is the leading cause of neonatal sepsis and meningitis in the United States and other developed countries (10, 11). Although GBS is not considered a frequent cause of perinatal infections in Mexico (3, 13), recent studies have found vaginal colonization rates of approximately 10% in pregnant women (12) and a neonatal infection rate of 1/1,500 live births with a case fatality rate of 38.5%, which is six times higher than that found in the United States (1, 10, 11–14). In contrast to developed countries, where type III is a predominant serotype, the most frequently identified serotypes in Mexico are Ia and Ia/c, with serotype III present at a low frequency. In addition, nontypeable strains that are rarely found in developed countries (<1%) (1) are reported at a 12% rate in Mexico (4, 5). Thus, the low frequency of GBS invasive neonatal infections in Mexico has been attributed to the low prevalence of type III strains along with increased levels of nontypeable GBS (12, 13).

In view of previous reports demonstrating variability in levels of type-specific antigen production in GBS isolates (6, 19), the present study examined a collection of strains from Mexico, including 49% nontypeable isolates as determined by the Lancefield hot-acid extraction technique (7, 8, 17). When type-specific antigens were extracted by the *N*-acetylmuramidase procedure (2, 6), the number of nontypeable isolates decreased to 9%. In addition, all the nontypeable isolates (five) were from asymptomatic carriers. These results indicate that the method of type-specific antigen extraction can markedly influence the interpretation of GBS epidemiological data.

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MATERIALS AND METHODS

Bacterial strains and growth medium. Fifty-seven GBS strains were isolated in Mexico from 43 asymptomatic infant and adult carriers and 14 infants and women with invasive disease (sepsis, meningitis, pneumonia, endometritis, and an abscess). All strains were no more than two passages from the clinical situation and were stocked in Todd-Hewitt broth (Difco) and stored at −70°C. Thawed cultures were routinely streaked onto sheep blood agar plates and incubated at 37°C overnight before use. All growth studies were performed in the chemically defined medium (FMC) containing 65 mM sodium phosphate buffer (9).

Preparation of typing antigen and serotyping procedures. Isolates were initially serotyped by using antigenic extracts prepared from whole cells by Lancefield's hot-HCl procedure as previously described (7, 8, 17). All strains reported as nontypeable by the Lancefield extraction procedure had undetectable levels of sialic acid (<0.01 μg of sialic acid/mg of cells [dry weight]). The antigens were obtained from cells grown in 10 ml of FMC to the mid-exponential phase of growth. A second procedure employed the mutanolysin extraction technique for whole cells (2, 6). In this procedure, isolates were first grown on 24-h blood agar plates and then inoculated into 10 and 100 ml of FMC and grown aerobically at 37°C in a circulating water bath. The pH and optical densities were monitored during growth. The pH was maintained between 6.5 and 7.0 by slow titration with 2.5 N NaOH. Cultures were grown to mid-exponential phase (0.2 mg of cells [dry weight]/ml) and immediately chilled in an ice bath. Cells were removed by centrifugation (9,000 × *g* for 20 min at 4°C), and the supernatant fluid was dialyzed overnight at 4°C against double-distilled water with four daily water changes and then lyophilized. Cell pellets were stored at −20°C until needed. For enzyme extraction, frozen pellets were thawed and washed in 5 ml (10-ml cultures) or 50 ml (100-ml cultures) of 0.03 M potassium phosphate buffer, pH 7.0. Washed cell suspensions were incubated for 1 h at 37°C in a 0.2-ml (10-ml culture) or 0.5-ml (100-ml culture) 5,000-U/ml *N*-acetylmuramidase (mutanolysin; Sigma) preparation. During the digestion process, there was usually an 80 to 90% decrease in optical density. Digested mixtures were centrifuged at 13,000 × *g* for 20 min at 4°C, and the supernatant material was dialyzed as described above and then lyophilized. Lyophilized materials from the supernatant material and cell pellets were dissolved in water to final concentrations of 10×, 100×, and 1,000×. Serotyping of the antigen extracts was performed by the capillary precipitin method using hyperimmune rabbit antiserum prepared as previously described (8).

Quantitation and comparison of type-specific antigens from asymptomatic and invasive Mexican isolates. Sialic acid is a useful marker for quantitation of type-specific polysaccharide antigens of GBS and was used to compare secreted and cell-associated antigens from strain 14 (a nontypeable asymptomatic isolate), strain 49 (a serotype III invasive isolate), and control strain GBS 110, a well-described neonatal meningeal isolate (9). Each strain was grown in 1 liter of

TABLE 1. Serotypes of 57 Mexican GBS strains determined by the capillary precipitin test using antigens prepared by hot-acid extraction and an enzymatic procedure

Serotype	No. of strains					
	Hot-acid extraction			Enzymatic extraction[a]		
	Carrier isolates	Invasive isolates	Total	Carrier isolates	Invasive isolates	Total
III	2	7	9	10	9	19
II	2	1	3	7	1	8
Ia	3	2	5	6	2	8
Ib	4	2	6	8	2	10
Ic	6		6	7		7
NT[b]	26	2	28	5		5
Total	43	14	57	43	14	57

[a] Cell-associated antigen from 10- and 100-ml cultures concentrated 10-, 100-, and 1,000-fold.
[b] NT, nontypeable.

FMC to early stationary phase (0.8 mg of cells [dry weight]/ml), with pH maintained between 6.5 and 7.0 by titration with 2.5 N NaOH. After centrifugation, the supernatant fluid was dialyzed against 0.01 M sodium acetate buffer (pH 6.5) at 4°C for 4 days with daily changes of buffer. The mutanolysin extraction procedures for type-specific polysaccharide antigens have been described previously (2, 6, 18). Extracts were lyophilized and dissolved in 2 ml (supernatant antigen) or 7 to 15 ml (cell-associated antigen) of water. The dissolved extracts were chromatographed on a DEAE-Sephacel column (18) and eluted with a continuous gradient of 0.02 and 0.3 M $(NH_4)_2CO_3$ (750 ml each). Sialic acid was estimated by the thiobarbituric acid assay following mild acid hydrolysis (15). Fractions indicating the presence of sialic acid were pooled, lyophilized, dissolved in double-distilled water, and serotyped as described above.

RESULTS

Serotyping using hot-acid-extracted versus enzymatically extracted antigens. As shown in Table 1, antigenic extracts prepared by Lancefield's hot-HCl extraction procedure yielded 28 nontypeable strains, 9 serotype III isolates, and 20 other serotypes. With enzyme-extracted antigens at several concentrations (Table 1), 23 of the 28 nontypeable strains were typeable. Ten of these strains were serotype III, and eight of these were from asymptomatic carriers. Two invasive strains that were initially classified as nontypeable with hot-acid extracts were now typeable as serotype III by the enzyme extraction procedure. The other nontypeable strains were classified as type II (five strains), type Ia (three strains), type Ib (four strains), and type Ic (one strain); all were from asymptomatic carriers. Only five strains could not be serotyped by using enzyme-extracted antigens (Table 1).

The results of serotyping eight strains which were initially classified as nontypeable are shown in Table 2. Concentrations of enzyme-extracted antigens required to serotype these strains were variable. For example, the lowest concentration of antigen (10×) was sufficient to serotype strain 3 (Table 2), while other strains (strains 2, 8, 16, and 24) required more-concentrated antigen preparations. Two strains (strains 14 and 16) could not be serotyped with any antigen preparation. Antigenic extracts of strain 29 precipitated with type Ia antiserum when 10×-concentrated cell-associated antigen extracts from 10-ml cultures were used. More-concentrated antigen extracts of this strain also had a positive reaction with type Ib antiserum, indicating that this strain was serotype Ic (1, 17). The other 29 strains which were typeable with hot-acid extracts were also serotyped with the lowest concentration of enzymatic extracts; there was complete agreement in serotypes between the two extraction procedures.

Quantitation of soluble extracellular and cell-associated type-specific antigen. The release of extracellular type-specific antigens by three strains (strains 14 [Ia], 49 [III], and GBS 110 [III]) grown in 1-liter FMC cultures was quantitated by estimation of sialic acid levels following elution on DEAE-Sephacel (Fig. 1) as previously described (18). Fractions containing only the high-molecular-weight form of the antigen (fractions 35 to 58) at various stages of polymerization were pooled, dialyzed, and lyophilized. Cell-associated type-specific antigen has been shown to be covalently associated with enzymatically solubilized components of the peptidoglycan layer (20) and eluted over a wider gradient (fractions 41 to 63) (Fig. 2) than secreted type-specific antigen. While GBS 110 produced more type-specific antigen on an equivalent cell dry weight basis than strain 49, strain 14 barely produced detectable levels of sialic acid-containing antigen. However, after the sialic acid-containing fractions were pooled, strain 14, previously shown to be nontypeable by both extraction procedures, was now typeable as Ia. These findings also demonstrate that for serotyping purposes, whole cells provide the greatest likelihood for obtaining sufficient type-specific antigen for examination.

TABLE 2. Serotyping of eight nontypeable[a] GBS strains using different concentrations of antigens isolated from supernatant fluids and extracted[b] from cells

Strain	Serotype[c]							
	10-ml culture concentrated:				100-ml culture concentrated:			
	10-fold		100-fold		100-fold		1,000-fold	
	Supernatant fluid	Cell surface	Supernatant fluid	Cell surface	Supernatant fluid	Cell surface	Supernatant fluid	Cell surface
2	NT	Ia	NT	Ia	NT	Ia	Ia	Ia
3	III	III	III	III	III	III	III	III
6	NT	NT	NT	NT	NT	NT	NT	NT
8	NT	NT	III	III	III	III	III	III
14	NT	NT	NT	NT	NT	NT	NT	NT
16	NT	NT	NT	Ib	NT	Ib	Ib	Ib
24	NT	NT	NT	Ib	NT	Ib	Ib	Ib
29	NT	Ia	NT	Ia	NT	Ic	NT	Ic

[a] Antigen extracts prepared by Lancefield's hot-HCl extraction procedure.
[b] Antigen extracts prepared by the *N*-acetylmuramidase procedure.
[c] Supernatant fluids and cells were obtained from mid-exponential-phase cultures. NT, nontypeable.

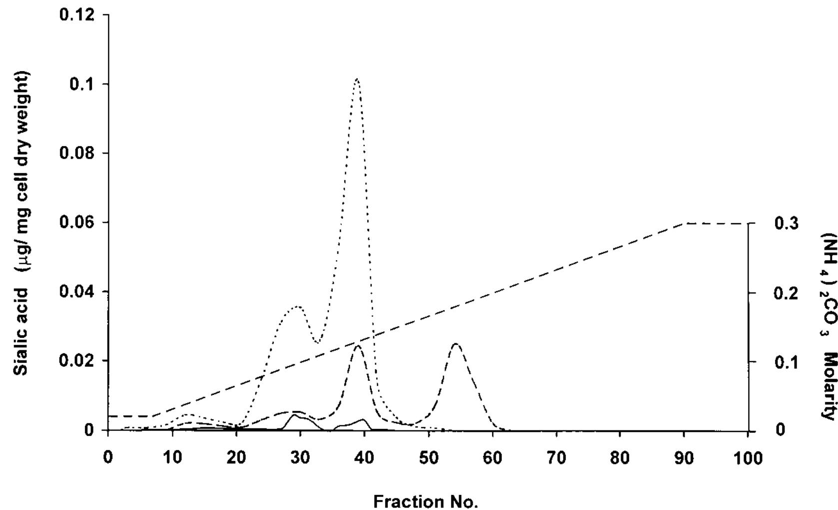

FIG. 1. Type-specific antigen as measured by the sialic acid moiety obtained from the supernatant fluids of 1-liter cultures. Concentrated material was eluted through a DEAE-Sephacel column (18) with a linear gradient of 750 ml each of 0.02 and 0.3 M $(NH_4)_2CO_3$. Fractions containing the high-molecular-form of the antigen were found in fractions 35 to 58. GBS isolates examined included strains 14 (Ia) (——), 49 (III) (------), and 110 (III) (······).

DISCUSSION

The type-specific capsular polysaccharide antigen of GBS is secreted as a soluble polysaccharide and is also covalently attached to the insoluble peptidoglycan layer of GBS (2, 5, 20). It is composed of repeating units of glucose, galactose, *N*-acetylglucosamine, and *N*-acetylneuraminic acid (sialic acid) (4, 16). Approximately 25% of type-specific polysaccharide consists of sialic acid, which is the main virulence and immunodeterminant portion of the antigen (1, 18). Of the serotypes associated with neonatal infections, serotype III is the most common and accounts for >90% of the cases of neonatal meningitis caused by GBS (1, 17).

Several methods have been used to extract cell surface antigens for grouping and typing studies; however, the Lancefield hot-HCl extraction procedure of whole cells has been the standard method (8). This technique and other chemical extraction procedures result in partially degraded antigens and decreased antigen yield (1, 5). The enzymatic method used in this study has been shown to provide maximum yields of nondegraded, high-molecular-weight polysaccharides (2, 6, 20).

Studies evaluating the role of GBS in perinatal disease in Mexico are limited, but available data indicate that in contrast to what is found in the United States, only 10% of pregnant Mexican women are colonized with GBS (12). A neonatal infection rate of 1/1,500 live births was reported, however, with a sixfold-higher fatality rate than that in the United States (12, 13). Other interesting differences between isolates from Mexico and the United States are the prevalence of serotypes Ia and Ia/c in Mexico, the high percentage of nontypeable strains in Mexico, and the low percentage of serotype III strains in Mexico (12, 13), based on the Lancefield hot-HCl antigen extraction technique. Thus, the low frequency of GBS invasive

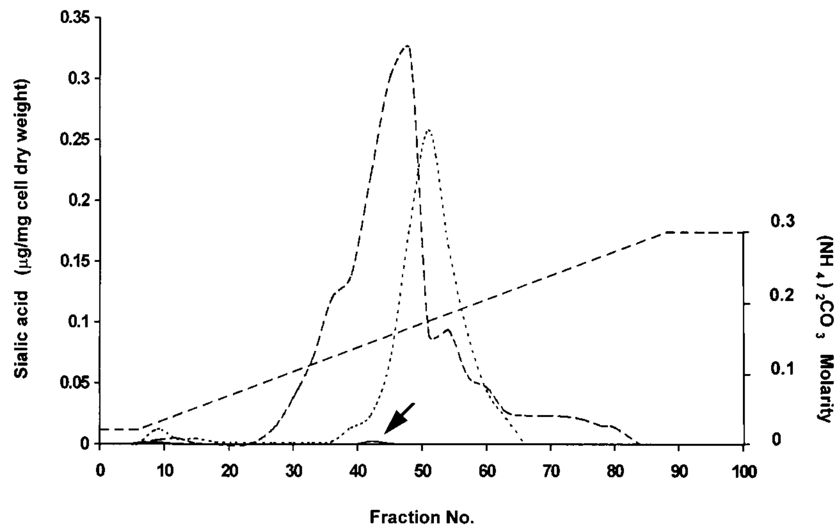

FIG. 2. Type-specific antigen as measured by the sialic acid moiety obtained by *N*-acetylmuramidase extraction of cells from a 1-liter culture. Fractions (41 to 63) contained the high-molecular-weight type-specific antigen. The elution conditions and symbols are as described for Fig. 1. The arrow indicates detectable levels of sialic acid in strain 14 (Ia).

disease in Mexico has been attributed, at least in part, to the high prevalence of nontypeable strains and low prevalence of serotype III isolates.

The present study suggests that these previous interpretations may be inaccurate in light of the findings in this study using the enzymatic extraction technique for antigen preparation. Twenty-three of the 28 nontypeable strains from Mexico were typeable by the enzyme extraction procedure. Of the invasive isolates examined, 9 of 14 (64%) were serotype III, indicating that this serotype is present at a higher percentage than previously reported and likely represents the major serotype in Mexico. Additional studies of GBS isolated throughout Mexico will be required to confirm these findings.

These findings indicate that many "nontypeable" strains may produce type-specific antigen, however, at levels that are undetectable by classical methods. Purification and concentration of antigens from nontypeable strains may be necessary before definitive conclusions regarding serotype distribution can be drawn, as demonstrated by the present study.

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