Influence of capsular neuraminic acid on properties of streptococci of serological group B

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Neuraminic acid is thought to be a critical virulence factor of group B streptococci. The present study was designed to further characterize a previously described type III group B streptococcus and its transposon-mutagenized asialo capsular mutant. The wild-type group B streptococcus grew as short chains with a uniform turbidity and had diffuse colonies in soft agar media. In contrast, the asialo mutant grew in fluid media as a granular sediment, formed significantly longer chains and had compact colonies in soft agar. These differences, possibly related to the surface charge of the bacteria, could also be demonstrated in salt aggregation tests and hexadecane adherence studies. The wild-type group B streptococcus showed hydrophilic, and the asialo mutant hydrophobic surface properties. Removal of neuraminic acid from the wild-type strain changed the surface properties from hydrophilic to hydrophobic. A similar masking effect of capsular neuraminic acid could be observed in adherence and phagocytosis experiments. In contrast to the wild-type strain, the asialo mutant adhered significantly more to buccal epithelial cells and was phagocytosed more by polymorphonuclear leucocytes. These altered properties might possibly be of importance for group B streptococcal pathogenicity.

Introduction

Streptococci of serological group B are well known as causative agents of bovine mastitis and as the leading cause of neonatal sepsis and meningitis (Hahn, 1980; Baker, 1980; Jelinkova, 1977). In an attempt to define virulence factors of group B streptococci various extracellular products have been isolated and studied. These included enzymes (Kjems *et al.*, 1980; Milligan *et al.*, 1978; Ferrieri *et al.*, 1980; Straus *et al.*, 1980) and haemolytically active substances (Marchlewicz & Duncan, 1981; Bernheimer *et al.*, 1979; Jürgens *et al.*, 1987). The pathogenic importance of some of these virulence factors has been questioned (Durham *et al.*, 1981; Orefici *et al.*, 1984; Weiser & Rubens, 1987). Lipoteichoic acid and the type-specific polysaccharide have also been described as virulence determinants of group B streptococci and lipoteichoic acid has been shown to mediate bacterial adherence (Nealon & Mattingly, 1985; Mattingly *et al.*, 1988; Teti *et al.*, 1987). However, group B streptococcal adherence without lipoteichoic acid involvement has been reported (Miyazaki *et al.*, 1988). The type-specific capsular polysaccharide antigen is recog-

nized as the antigenic determinant that induces production of protective antibodies. It also displays antiphagocytic properties (Baltimore *et al.*, 1979; Edwards *et al.*, 1982; Yeung & Mattingly, 1984). The capsule of all streptococcal serotypes contains sialic acid residues. This is more pronounced in those of serotype Ia, II and III (Shigeoka *et al.*, 1983; Molinari *et al.*, 1987).

In recent studies Wessels *et al.* (1989) constructed a transposon mutant strain of a type III group B streptococcus which expressed a capsular polysaccharide lacking sialic acid. The present report describes growth patterns and further characteristics of this type III group B streptococcus and the asialo capsular mutant. This included adherence properties of the cultures and the behaviour of the bacteria in phagocytosis experiments.

Methods

Bacterial cultures. Group B streptococcal strain COH 31 r/s and the asialo capsular mutant COH 31-21 used in this study were kindly provided by M. Wessels (Channing Laboratory, Boston, MA, USA). The mutant strain COH 31-21 was constructed by transposon mutagenesis with Tn916, a 16.4 kb transposon encoding tetracycline resistance (Wessels *et al.*, 1989). Strain COH 31 r/s was maintained on sheep blood agar, COH 31-21 on sheep blood agar containing 30 µg tetracycline ml⁻¹. Serogrouping was performed with autoclaved

Abbreviations: FITC, fluorescein isothiocyanate; HBSS, Hanks' balanced salt solution; PMN, polymorphonuclear leucocytes.

extracts and group B streptococcal antisera (Wellcome) in immunodiffusion reactions. The cultures were additionally tested for CAMP reactivity (Lämmler & Blobel, 1987) and pigment production. The latter was performed in GBS Islam Agar (Oxoid) supplemented with 50 ml sterile horse serum 1^{-1} as stab cultures in screw-capped tubes. For serotyping, the type-specific antigens were obtained by extraction of the bacteria in 0.2 M-HCl for 2 h at 52 °C. The antigens were tested with monospecific antibodies in coagglutination and immunodiffusion reactions as described previously (Wibawan & Lämmler, 1990a). For growth in fluid media, the bacteria were cultivated in Todd-Hewitt Broth (THB, Gibco) for 24 h at 37 °C without shaking. The bacteria were subsequently transferred onto microscope slides, Gram-stained and examined for chain length.

Soft agar and salt aggregation test. Cultivation of the group B streptococci in soft agar and detection of salt aggregating properties of the bacteria were performed as described previously (Wibawan & Lämmler, 1990b). The soft agar medium contained Brain Heart Infusion (BHI, Gibco) and 0.15% agar. After inoculation of the bacteria, the soft agar was incubated for 24 h at 37 °C and examined. For salt aggregation, the group B streptococci were adjusted photometrically (620 nm) to 10% transmission (10^9 cells ml^{-1}) in 2 mM-sodium phosphate buffer (pH 6.8) and mixed with equal volumes of ammonium sulphate (1.6–2.8 M). Distinct cell aggregation was regarded as a positive reaction. To exclude self-aggregation, a bacterial suspension in the above-mentioned buffer served as a control.

Hydrocarbon adherence test. The method for measuring adherence of the bacteria to a hydrocarbon was basically as described by Rosenberg (1984). For this, the washed cells were suspended in 3 ml PBS ($g\ l^{-1}$: Na_2HPO_4 , 12.14; NaCl, 4.35; pH 7.5) to an OD_{540} of 0.5 and mixed with 0.8 ml *n*-hexadecane (Sigma) for 30 s. After phase separation (20 min at room temperature) OD_{540} was measured. The bacterial suspension without hexadecane was used to measure the initial optical density. The data were expressed as the percentage (mean of three separate determinations) of bacteria that adhered to hexadecane.

In parallel experiments the bacteria were adjusted photometrically to 10% transmission (10^9 cells ml^{-1}) and treated with 0.1 units neuraminidase (neuraminidase type V from *Clostridium perfringens*, Sigma). Subsequently the bacteria were washed and used in the hydrocarbon adherence test as described above.

Adherence to buccal epithelial cells. Human epithelial cells were obtained from buccal mucous membranes by scraping with a wooden spatula, washed in PBS and adjusted to 10^5 cells ml^{-1} , measured with a haemocytometer. For staining, the bacteria were cultivated in THB, centrifuged, washed with 0.1 M-sodium bicarbonate buffer (pH 9.2), adjusted photometrically (620 nm) in the same buffer to 10% transmission (10^9 bacteria ml^{-1}) and incubated with fluorescein isothiocyanate (FITC, 1 mg ml^{-1} in 0.1 M-sodium bicarbonate buffer, pH 9.2; Sigma) at a final concentration of 0.25 mg per ml bacteria for 1 h at 37 °C. To remove unbound FITC, the bacteria were washed repeatedly in PBS. The adherence assay was performed by incubation of 0.5 ml of the FITC-stained bacterial suspension (diluted 1:10 in PBS) and 0.5 ml of the epithelial cells for 1 h at 37 °C with shaking. The epithelial cells were purified from unbound bacteria by density centrifugation in a 25% (diluted in PBS) density medium (Percoll, Pharmacia-LKB) and washed in PBS. Determination of bacterial adherence to the epithelial cells was performed on a microscope slide with a fluorescence microscope. The values given in Results and Discussion represents the number of bacteria that adhered to 50 epithelial cells.

Phagocytosis assay. The phagocytosis assay was basically performed as described by Pruzanski *et al.* (1983). Human polymorphonuclear leucocytes (PMN) were isolated from venous blood by density centrifugation (Histopaque 1077, Sigma). The erythrocytes of the pellet

were lysed by the addition of 0.87% ammonium chloride, pH 7.2 (1:5, v/v), and the leucocytes washed with Hanks' balanced salt solution (HBSS, Gibco). The final leucocyte suspension was adjusted to 5×10^6 cells ml^{-1} , measured with a haemocytometer. The viability of the leucocytes was determined by the trypan blue dye exclusion test. The bacteria were cultivated, washed in PBS and HBSS, adjusted photometrically to 10^9 bacteria ml^{-1} in HBSS, diluted 1:10 (v/v) in the same buffer and opsonized with human serum (10%) for 40 min. After ultrasonication (3 s, 20 kHz) the bacteria were used in the phagocytosis experiments. The opsonized bacteria were incubated with the adjusted leucocytes (1:1, v/v) for 1 h at 37 °C with shaking. After washing with HBSS, the suspension was stained with 200 μ l acridine orange (20 μ g ml^{-1} in HBSS), washed with ice-cold HBSS, suspended in 50 μ l HBSS and examined immediately under oil immersion in a Zeiss fluorescence microscope. In parallel experiments, 200 μ l crystal violet (50 μ g ml^{-1} 0.15 M-NaCl) was used for quenching extracellular adhering bacteria. A total of 100 neutrophils were examined for the determination of phagocytic activity. In addition, the number of internalized bacteria per 50 PMN was counted and defined as phagocytic capacity. The internalized bacteria were not differentiated into viable and non-viable cells.

The epithelial cell adherence test and the phagocytosis experiments were performed on four separate occasions.

Student's *t*-test was used for statistical analysis of the adherence properties and the two-factorial analysis of variance for the phagocytosis experiments.

Results and Discussion

Both cultures used in the present investigation reacted with group B specific antiserum, gave a positive CAMP reaction in the zone of staphylococcal β -lysin and were pigmented on GBS-Islam agar. Wild-type strain COH 31 r/s reacted with type III specific antibodies in coagglutination and immunodiffusion reactions. No reactions with type III specific antibodies could be observed with transposon-mutagenized strain COH 31-21.

The technique of transposon mutagenesis has proved to be a useful tool for studying individual group B streptococcal characteristics and this might help to further our understanding of virulence factors of this bacterial species. Transposon mutagenesis has already been used to demonstrate the importance of group B streptococcal β -haemolysin (Weiser & Rubens, 1987) and to construct a type III group B streptococcus which lacks capsular polysaccharides (Rubens *et al.*, 1987). In recent studies of Wessels *et al.* (1989) type III group B streptococcal mutant COH 31-21 was described and was shown to express a capsular polysaccharide which differed from the wild-type strain only in that the mutant's capsule lacked sialic acid. The loss of capsular sialic acid was related to a loss of virulence. This was demonstrated in a neonatal rat model.

It is possible that the insertion of the transposon also changed other phenotypic characteristics in mutant strain COH 31-21. However, as already indicated by Wessels *et al.* (1989), there is no concrete evidence for this assumption.

Table 1. *Properties of group B streptococcus COH 31 r/s and the asialo capsular mutant COH 31-21*

Property	COH 31 r/s	COH 31-21 (asialo mutant)
Group antigen	B	B
CAMP	+	+
Pigment	+	+
Serotype	III	—
Growth in fluid media	Uniform turbidity	Granular sediment
Growth in soft agar	Diffuse	Compact
Salt aggregation		
0.8*	—	—
1.0*	—	+
1.2*	—	+
1.4*	+	+
Adherence (%) to hexadecane		
Before neuraminidase treatment	29	84
After neuraminidase treatment	62	76
Adherence to epithelial cells (bacteria per 50 cells)†	530 (486–541)	642 (611–675)
Phagocytic activity (% phagocytosis)†		
Unopsonized	35 (24–42)	53 (38–63)
Opsonized	31 (13–41)	74 (71–78)
Phagocytic capacity (bacteria per 50 PMN)†		
Unopsonized	101 (78–119)	137 (102–175)
Opsonized	100 (81–128)	194 (181–217)

* Final molarity of ammonium sulphate in the salt aggregation assay.

† $P < 0.01$.

In the present investigation the transposon-mutagenized asialo capsular mutant (COH 31-21) and its parental wild-type group B streptococcus (COH 31 r/s) were found to differ significantly in their growth patterns in fluid media and in soft agar. Strain COH 31 r/s grew with uniform turbidity in the fluid media; the asialo capsular strain COH 31-21 grew as a granular sediment with clear supernatant. The growth pattern in fluid media correlated with the chain length of the cultures. Strain COH 31-21 grew with longer chains (generally > 40 cocci per chain) than strain COH 31 r/s (generally < 20 cocci per chain). It was of interest that the streptococcal-specific growth in chains seemed to be at least partly related to capsular neuraminic acid.

Strain COH 31 r/s had diffuse colonies in soft agar (Fig. 1) aggregated in 1.4 M-ammonium sulphate solution and adhered weakly to hexadecane droplets. In contrast, strain COH 31-21 had compact colonies in soft agar (Fig. 1), aggregated in 1 M-ammonium sulphate and adhered strongly to hexadecane (Table 1). Pre-treatment with neuraminidase significantly enhanced the hexadecane adherence of strain COH 31 r/s. Treatment of strain COH 31-21 with neuraminidase had no effect.

Growth in fluid media and soft agar together with salt aggregation studies had already been used to differentiate between group B streptococci with and without surface protein antigens X and R (Wibawan & Lämmler, 1990b). However, the results of the present study seemed to make it more likely that these surface properties are not only related to surface antigens X or R, but also to

Fig. 1. Typical diffuse colony formation of strain COH 31 r/s (left) and compact colony formation of strain COH 31-21 (right) in soft agar media.

hitherto unknown hydrophobic surface antigens and to the occurrence of capsular neuraminic acid.

Bacterial adherence to hydrocarbons such as hexadecane, octane or xylene provides a simple and rapid method to determine cell surface hydrophobicity (Rosenberg *et al.*, 1980). The adherence of group A streptococci to hexadecane seemed to be mediated by surface proteins or glycolipids complexed with surface protein structures (Ofek *et al.*, 1983).

It was of interest that, similar to the neuraminic acid of group B streptococci, the hyaluronic acid capsule of group A streptococci seemed to mask these hydrophobic components. Sialylation of the group B streptococcal capsule represents an important virulence determinant (Shigeoka *et al.*, 1983; Wessels *et al.*, 1989). The determination of hexadecane adherence could possibly be used as a rapid screening test to determine various degrees of capsular neuraminic acid expressed by group B streptococci.

Group B streptococcal strain COH 31 r/s adhered to human epithelial cells with a mean value of 530 FITC-labelled bacteria per 50 epithelial cells. Strain COH 31-21 adhered to the epithelial cells with a mean value of 642 bacteria per 50 cells (Table 1). The adherence to epithelial cells, the initial step in infections with group B streptococci, seemed to be inhibited by capsular neuraminic acid. This corresponded to the findings of Zawaneh *et al.* (1979). Similarly Nealon & Mattingly (1985) and Bagg *et al.* (1982) reported that surface structures responsible for attachment to buccal cells seemed to be masked by capsular neuraminic acid.

Phagocytosis experiments revealed that group B streptococcus COH 31 r/s was phagocytosed less than group B streptococcus COH 31-21. This was expressed in the number of PMNs that phagocytosed the bacteria (phagocytic activity) and in the total number of bacteria internalized by phagocytosis (phagocytic capacity). The differences in phagocytosis between COH 31 r/s and COH 31-21 and between unopsonized and opsonized COH 31-21 were significant (Table 1). These differences might be explained by a lack of opsonization of the wild-type group B streptococcus (Shigeoka *et al.*, 1983; Edwards *et al.*, 1982). Similar differences in opsonization and phagocytosis of wild-type and asialo mutant group B streptococci were recently described by Rubens (1991); (C. E. Rubens. New insights into the pathogenesis of group B streptococcal infections. Oral presentation at 91st General Meeting of the American Society for Microbiology, 5-9 May 1991, Dallas, Texas, USA).

The wild-type group B streptococcus used in this study has been reported to possess relatively small amounts of capsular neuraminic acid (Wessels *et al.*, 1989). The use of a neuraminic-acid-rich wild-type group B streptococcus and an isogenic asialo mutant further indicates the

effect of sialylation on bacterial surface properties and on the influence capsular neuraminic acid might have on group B streptococcal pathogenicity.

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