Role of group B streptococcal capsular polysaccharides in the induction of septic arthritis

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The ability of different serotypes of group B streptococci (GBS) to induce septic arthritis in mice was compared. Types II, III, IV, V, VI and VII GBS were investigated. A highly capsulate strain of type III GBS, COH1, and its mutants, COH1-11 (lacking capsular sialic acid) and COH1-13 (non-capsulate), obtained by transposon insertional mutagenesis, were used to assess the role of type-specific polysaccharide on the induction of arthritis. At an intravenous dose of \(10^7\) cfu/mouse, reference strains of types II, III, IV, VI and VII and type III strain COH1 induced arthritis with an incidence ranging from 70 to 90%. For type V and strain COH1-11, \(10^8\) cfu/mouse was required to obtain a 50% incidence of arthritis; lesions were not evident with strain COH1-13. The presence of the capsule played a major role in the induction of GBS septic arthritis. The presence and amount of sialic acid in capsular polysaccharide influenced the incidence of articular lesions. The bacterial dose affected the manifestations of arthritis; the less virulent strains of GBS also induced articular lesions when an adequate number of micro-organisms reached the joints.

Introduction

Group B streptococci (GBS) are a leading cause of life-threatening infection in neonates and young infants [1]. Invasive neonatal GBS infection has either an early (usually the first 24 h after birth) or late (7 days after birth) onset. Early onset disease is acquired from the mother by vertical transmission from the uterus during labour or by direct contact at delivery [2–4]. Late onset disease may be acquired at birth from the mother or later in life from other individuals [5, 6]. All GBS serotypes are capable of causing infections in man. However, type III is the most frequently isolated serotype from infants with late onset disease (80–90% of cases) [1, 7]. Common manifestations of GBS disease in neonates include pneumonia, septicemia, meningitis, bacteremia, and bone or joint infections, such as septic arthritis and osteomyelitis [1, 8–10]. Invasive disease due to GBS in adults has also been recognised [11, 12].

GBS are facultative \(\beta\)-haemolytic, gram-positive cocci that possess a distinct polysaccharide capsule. A common feature of the GBS type polysaccharide is the structure of the high-mol.wt polymers with a repeating unit composed of glucose, galactose, N-acetylglucosamine and N-acetylneuraminic acid (sialic acid) [13–18]. All these type polysaccharides are immunologically distinct [13, 15–18]. Mechanisms of host response against GBS are based largely upon the recognition of capsular antigen specificity by antibodies [19, 20]. Furthermore, it has been shown that terminal side-chain sialic acid residues of the capsular polysaccharide inhibit the activation of the alternative complement pathway in the absence of type-specific capsular antibody, allowing the organisms to impede phagocytosis [21–23].

Septic arthritis is described as a clinical manifestation of late onset GBS disease in neonates [1, 6, 8] and requires prolonged antibiotic treatment to ensure an uncomplicated outcome. In adults, septic arthritis due to GBS has also been documented [11, 24] and is often associated with age and risk factors, such as diabetes mellitus, cancer, cardiovascular disease, chronic renal insufficiency, alcoholism, intravenous drug abuse, HIV infection, neurological disease and cirrhosis [25]. A previous study described an experimental model of type IV GBS systemic infection in mice [26]. Some clinical features of this infection closely resembled GBS infection in man, in particular, the appearance of diffuse septic arthritis.
The present study was designed to investigate the ability of different GBS serotypes to induce arthritis in mice. Although the role of type-specific capsular polysaccharide as a virulence factor in systemic infection has been demonstrated [27], its importance in the manifestation of arthritis has not yet been shown. In addition to the reference strains of several GBS serotypes, two isogenic mutants of a type III GBS clinical isolate were employed, one did not produce capsular polysaccharide whilst the other expressed a capsular polysaccharide lacking sialic acid residues [28–31].

Materials and methods

Mice

Female outbred CD1 mice, 8–10 weeks old, were obtained from Charles River Breeding Laboratories, Calco, Milan, Italy.

Bacterial strains

GBS reference strains included NCTC 11079 (type II), NCTC 11080 (type III), and strains 1/82 (type IV), 10/84 (type V), 118754 (type VI) and 7271 (type VII) obtained from the Czech National Type Culture Collection (Prague). Strain COH1, a highly capsulate type III organism isolated from an infant with bacteraemia [28] and two isogenic mutants of COH1 derived by transposon-insertional mutagenesis [29–31] – COH1-11, lacking capsular sialic acid, and COH1-13, lacking type III capsular polysaccharide – were kindly supplied by M. Wessels (Channing Laboratories, Boston, MA, USA). All GBS strains were grown overnight at 37°C in Todd-Hewitt broth (THB, Oxoid, Basingstoke, Hants) and samples were stored at −70°C until use. For experimental infections, micro-organisms in the log or stationary phase of growth (overnight culture) were used. After centrifugation at 700 g for 10 min, supernates were discarded and GBS cells were resuspended in serum-free RPMI 1640 medium (Gibco, Life Technologies, Milan, Italy). The turbidity and inoculum size were estimated at 540 nm in a Beckman DV-68 spectrophotometer (Beckman Instruments, Fullerton, CA, USA). The number of live bacterial cells was confirmed by the number of cfu on Islam agar (Oxoid) plates containing inactivated horse serum 5% v/v and incubated under anaerobic conditions at 37°C for 24 h. The appropriate number of bacteria was diluted in RPMI medium and 0.5 ml was injected intravenously (i.v.) into the tail vein of each mouse.

Determination of cell-associated sialic acid content

As sialic acid is a characteristic component of all known GBS type polysaccharides, its concentration was determined in all strains examined. Sialic acid was extracted and assayed as described previously [32]. Briefly, bacterial shake cultures were grown in THB at 37°C for 18 h with agitation and the cells were recovered and lyophilised. Cells were then incubated in 0.2 M H₂SO₄ to cleave the sialic acid from the capsular polysaccharide. After centrifugation at 7000 g for 20 min, the sialic acid content was determined in supernates by the thiobarbituric acid method [33] and expressed as μg/mg of dry cell weight.

Virulence determination

To evaluate the virulence of the different GBS serotypes, groups of 20 CD1 mice were inoculated i.v. with 10⁷–10⁸ cfu/mouse and mortality was recorded at 24-h intervals for 60 days. The 50% lethal dose (LD50) was calculated by a standard method [34] and represented the mean of three separate experiments.

Experimental GBS infection

Septic arthritis was induced in mice as described previously [26]. Briefly, mice challenged i.v. with 10⁷ or 10⁸ GBS were examined twice or more during day 1 after inoculation, and then daily for 2 months to evaluate the clinical features of the disease, in particular, the presence of joint inflammation. The time of onset, incidence, number of joints involved, duration of arthritis and ankylosis were observed.

Histological studies

Groups of mice inoculated i.v. with 10⁷ or 10⁸ cfu of the different GBS serotypes were examined every 2 days for histopathological features of arthritis. Joints were removed aseptically, fixed in formalin 10% v/v for 24 h and then decalcified in trichloroacetic acid 5% v/v for 7 days, dehydrated, embedded in paraffin, sectioned at 5–7 μm and stained with haematoxylin and eosin.

GBS growth in the blood and joints

Blood and joint infections were determined by the number of GBS cfu at different intervals following i.v. inoculation with the different serotypes. Blood samples were obtained by retro-orbital sinus bleeding before dissection. Ten-fold dilutions were made in RPMI medium and 0.2 ml of each dilution was plated in triplicate on to Islam agar and incubated anaerobically at 37°C for 24 h. The numbers of cfu were determined and results were expressed as the number of cfu/ml of blood. The affected joints were removed, ground in a mortar, and resuspended in 1 ml of sterile RPMI medium. All samples were diluted and plated on Islam agar and results were expressed as the number of cfu/ml of joint homogenate.
Statistical analysis

Differences in the number of cfu between the groups of mice treated with various GBS strains and differences in their sialic acid content and LD50 were analysed by Student’s t test. A comparison of the incidence of arthritis was performed by the χ² test. Each experiment was repeated three to five times. A p value of ≤0.05 was considered significant.

Results

**LD50 and sialic acid content of GBS strains of different serotypes**

The LD50 and sialic acid content of GBS strains at stationary growth phase are shown in Table 1. Significant differences in LD50 were not evident amongst reference strains of GBS serotypes II, III, IV, VI and VII, with the exception of the serotype V strain which had a significantly higher LD50 (p<0.001). The type III clinical isolate COH1 had the lowest LD50, whilst that of COH1-13 (non-capsulate) could not be evaluated because death did not occur, even at higher concentrations (>10⁷ cfu/mouse). Loss of capsular sialic acid in the type III strain COH1-11 resulted in an almost 20-fold enhancement of LD50 in comparison with the wild-type strain COH1. As previously demonstrated for type IV GBS [32], the virulence of GBS strains of different serotypes was not significantly influenced by the growth phase of the inoculum. Differences in LD50 between strains grown to log or stationary phase were always <10% (data not shown). The amount of cell-associated sialic acid of the different GBS serotypes appeared to correlate with virulence. The reference strains of type II, III, IV, VI and VII had similar sialic acid contents and LD50. In contrast, the type V GBS strain that had the lowest amount of sialic acid had the highest LD50. The type III clinical isolate COH1, which expressed a significantly (p<0.001) greater amount of sialic acid, had the lowest LD50.

**Induction of septic arthritis**

At 10⁷ cfu/mouse all GBS serotype strains induced articular lesions with the exception of the non-capsulate mutant strain COH1-13. A clinical onset of arthritis occurred within 2 days of infection. As shown in Fig. 1, the highest incidence of arthritis was recorded with type VII GBS and the type III clinical isolate COH1 (90%), whilst with types II, III, IV and VI the percentage of mice with articular lesions ranged from 77 to 80%. The lowest incidence of arthritis (15%) was shown with type V GBS, whilst the type III mutant strain COH1-11, lacking sialic acid, induced articular lesions in 20% of the animals. In mice inoculated with 10⁷ cfu of type V or strain COH1-11, complete recovery occurred within 2 weeks. The i.v. inoculation of 10⁶ cfu/mouse enhanced the incidence of articular lesions. However, at this dose mice inoculated with all GBS serotype strains, except type V and strain COH1-11, died within 5–7 days after infection (data not shown). All mice were negative with both doses of the non-capsulate strain COH1-13.

**Recovery of GBS from blood and joints**

In-vivo growth experiments with the different GBS serotype strains, the type III clinical isolate COH1 and its mutants COH1-11 and COH1-13, were undertaken by quantitative monitoring of bacteraemia and GBS growth in the joints 1, 5 and 10 days after i.v. injection of 1×10⁷ cfu/mouse and for serotype V and strain COH1-11 after i.v. injection of 1×10⁶ cfu/mouse (Fig. 2). The two mutants, COH1-11 and COH1-13, were cleared rapidly from mice within 10 days. In blood, the number of cfu of all GBS serotype strains progressively decreased from day 1 to day 10. In joints, the number of cfu of type III COH1 and the reference GBS type strains, with the exception of type V, progressively increased. For type V GBS, a low number of cfu was observed in the joints with clearance of the micro-organisms within 10 days. In contrast, when mice were inoculated i.v. with 1×10⁶ cfu/mouse of type V and

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**Table 1. LD50 and cell-associated sialic acid content of different GBS serotype strains**

<table>
<thead>
<tr>
<th>GBS strain</th>
<th>Mean (SD) LD50</th>
<th>Mean (SD) sialic acid content</th>
</tr>
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<tbody>
<tr>
<td>II (NCTC 11079)</td>
<td>2.15 × 10⁷ (0.20) × 10⁷</td>
<td>5.5 (0.1)</td>
</tr>
<tr>
<td>III (NCTC 11080)</td>
<td>1.93 × 10⁷ (0.23) × 10⁷</td>
<td>4.9 (0.2)</td>
</tr>
<tr>
<td>IV (1/92)</td>
<td>1.83 × 10⁷ (0.23) × 10⁷</td>
<td>4.5 (0.3)</td>
</tr>
<tr>
<td>V (10/84)</td>
<td>3.16 × 10⁷ (0.7)</td>
<td>1.8 (0.3)</td>
</tr>
<tr>
<td>VI (118754)</td>
<td>1.83 × 10⁷ (0.23) × 10⁷</td>
<td>3.8 (0.4)</td>
</tr>
<tr>
<td>VII (7271)</td>
<td>1.93 × 10⁷ (0.23) × 10⁷</td>
<td>4.8 (0.2)</td>
</tr>
<tr>
<td>III (COH1)</td>
<td>1.25 × 10⁷ (0.22) × 10⁷</td>
<td>10.1 (0.3)</td>
</tr>
<tr>
<td>III (COH1-11)</td>
<td>2.39 × 10⁶ (0.22) × 10⁷</td>
<td>Undetectable</td>
</tr>
<tr>
<td>III (COH1-13)</td>
<td>&gt;10⁷</td>
<td>Undetectable</td>
</tr>
</tbody>
</table>

LD50 values represent the means±SD of three separate experiments. Sialic acid content of different strains was expressed as µg/mg of dry cell weight. Values are the means±SD of three separate determinations. The sialic acid content of strains COH1-11 and COH1-13 was <0.9 µg/mg of dry cell weight, which corresponded to the detection limit of the assay.

*p<0.001 (type V versus reference strains of other serotypes).

+p<0.001 (strain COH1-11 versus type III COH1).

+p<0.001 (strain COH1 versus all strains).
Fig. 1. Incidence of articular lesions induced in CD1 mice by i.v. inoculation of $10^7$ cfu/mouse (□) or $10^8$ cfu/mouse (●) of reference GBS type strains II-VII, type III clinical isolate COH1 and its mutant strains COH1-11 and COH1-13. Values are the means and SD of three separate experiments. In each experiment, 40 mice were used. *p < 0.001 (type V versus reference strains of other serotypes and strain COH1); †p < 0.001 (strains COH1-11 and COH1-13 versus type III COH1 and strain COH1-13 versus COH1-11).

Histopathology

In the joints of mice inoculated i.v. with $10^7$ cfu of type II, III, IV, VI, VII reference strains and the type III clinical isolate COH1, an acute exudative synovitis and a polymorphonuclear leucocyte-monocyte infiltrate of the subsynovial and periarticular connective tissues were observed 48 h after infection. One week later, the articular cavity was filled with purulent exudate, and joint destruction progressed rapidly until fibrous ankylosis was observed on day 60. The joints of COH1-13-infected mice were always negative for lesions, whilst with type V and strain COH1-11 only transient inflammation was observed. At a dose of $10^8$ cfu/mouse, joints of mice inoculated with the non-capsulate strain COH1-13 were negative; with type V strain and the type III strain COH1-11, the histopathological features of the articular lesions were similar to those observed with the other type strains.

Discussion

GBS capsular polysaccharides play crucial roles as major virulence factors for the organism. In particular, the presence of sialic acid residues as side-chain termini of the polysaccharides is a key component of virulence [14, 21, 23, 35, 36].

In the experimental model described above, no significant differences were found in the LD50 between reference strains of types II, III, IV, VI and VII. The type V reference strain showed the lowest virulence. Marked differences in LD50 were observed with the highly capsulate type III COH1 strain and its isogenic mutants (COH1-11 and COH1-13) with altered capsular expression. It is important to point out that type III strain COH1 expressed a large amount of capsular polysaccharide and was a wild-type isolate typical of those associated with neonatal sepsis [28, 31]. For this reason, its mutants (COH1-11 and COH1-13) provided a useful model for studying the effect of capsular alteration. The results from this study provided further evidence of the role of the capsule and capsular sialic acid as major GBS virulence factors [23, 31, 37].

As previously documented, an i.v. dose of $1 \times 10^7$ type IV GBS is required to produce septic arthritis in c. 80% of the animals [26]. Induction of articular lesions is not related to the GBS strain of serotype IV employed and arthritis is not induced with inoculation of heat-inactivated bacteria (even at high doses) or sonicated cell extracts containing group- and type-specific polysaccharides [26]. Strains of the different serotypes examined in this study, with the exception
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Fig. 2. Growth kinetics of different GBS type strains II (●), III (□), IV (●), V (●), VI (▲) and VII (●), type III clinical isolate COH1 (○) and its mutants COH1-11 (△) and COH1-13 (○) in the blood and joints of CD1 mice. Mice were inoculated i.v. with 10⁷ cfu/mouse on day 0. Values represent the means and SD of three separate experiments. Eight mice per group were killed each time. The number of cfu/ml of blood or joint homogenate are reported. p values > 0.001 have been omitted. *p < 0.001 (strains COH1-11 and COH1-13 versus type III COH1 and type III GBS reference strain). †p < 0.001 (types II, V and VII versus types IV and VI). ‡p < 0.001 (type V GBS versus other serotypes).

of type V and the type III mutant strain COH1-11, were able to induce septic arthritis at the dose of 10⁷ cfu/mouse with an incidence rate that was not significantly different from that obtained with type IV GBS. For type V and type III COH1-11 a dose of 10⁸ cfu/mouse was needed to obtain at least a 50% incidence of articular lesions. The variability could be due to the degree of capsulation, sialylation of the capsule and the number of micro-organisms in the joints.

Many authors have shown that the presence and
amount of capsule and capsular sialic acid on capsular polysaccharide strongly influence phagocytosis of the micro-organisms in vitro [14,31,38]. Poorly capsule strains or strains lacking sialic acid are phagocytosed and killed even in the absence of specific antibodies, whereas highly capsule strains resist phagocytic killing [14,31]. The type V GBS strain used in this study had a low sialic acid content (1.8 μg/g dry cell weight) and a thin layer of capsular material [35]. Type III GBS strain COH1-11 lacked sialic acid and had a diminished level of capsule production [31]. Therefore, the type V and type III COH1-11 strains were easily phagocytosed in vivo. The number of bacteria that survived in the blood and reached the joints with a dose of 10^7 cfu/mouse was not sufficient to establish permanent arthritis. This hypothesis was confirmed by the number of cfu in the joints. It was evident that both resident and recruited phagocytes were able to eliminate the low number of GBS present at the site of infection.

Histological studies confirmed the presence of polymorphonuclear leucocyte-monocyte infiltration in the joints of mice injected i.v. with 10^7 cfu of the type V strain or strain COH1-11 2 days after infection; complete recovery was observed within 10 days. On the other hand, with an i.v. challenge of 10^9 cfu/mouse, the number of cfu in the joints was similar to that obtained with strains of other serotypes and septic arthritis was observed. In this case, a proportion of the bacteria was killed in blood, but the number of streptococci that evaded phagocytosis was sufficient to induce articular lesions. The non-capulate strain COH1-13 did not induce arthritis at any dose. This correlated with the finding that micro-organisms and lesions were not found in the joints.

It appears, therefore, that all GBS serotypes are able to induce septic arthritis. This is important because septic arthritis is a severe and frequent clinical manifestation of GBS infection in infants and adults [8–10,24]. Both GBS septic arthritis and osteomyelitis are manifestations of late onset disease in neonates [1]. GBS are isolated from specimens of blood and bone or joint aspirate [1]. In adults, supplicative arthritis may occur as a localised infection or as a manifestation of generalised septicemia [24,39]. Synovial fluids are the most reliable cultures for diagnosis [24].

GBS serotypes isolated from infants with septic arthritis are usually of type III, whereas those associated with osteomyelitis are of type III, Ib/c and Ia/c [1,8,10]. Types Ia/c and V have been isolated in adults [40]. In this study, type V was found to be less arthritogenic in mice. Serotype V has also been isolated from patients with severe chronic disease (diabetes, cancer, or cirrhosis) [25]. Therefore, it may be possible that an impaired human immune system allows this serotype to cause infection, even if it appears to be less virulent. For this reason, it would be of interest to study mice with altered immune systems so as to gain a better understanding of the emergence of serotype V among such patients.

In conclusion, these results clearly indicate that GBS must be capsule to induce arthritis. However, the amount of the capsule and the presence and amount of sialic acid in the capsular polysaccharide influenced the incidence of articular lesions. This is consistent with the observation that strains isolated from infants with late onset disease produced more capsular antigen than strains from colonised asymptomatic infants [34]. With the type VII GBS reference strain and the type III clinical isolate COH1 the incidence of arthritis was identical (90%), although the sialic acid content of the two strains differed (4.8 and 10.1 μg/mg dry cell weight, respectively). Therefore, we hypothesise that perhaps another surface bacterial component, apart from sialic acid, or a cellular product of these micro-organisms could contribute to the induction of articular lesions. The bacterial concentration also played an important role in the establishment of arthritis. Less virulent strains of GBS appeared to induce articular lesions when an adequate number of micro-organisms reached the joints.

We are grateful to Eileen Mahoney Zannetti for dedicated secretarial and editorial assistance and to M. Pataracchia and Giovanna Alfarone for technical assistance.

References
