RESEARCH LETTER

The transcription of the neuD gene is stronger in serotype III group B streptococci strains isolated from cerebrospinal fluid than in strains isolated from vagina

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Abstract

Group B streptococci (GBS) are a major cause of neonatal meningitis, and sialic acid is a determinant of the development of meningitis. The transcription level of the neuD gene, used as a marker of neu gene expression and capsular production, was significantly higher in serotype III GBS strains isolated from meningitis than from vaginal carriage. This was irrespective both of the phylogenetic position of strains and of the presence of a thymine at position 264 in the neuD gene. Differences in neuD gene transcription may explain in part why particular isolates among the GBS strains colonizing the vagina can cause meningitis.

Introduction

Group B streptococci (GBS) asymptotically colonize the vagina of 15–36% of women (Hansen et al., 2004; Muris et al., 2010). They can be transmitted to the newborn during passage through the birth canal (Hansen et al., 2004), resulting in many cases of invasive neonatal infections including meningitis. Serotype III GBS is the leading cause of neonatal meningitis and is responsible for 86% of GBS-associated neonatal meningitis. Most of these strains belong to the sequence type 17 (ST17) that is responsible for more than 80% of cases of GBS neonatal meningitis (Tazi et al., 2010). Consequently, the ST17 GBS clone is considered to be highly virulent. The second GBS clone implicated in neonatal meningitis is ST19 (Manning et al., 2009). The differences in the ability of strains to invade the central nervous system of neonates have not been fully explained.

Capsular sialic acid (Sia) is a major virulence factor in bacteria with meningeal tropism, including GBS, Escherichia coli K1, and Neisseria meningitidis (Wessels et al., 1989; Severi et al., 2007). By molecular mimicry of mammalian sialic acid, GBS Sia favors immune escape, interacting, for example, with Sia-recognizing immunoglobulin superfamily lectins (Siglec) expressed by host leukocytes (Carlin et al., 2009). The neu genes, in the cps operon, are responsible for Sia synthesis and activation. One of them, neuD, unlike the other neu genes, does not have any mammalian homologs (Lewis et al., 2006). This gene encodes an O-acetyltransferase that O-acetylates Sia. NeuD also has a role in capsular sialylation, independent of its O-acetyltransferase activity (Lewis et al., 2006). Therefore, neuD is a good marker of neu gene transcription and capsular expression in GBS (Lartigue et al., 2011). Moreover, a single-nucleotide polymorphism in neuD may determine whether GBS displays a ‘high’ or
‘low’ Sia-O-acetylation phenotype (OAc) (Lewis et al., 2006).

We previously evaluated the inter- and intraphylegetic lineage variability of capsular Sia expression in serotype III GBS belonging to the vaginal flora of asymptomatic pregnant women by investigating the levels of neuD transcription (Lartigue et al., 2011). We found large differences in the levels of transcription between isolates. ST17 strains transcribed the neuD gene earlier and at a higher level than ST1 and ST19 strains; but about a third of ST19 strains had a Sia concentration comparable with that in ST17 strains. Here, we report an analysis of neuD transcription in serotype III GBS strains isolated from cases of neonatal meningitis. Our goal was to compare the level of neuD gene transcription in CSF strains to that observed previously for vaginal strains examined in the same conditions (Lartigue et al., 2011).

Materials and methods

Bacterial strains and growth conditions

Thirty-five serotype III GBS strains, isolated since 2002 in France, were used: 18 were from CSF of neonates with meningitis and 17, used for comparison, were from cases of vaginal carriage during pregnancy (Lartigue et al., 2011). These strains were selected from a collection of 111 serotype III strains on the basis of MLST classification to represent the genetic diversity of serotype III strains. All the serotype III CSF strains belonged to the two major clones implicated in neonatal meningitis (eight were ST17 and ten ST19), whereas vaginal strains were ST17 (six strains), ST19 (eight strains), and ST1 (three strains). The ST23 strain NEM 316 was used as a reference (Glaser et al., 1989). GBS strains were grown in Todd Hewitt broth. Samples were collected from cultures in early-logarithmic (EL; OD600 nm = 0.3), in mid-logarithmic (ML; OD600 nm = 0.7), and in late-logarithmic (LL; OD600 nm = 1.2) growth phases.

DNA extraction, amplification, and sequencing of neuD gene

Bacterial genomic DNA, extracted and purified by conventional methods (Sambrook et al., 1989), was used as the template for PCR assays. Primers for amplifying the neuD gene [neuD ACC ACT GAA ATA CCG CGT TC and neuDR CCT CCT CTG TAT TGG AGG (Eurogentec®)] were designed with Primer3 software (http://fokker.wi.mit.edu/primer3/). PCRs were performed with an Applied Biosystems 2720 apparatus, using 1 U Ampli Taq DNA polymerase from Applied Biosystems® in a 50-μL 1X Gene Amp buffer containing 1.5 mM MgCl2, 200 μM of each deoxy-nucleoside triphosphate, 200 μM of each primer, and 20 ng of genomic DNA. Cycling conditions were as follows: 1 cycle of 10 min at 94 °C; 35 cycles of 30 s at 94 °C, 30 s at 55 °C, and 1 min per kb at 72 °C; and a final extension of 10 min at 72 °C. PCR products were separated in 1% agarose gels for 1 h at 10 V per cm of gel. Then, PCR products purified with the QIAquick Gel extraction kit (Qiagen®) were sequenced on both strands using the BigDye® Terminator v3.1 cycle sequencing kit from Applied Biosystems® and the ABI PRISM® 310 Genetic Analyzer.

RNA extraction and quantitative reverse transcription PCR (RT-PCR)

The relative transcription level of the neuD gene in GBS strains was evaluated by quantitative RT-PCR (Lartigue et al., 2011). Experiments were performed in triplicate. GBS cells were mechanically disrupted by shaking with glass beads (Tissue Lyser, Qiagen, Courtaboeuf, France). Total RNA was extracted with the Qiagen RNAsy Plus Mini kit (Qiagen), according to the manufacturer’s instructions. Residual DNA was removed by treatment with TurboDNA free kits (Ambion, Austin). A QuantiTect Reverse Transcription kit (Qiagen) was used for RT, according to the manufacturer’s instructions. Quantitative real-time PCR was then performed with a LightCycler 480 (Roche Diagnostics, Basel, Switzerland).

The quantity of neuD cDNA was normalized by the ΔΔCt method. This method involves normalization to both an internal standard gene (gyrA) in each test strain and to the reference strain, NEM316, grown to the same phase as the sample strain (EL, ML, or LL). First, the ΔCt, which is the threshold cycle (Ct) difference between the neuD gene and the gyrA gene, was calculated for each strain. Next, ΔΔCt was calculated as the difference in ΔCt between the sample strain and the reference strain NEM316, both in the same growth phase (EL, ML or LL). The results are expressed according to the formula 2−ΔΔCt.

Statistical analysis

Data were analyzed with ANOVA or nonparametric Kruskal–Wallis (KW) test when normality conditions were not respected.

Results

NeuD polymorphism and anatomic origin of strains

We sequenced the complete neuD gene in all the 35 serotype III GBS strains isolated from CSF and vagina display (GenBank accession numbers KF731780-KF731814, see

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Supporting Information, Table S1), and looked for whether the genotype corresponded to the ‘high’ or ‘low’ OAc as described by Lewis et al. (2006). All strains belonging to ST17, ST19, and ST1 and isolated from the vagina or from CSF had a thymine at position 264 described as a marker of ‘high OAc’; the ST23 NEM316 reference strain had a guanine at that position described as a marker of ‘low OAc’. This polymorphism leads to an amino acid coding change at position 88, with phenylalanine in ‘high OAc’ strains and cysteine in ‘low OAc’ strains. All ST19 strains isolated from CSF or vagina had a single silencing nucleotide change (C→T) at position 405, and the three vaginal ST1 strains had two single silencing nucleotide changes at positions 321 (G→A) and 555 (C→A). Therefore, these mutations may be specifically associated with particular phylogenetic lineages.

Quantification of neuD transcripts

The mean level of transcription of the neuD gene in serotype III strains isolated from the CSF differed from that in strains from vagina: The mean level of neuD transcription was significantly higher in CSF strains than in vaginal strains in all three growth phases: (EL: 1.58 ± 1.24 vs. 0.50 ± 0.39; *P < 0.01; KW), (ML: 2.13 ± 0.85 vs. 0.94 ± 0.85; #P < 0.001; ANOVA), and (LL: 3.20 ± 1.47 vs. 0.59 ± 0.49; *P < 0.001; KW). The strains from vagina were of three different STs (ST17, ST19, and ST1), whereas strains from CSF were all ST17 or ST19. Therefore, the observed differences may be related to the greater genetic diversity of the serotype III GBS in the vaginal flora: STs only found in the vagina may transcribe the neuD gene more weakly, whereas those found in both the vagina and CSF may show strong transcription of the gene. We therefore compared the level of neuD gene transcription of CSF strains and vaginal strains belonging only to ST17 and ST19 (Fig. 1a). In this subpopulation, the neuD gene was significantly more strongly transcribed by CSF strains than by vaginal strains: (EL: *P < 0.01, KW), (ML: **P < 0.01, KW), and (LL: *P < 0.001, ANOVA).

Most of the serotype III GBS strains studied belong to ST17 and ST19, whatever their origin (32/35 strains). The neuD gene transcript was normalized to the amount of 16S rRNA, and in each strain, the neuD gene transcript was quantified separately in two growth phases: (EL: 1.14 ± 0.94 vs. 0.89 ± 1.20; *P = 0.518; ANOVA), (ML: 1.78 ± 1.02 vs. 1.59 ± 1.07; #P = 0.604; ANOVA), and (LL: 2.43 ± 1.95 vs. 1.86 ± 1.82; **P = 0.353; ANOVA). Nevertheless, within each of these two major serotype III phylogenetic lineages, the neuD transcription level differed according to the anatomic origin of strains. In ML and LL phases, ST17 CSF strains had higher mean neuD transcription levels than ST17 vaginal strains: (ML: *P < 0.05; ANOVA), (LL: **P < 0.001; ANOVA; Fig. 1b). Similarly, ST19 CSF strains had a higher neuD mean transcription levels than ST19 vaginal strains in the three growth phases: (EL: *P < 0.01, KW), (ML: *P < 0.05, KW), and (LL: **P < 0.001, ANOVA; Fig. 1c).

Discussion

Serotype III GBS is a major cause of neonatal meningitis. Sia contributes to immune evasion and is an important virulence factor of GBS. In this species, the neu genes encode the Sia biosynthetic pathway. The transcription of the neuD gene, a gene that belongs to the cps operon, could be considered as reflecting Sia expression in GBS populations (Lartigue et al., 2011). Indeed, in this previous study, we showed that the levels of neuD transcription in populations of serotype III GBS isolated from vagina and belonging to various phylogenetic lineages varied in parallel with the expression of Sia as determined enzymologically. In addition, the neuD gene was transcribed earlier and at a higher level in vaginal ST17 strains than in ST1 and ST19 strains, and about a third of ST19 strains had an Sia concentration comparable to that in ST17 strains. Therefore, differences in Sia production in the various lineages that compose the serotype III GBS vaginal population might be one of the factors that explain the different rates of involvement of strains of the various lineages in meningitis in neonates. Here, we report further evidence that this is the case: (1) The mean level of neuD transcription was higher in meningitis than in vaginal serotype III GBS strains whatever the phylogenetic

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Fig. 1. Comparison of relative levels of neuD gene transcription in GBS strains isolated from the CSF (from cases of neonatal meningitis) and from the vagina (from cases of vaginal carriage). Relative levels of neuD gene transcription were evaluated in EL, ML, and LL growth phases. Strains from cases of meningitis were, as is usual, ST17 and ST19. The amount of neuD gene transcript was normalized to the amount of gyrA transcript and is expressed relative to the level of transcription in the GBS strain NEM316. The bar inside each box indicates the median, the bottom and the top of each box are the 25th and the 75th percentiles, and the ends of the whiskers represent the minimum and the maximum relative neuD transcription levels for the three growth phases. Mean transcription levels and standard deviations of the values for neuD gene transcription in CSF and vaginal GBS strains are given below the graph. Significant differences in the mean values for neuD gene transcription, calculated by ANOVA or KW test (see methods), are marked as follows: *P < 0.05, **P < 0.01, P < 0.001. (a) In ST17 and ST19 GBS strains. (b) In ST17 GBS strains. (c) In ST19 GBS strains.
Relative transcription level of *neuD* gene

### ST17 and ST19 strains

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<th>EL</th>
<th>ML</th>
<th>LL</th>
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<tbody>
<tr>
<td><strong>CSF</strong></td>
<td>1.38 ± 1.24</td>
<td>2.13 ± 0.85</td>
<td>3.20 ± 1.47</td>
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<td><strong>VAGINA</strong></td>
<td>0.50 ± 0.45</td>
<td>1.07 ± 0.95</td>
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### ST17 strains

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<tr>
<td><strong>CSF</strong></td>
<td>1.34 ± 1.16</td>
<td>2.23 ± 0.84</td>
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<tr>
<td><strong>VAGINA</strong></td>
<td>0.85 ± 0.42</td>
<td>1.12 ± 0.94</td>
<td>0.95 ± 0.69</td>
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### ST19 strains

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<tr>
<td><strong>CSF</strong></td>
<td>1.41 ± 1.41</td>
<td>2.05 ± 0.91</td>
<td>3.01 ± 1.70</td>
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<tr>
<td><strong>VAGINA</strong></td>
<td>0.25 ± 0.25</td>
<td>1.03 ± 1.01</td>
<td>0.42 ± 0.25</td>
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classification of the strain, and; (2) the level of neuD transcription did not differ between GBS strains of the phylogenetic lineages ST17 and ST19 isolated from meningitis. Thus, differences in neuD transcription do not seem to be related to the phylogenetic position of GBS strains in ST17 and ST19, the two major lineages involved in neonatal meningitis caused by serotype III GBS; rather, these differences correspond to the anatomic origin of the strain. Strong neuD transcription, and thus high-level transcription of neu genes, may be one of the factors that confer the ability to cause meningitis on particular GBS strains.

Lewis et al. (2006) reported that the neuD gene encodes a Sia-O-acetyltransferase that is itself required for capsular polysaccharide sialylation; they suggested that a single-nucleotide polymorphism in neuD could determine whether a GBS strain displays a ‘high’ or ‘low’ OAc. In our study, the nucleotide sequences of the neuD genes of all the 35 strains studied had a thymine at position 264, described as a marker of ‘high’ OAc by Lewis et al. (2006). This feature was observed whatever the anatomic origin (vagina or CSF) and whatever the phylogenetic position (ST1, ST17, ST19) of the strains and irrespective of the level of neuD transcription. Therefore, this particular mutation did not appear to be a characteristic of the strains at high risk of invading the central nervous system of neonates. However, differences in neuD transcription may lead to quantitative differences in capsular sialylation (Lartigue et al., 2011). Sia has a major role in GBS virulence (Wessels et al., 1989), promoting immune escape. Furthermore, Sia is a determinant of the development of meningitis caused by other bacteria, including E. coli K1 (Kim et al., 1992). Severe bacteremia seems to be a critical determinant for meningeval invasion, as is the case for E. coli K1, so, as Sia favors escape from host defenses, it may allow the pathogen to attain a threshold level of bacteremia necessary for invasion of the meninges (Kim et al., 1992). Therefore, ‘high Sia GBS strains’ in each ST may have a survival advantage favoring diffusion through the blood–brain barrier. Finally, the difference in the strength of neu gene transcription between vaginal and meningitis GBS strains may explain, in part, the ability of a subpopulation of serotype III GBS strains in the vaginal GBS population to cause meningitis when transmitted to the neonate. The reasons for the observed diversity of neu gene transcription in strains belonging to the same phylogenetic lineage remain, however, unclear.

References


Supporting Information
Additional Supporting Information may be found in the online version of this article:

Table S1. Accession numbers of gene sequences.