Opsonisation of group B streptococci and restriction endonuclease digestion patterns of their chromosomal DNA

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Summary. Isolates of group B streptococci (GBS) from neonates with early-onset septicaemia are associated with particular restriction endonuclease digestion patterns (RDP types Ia-3 and III-3) of chromosomal DNA. Opsonophagocytosis of serotype Ia and serotype III GBS isolates was studied by the luminol-enhanced phagocytic chemiluminescence (CL) assay. Pools of serum containing GBS type-specific antibody levels equivalent to or just above levels typically found in sera from mothers of infected infants were used. CL intensities induced by GBS isolates of RDP types Ia-2, Ia-3 and III-3 were lower than those of the other RDP types of the same serotype. Opsonophagocytosis was more efficient with serum containing higher concentrations of type-specific antibodies but for RDP type III-3 strains these differences were much less marked than for other RDP types. CL intensity did not correlate with cell surface charge, hydrophobicity or sialic acid content of GBS. Results demonstrate that certain GBS RDP types are more resistant to opsonophagocytosis and suggest that potentially virulent strains with genetic homogeneity may exist.

Introduction

Group B streptococci (GBS) are important pathogens in neonatal systemic infections. The type-specific capsular polysaccharides of GBS consist of repeating units with sialic acid (SA) as a terminal residue. These SA residues, by their strong affinity for the inactivating protein, factor H, a component of the amplification loop, block the alternative pathway of complement. Opsonisation of GBS has been shown to depend on the alternative complement pathway which can be activated by the presence of type-specific antibody. The latter binds and neutralises the SA moieties, allowing the formation of the convertase C3bBb of the C3b amplification loop. Recent work in the neonatal rat model has demonstrated that both the complete loss of the capsular polysaccharide and the selective loss of capsular SA residues by transposon mutagenesis result in a significantly higher LD50 value than in the wild-type strain. The important role of type-specific antibody has been emphasised by the low concentrations of type-specific antibody found in sera from mothers of infected infants.

Recently, on the basis of numerical analysis of restriction endonuclease HindIII digestion patterns (HindIII RDP), we reported that strains from neonates with early-onset septicaemia belong to two particular RDP types (RDP Ia-3 and III-3). The present study investigated the opsonophagocytosis of serotype Ia and serotype III GBS isolates of different RDP types.

Materials and methods

Bacterial strains

GBS isolates, selected from a previous study of RDP typing and including 10 isolates from infected infants and 14 vaginal isolates from healthy carriers, are listed in the table. Overnight cultures (2 ml) in Todd-Hewitt Broth (BBL, Microbiology Systems, Cockeysville, MD, USA) were inoculated into fresh Todd-Hewitt broth (200 ml) and incubated at 37°C to late-exponential growth phase (OD600, 0.6±0.01). Bacterial cells were pelleted and washed twice with normal saline, resuspended in normal saline (OD600, 12), and frozen at −80°C in 1-ml volumes until required.

Serotyping

Strains were grouped by the Phadirect Streptococcus Test (Pharmacia Diagnostics, Uppsala,
Sweden) and were serotyped by the capillary precipitin method with antisera (Denka Seiken, Tokyo, Japan) to the polysaccharide antigens, and to the protein antigens c, R and X.

**RDP typing**

Extraction of chromosomal DNA, detection of plasmid DNA, digestion with restriction endonuclease HindIII, agarose gel electrophoresis, densitometric scanning and numerical analysis were performed as described previously.\(^{10-12}\) Pearson's product moment correlation coefficients between all possible pairs of scanning patterns were calculated on the basis of the OD value of the overlapping portion between a pair of the patterns. The patterns were then clustered by the method of unweighted pair group method using average (UPGMA).\(^{13}\)

**Preparation of sera**

Serum was collected from healthy donors of blood type AB and frozen at \(-80^\circ\text{C}\). Antibodies to serotypes Ia and III capsular polysaccharide antigens were determined by Dr S. Ikuta, Toyo-Jozo Co., Tokyo, with an ELISA kit (antigen-antibody-antiIgG labelled with alkaline phosphatase system) (Toyo-Jozo Co.). In this diagnostic kit, a polyethylene glycol-treated human IgG preparation (lot T081D; Glovenin-I, Takeda Chemical Industries Ltd, Osaka, Japan) was employed as the standard, and the concentration (U/ml) of each type-specific antibody was defined as \(1/d \times 10^4\), where \(d\) is the dilution value of the standard at which its OD value (at 405 nm) is the same as that of the serum sample tested. Two pools of sera were prepared by mixing: serum pool M with moderately high antibody levels to GBS type Ia (10-1 U/ml) and to GBS type III (10-6 U/ml), and serum pool L with low antibody levels to GBS type Ia (5-1 U/ml) and to GBS type III (5-3 U/ml). Complement activity, measured by the sensitised sheep erythrocyte lysis assay,\(^{14}\) was 30-5 U/ml for serum pool M and 37-7 for serum pool L.

**Opsonisation of bacterial cells**

Frozen bacterial cells were washed twice and resuspended in Hank's Balanced Salts Solution (HBSS) without phenol red to various concentrations (OD\(_{600}\)). Bacterial suspensions (110 \(\mu\)l) were opsonised by mixing with an equal volume of serum and incubating for 30 min at 37°C with agitation. HBSS (330 \(\mu\)l) was then added and opsonised suspensions were left in an ice-water bath until required.

**Preparation of polymorphonuclear leukocytes (PMNL)**

Venous blood samples were collected from healthy donors and anticoagulated with preservative-free heparin (10 U/ml of blood). PMNL were isolated by the gradient-centrifugation method with Polymorphprep (Nycomed Pharma As, Torshov, Norway) according to the manufacturer's instructions, and were then washed in HBSS with heparin (10 U/ml of HBSS). Remaining erythrocytes were removed by hypotonic lysis. PMNL were washed and resuspended in HBSS to a concentration of \((2.0 \pm 0.1) \times 10^6$/ml.

**Luminol-enhanced chemiluminescence (CL) assay**

PMNL suspensions (250 \(\mu\)l) were mixed with an equal volume of 4 \(\times 10^{-4} \text{ M} 5\)-amino-2,3-dihydro-1,4-phthalazinedione (luminol, Sigma) in HBSS in a reaction tube. After pre-incubation of this mixture at 37°C for 10 min, 500 \(\mu\)l of opsonised bacterial suspension was added and CL intensity was measured in a photometer (BLR-201, Aloka Co., Tokyo).

**Cell-surface hydrophobicity**

Cell-surface hydrophobicity was determined by the xylene affinity method\(^{16}\) with minor modifications. Bacterial cells were washed and resuspended in PUM buffer (30 mM urea and 0.8 mM MgSO\(_4\)·7H\(_2\)O in 75 mM phosphate buffer, pH 7.1) to an OD\(_{600}\) of 0.5; 3 ml of bacterial suspension were pre-incubated at 37°C, 300 \(\mu\)l xylene was added, and the mixture was vortex mixed for 30 s. After standing for 10 min, the xylene phase was separated by centrifugation at 200 \(g\) for 1 min, and the OD\(_{600}\) of the aqueous phase was measured. The affinity for xylene (transfer rate) was expressed as a percentage of the OD value of the control material treated by the same procedure without the addition of xylene.

**Cell-surface charge**

Cell-surface charge was measured by the hydroxyapatite (HA) adherence method\(^{16}\) with minor modifications. Bacterial cells were washed and resuspended in phosphate buffered saline (PBS, pH 7.4) to an OD\(_{600}\) of 0.6; 500 \(\mu\)l of a washed HA (8 \(\mu\)m diameter; Tonen Co., Tokyo) suspension (in PBS 20% w/v) was mixed with 2.5 ml of bacterial suspension (pre-incubated at 37°C) and vortex mixed for 30 s. After standing for 10 min, the HA particles were pelleted by centrifugation at 200 \(g\) for 1 min and the OD\(_{600}\) value of the supernate was measured. The adhesiveness towards HA (transfer rate) was expressed as a percentage of the OD value of control material treated in the same way except that 500 \(\mu\)l of PBS was substituted for the HA suspension.

**Cell-wall SA**

Cell-wall SA was extracted from freeze-dried bacterial cells by acid-heat treatment as described previously.\(^{17}\) SA was measured by the thiobarbituric acid
OPSONISATION AND RDP TYPE OF GBS

Phenon Strain Densitometric RDP No. 1 0.95 0.90 0.85
17 18 19 20
13
14 15 16
5
6 7 8
21
22 23
24
1 2 3
4
9 10 11 12
Phage lambda

Fig. 1. Dendrogram of cluster analysis based on HindIII RDPs. Similarities were determined by the correlation coefficient and UPGMA clustering. The migration distance of each RDP was corrected to that of the reference (RDP of a phage \( \lambda \) DNA-HindIII digest). Similarity was calculated over the mol. wt range (15.27 – 2.88) \( \times 10^{9} \), these two limits corresponding to the left and right peaks of the reference. Phenons (RDP types) were formed at the 0.94 similarity level.

method with N-acetylneuraminic acid as the standard.\(^{18}\)

Results

RDP type

Plasmid DNA was not detected in DNA samples of any of the strains tested. Numerical analysis results of HindIII RDPs, based on the determinations of similarity through the correlation coefficients and UPGMA clustering, are summarised in fig. 1. In accordance with previously described criteria,\(^{10}\) phenons formed at the 0.94 similarity level were designated as RDP types.

Conditions for luminol-enhanced CL assay

To establish optimal conditions for the luminol-enhanced CL assay, preliminary experiments were
performed with six strains. The reproducibility of CL intensity was most affected by variations in PMNL preparation and bacterial cell concentration; absolute values varied markedly even among PMNL preparations from a single donor. However, relative values (%) of CL intensity between strains were reproducible (CV, < 6.5) and for the remainder of the study CL intensity was expressed as a percentage value of that of a control strain (strain 1, table). CL intensity increased in proportion to the rise in bacterial cell concentration (fig. 2), but decreased at concentrations above 0.12 OD_{600} (data not shown). Within the bacterial concentration range of 0.04–0.08 OD_{600} there was a linear correlation between cell concentration (log OD value) and peak CL intensity. In contrast, the correlation of bacterial cell concentration with the integral values or the maximum slope of CL intensity was relatively poor. In the main study, final bacterial concentrations were adjusted to an OD_{600} of 0.06.

**GBS RDP types and CL intensity**

The relationship between GBS RDP type and ability to stimulate PMNL CL is shown in fig. 3. Statistically significant differences were observed in peak CL intensities induced by different RDP types within both serotypes Ia and III (F-test, p < 0.01). Amongst serotype Ia isolates, the four RDP Ia-1 strains, opsonised with either serum pool, induced higher CL levels than RDP types Ia-2 and Ia-3 strains. For serotype III isolates there was more strain variation within RDP types, but the four RDP type III-2 strains consistently induced higher CL peaks than other strains.

**CL intensity and GBS surface properties**

CL intensity did not correlate with the origin of bacterial strains (infected infants or colonised healthy women), SA cell-wall content, hydrophobicity or cell-surface charge (results not shown). However, except for RDP Ia-3 strains and one RDP III-1 strain, cell-
measured by a radio-immunoassay.

3.0

specific antibody levels among mothers of infected infants. We have reported previously that most strains from neonates with early-onset septicaemia belonged to serotype II1 isolates. Certain other factors are likely to influence susceptibility to infection, including the concentration of type-specific antibodies in mothers of infected infants, whereas the type-specific antibody concentrations in serum pool M were adjusted to approximate to the cut-off level above which maternal antibody appeared to protect infants from infection.

The CL assay demonstrated clear differences in susceptibility to opsonisation between different GBS RDP types. Amongst serotype Ia isolates, RDP Ia-1 strains stimulated higher CL responses when opsonised with serum from pool L than RDP Ia-2 and Ia-3 strains opsonised with serum from pool M. Similar differences were found amongst serotype III isolates. The quantity of type-specific antibody in serum showed a positive correlation with the intensity of CL induced by opsonised organisms. However, for GBS RDP III-3 strains, the difference between the low-level and high-level antibody pools in ability to opsonise these strains was less pronounced than for other RDP types.

In the present study we did not investigate the role of different serum factors in opsonisation of GBS strains. Hastings and Easmon reported that opsonisation of GBS type III isolates was complement-dependent but that there was considerable variation in the requirements of individual strains for the classical and alternative pathways. Anderson et al. demonstrated that, for GBS type III strains, although the concentration of type-specific antibody correlated with the opsonic capacity of serum, the major determinant of opsonisation was activation of the alternative pathway. The most likely explanation for these observations is that type-specific antibody binds and neutralises the SA-containing immunodeterminants resulting in impaired factor H binding to C3b, which, in turn, would then allow the formation of a convertase C3bBb and the deposition of C3b on to the bacterial cell surface. The finding that there is considerable variation in the opsonic requirements of individual GBS strains suggests that protective antibody might, in certain circumstances, be strain-specific rather than merely type-specific. Since serological homogeneity within the same GBS serotype has been well demonstrated, we tried to identify differences in cell-surface characteristics between RDP types. Attempts to relate resistance to opsonisation with cell-surface characteristics, such as affinity for xylene or adhesiveness to HA, may not be adequate. However, the similarity of cell-surface characteristics within each RDP type suggested that the structure of the cell surface varies with RDP type.

In neonatal GBS infections, one well recognised risk factor is a low concentration of type-specific antibodies in the serum of mothers of infected infants. We have reported previously that most strains from neonates with early-onset septicaemia belonged to RDP Ia-3 and III-3, whereas serotype III strains from meningitis cases have not been linked to a particular RDP type.

The present study has demonstrated that amongst serotype Ia and III isolates, certain RDP types, in
particular RDP type III-3, are more resistant to opsonisation than other RDP types suggesting that potentially virulent strains of GBS, with genetic homogeneity, may exist.

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