Impaired opsonophagocytosis of serotypes Ib and II of group B streptococci as compared with serotypes Ia and III: role of the alternative pathway of complement in opsonisation of serotype III of group B streptococci

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SUMMARY Using the technique of phagocytic chemiluminescence, we have shown that serotypes Ib and II of group B streptococci are resistant to opsonophagocytosis. The resistant strains became susceptible to opsonophagocytosis by trypsin treatment, but neuraminidase had no effect. Several studies have failed to define a significant role for the alternative pathway of complement in opsonisation of group B streptococci. By simple chelation and heat inactivation studies, we have shown that the alternative pathway of complement is activated by serotype III of group B streptococci.

Group B streptococci continue to be a major cause of morbidity and mortality in neonates and in patients with compromised host defence mechanisms.^{1,2} There are strain specific differences in opsonic requirements for type II and III group B streptococci.^{3,4} Using the technique of chemiluminescence we have examined a number of sera for opsonic activity and a number of healthy donor leucocytes for opsonophagocytic function to serotypes Ia, Ib, II, and III of group B streptococci. Our results show the phenomenon of resistance to opsonophagocytosis by serotypes Ib and II despite donor leucocyte and serum variation. In experiments to determine the chemical nature of anti-phagocytic factors of group B streptococci, the serotypes were cultured with type V neuraminidase from *Clostridium perfringens* and pancreatic trypsin. After enzymatic treatment the serotypes were opsonised with pooled normal human serum and subjected to phagocytosis by polymorphonuclear leucocytes.

Sialic acid acts as a non-activating surface for the alternative pathway of complement activation since it increases the affinity of cell bound C3b for β 1H but

not B.⁵ This blocks the formation of the alternative pathway C3 convertase C3bBb. Several studies have failed to define a significant role for the alternative pathway of complement in opsonophagocytosis of group B streptococci.^{3,6} We have carried out simple chelation studies using MgEGTA and heat inactivation studies with serotype III of group B streptococci to ascertain the role of the alternative pathway of complement in defence against group B streptococci infection.

Material and methods

BACTERIA

Reference strains of group B streptococci types Ia (8190), Ib (8180), II (11079), and IIIa (8184) (of human origin) were supplied by the National Collection of Typed Cultures, Colindale Avenue, Colindale, London, NW9. They were grown overnight at 37°C in Todd-Hewitt broth (Oxoid), which was buffered to final pH 7.8. They were washed three times in Hank's balanced salt solution without phenol red (HBSS) and then heat killed at 60°C for 7 min. The strains were counted in a Thoma counting chamber and resuspended to 1×10^9 group B streptococci/ml in HBSS.

ENZYMES AND TRYPSIN INHIBITOR

A highly active preparation of pancreatic trypsin was obtained from Difco Laboratories (Surrey); neuraminidase type V purified from *C. perfringens* and trypsin inhibitor type I-P from beef pancreas were supplied by Sigma Chemicals.

SERUM

This was obtained from healthy adults or sick children. Aliquots of the sera were stored individually or as pools of healthy adult sera at -70°C . Inactivation of complement activity was achieved by heating the serum to 56°C for 30 min. Classical pathway activation was prevented by treating the serum with 10 mM MgCl_2 and 10 mM ethylene glycol tetraacetate (EGTA). Alternative pathway complement activity was inhibited by heating the serum to 50°C for 20 min, thus inactivating factor B.

POLYMORPHONUCLEAR LEUCOCYTES

Normal human polymorphonuclear leucocytes were separated from heparinised blood by dextran sedimentation of red cells (6 ml dextran 110: 10 ml heparinised blood). After 60–90 min at room temperature, the polymorphonuclear leucocytes were harvested from the supernatant, diluted in HBSS without phenol red, and centrifuged at 120 g at $+4^{\circ}\text{C}$ for 15 min. The cell pellet was reacted with 5 ml 0.83% tris buffered ammonium chloride for 7–10 min at $+4^{\circ}\text{C}$ to remove contaminating erythrocytes. After two washes in HBSS, the polymorphonuclear leucocytes were finally resuspended to a concentration of $5 \times 10^6/\text{ml}$ in HBSS and kept cold.

OPSONISATION OF GROUP B STREPTOCOCCI

After two washes in HBSS, group B streptococci at a concentration of $4 \times 10^6/\text{ml}$ were rotated at 37°C with 100 μl serum and 100 μl HBSS to achieve the desired dilution of serum for the required length of time. Opsonisation was stopped by addition of ice cold HBSS. The particles were then washed twice and resuspended in HBSS to provide a concentration of about $1 \times 10^6/\text{ml}$.

TREATMENT OF SEROTYPES WITH TRYPSIN, TRYPSIN INHIBITOR, AND NEURAMINIDASE

The serotypes were cultured in Todd-Hewitt broth supplemented with 0.5% trypsin and 0.9 U/ml neuraminidase; serotypes Ib and II were also cultured with trypsin plus inhibitor at final concentrations of 0.1%, 0.5%, and 1% made up in HBSS without phenol red. After overnight culture, the organisms were washed twice in chilled HBSS and opsonised with a working dilution (14%) of pooled normal human serum. After a further two washes in chilled HBSS, the organisms were suspended in

HBSS at the working concentration ($1 \times 10^6/\text{ml}$). Chemiluminescence assay was carried out with these organisms. A control of unopsonised organisms treated with trypsin and neuraminidase was set up for chemiluminescence assay at the same time.

CHEMILUMINESCENCE ASSAY

Luminol dependent phagocytic chemiluminescence was measured at 37°C in a picolite luminometer (Packard Instruments). Fifty microlitres of 10^{-5} luminol (Sigma) (5-amino-2, 3 dihydro 1, 4 phthalizinedione made up in phosphate buffered saline) and 50 μl of polymorphonuclear leucocyte suspension were added to a reaction vial, which was transferred to the luminometer chamber. Fifty microlitres of opsonised or unopsonised bacteria and 20 μl of HBSS were then added to the reaction vial, and the light generated was recorded on a digital print out every 6 min. Chemiluminescence was recorded in triplicate and a mean value obtained.

VISUALISATION OF PHAGOCYTOSIS

Quantitation of ingestion of opsonised or unopsonised organisms was attempted by centrifuging the reaction mixture after recording chemiluminescence, removing the supernatant, and resuspending the pellet in a drop of HBSS. An aliquot of this suspension was dropped on to a microscope slide and air dried; slides were fixed for 10 min in 100% methanol and stained with Giemsa (1/50 in buffered distilled water) for 1 h. The slides were examined microscopically under oil immersion (Fig. 1). An attempt to quantitate the degree of phagocytosis by counting the percentage of polymorphonuclear leucocytes ingesting or the number of bacteria ingested per polymorphonuclear leucocyte was extremely difficult, but there was a clear distinction between the appearance of preparations in which there had been phagocytosis and those in which no ingestion had occurred. In positive smears most of

Fig. 1 Ingestion of type III group B streptococci by neutrophils from a normal adult.

Fig. 2 Effect on phagocytosis and luminol enhanced chemiluminescence of group B streptococci III opsonised with varying concentrations of serum for varying lengths of time. Percentages in each graph refer to serum concentration.

the organisms appeared to be intracellular and almost all polymorphonuclear leucocytes had numerous ingested bacteria. This correlated with a high degree of chemiluminescence. In contrast, negative preparations contained bacteria which were predominantly unassociated with polymorphonuclear leucocytes, and only an occasional leucocyte contained organisms. This in turn correlated with a greatly diminished chemiluminescence. All slides were interpreted without knowing what the phagocytic mixture contained.

Results

Effect on phagocytosis and, luminol enhanced chemiluminescence of group B streptococci III opsonised with varying concentrations of serum for varying lengths of time

The combined effects of incubation time and serum concentration used to opsonise group B streptococci III on its subsequent phagocytosis and chemiluminescence response are shown in Fig. 2. In this experiment many different serum concentra-

Fig. 3 Temporal tracings of luminol enhanced chemiluminescence with variable ratios of bacteria: polymorphonuclear leucocytes (PMNS). Concentration of luminol $10^{-5}M$, concentration of PMNS = $5 \times 10^6/ml$.

tions were used while keeping the number of group B streptococci III constant. Fig. 2 shows that opsonisation of group B streptococci is time dependent and that in normal sera a plateau is reached at 15 min. Fig. 2 also shows that light emission from polymorphonuclear leucocytes increases with phagocytosis of group B streptococci III opsonised with increasing serum concentrations. In subsequent experiments a 14% serum concentration was chosen as the working dilution of serum, and the opsonisation time was 15 min.

Relation of group B streptococci to polymorphonuclear leucocyte ratio with luminol enhanced chemiluminescence

The effect of varying the group B streptococci to polymorphonuclear leucocyte ratio on chemiluminescence was evaluated using serial twofold dilutions of type III group B streptococci preopsonised with 14% pooled human serum (obtained from 10 normal individuals, five men and five women) with a constant number of polymorphonuclear leucocytes ($5 \times 10^6/ml$) and $10^{-5}M$ luminol. The number of organisms presented to the polymorphonuclear leucocytes had a striking effect on the resulting chemiluminescence (Fig. 3). Very low, almost undetectable peaks were obtained with

Fig. 4 Variations in the uptake of different serotypes of group B streptococci by normal adult polymorphonuclear leucocytes.

lower numbers of bacteria. In contrast, higher numbers of organisms increased the peak in chemiluminescence. In subsequent assays the concentration of group B streptococci was adjusted so as to give a group B streptococci to polymorphonuclear leucocyte ratio of 200:1—that is, 1×10^9 organisms (optical density 0.9 at 620 nm). This ratio produced a significant but not a maximum chemiluminescence response.

Fig. 5 Serotype specific variations in opsonisation.

Variations in the uptake of different serotypes of group B streptococci by normal adult polymorphonuclear leucocytes

Fig. 4 shows the consistency of the resistance to phagocytosis by serotypes Ib and II (opsonised with pooled normal human serum) despite donor leucocyte variation. By contrast, serotypes Ia and III were readily taken up by normal polymorphonuclear leucocytes with a concomitant rise in chemiluminescence and greatly increased intracellular group B streptococci as seen under the microscope.

Serotype specific variations in opsonisation

A number of sera from normal adults and sick children were examined for opsonins to group B streptococci types Ia, Ib, II, and III. Using polymorphonuclear leucocytes from one normal adult, we found that types Ia and III were opsonised and phagocytosed to a varying degree as shown by an increased chemiluminescence. A consistent failure to generate opsonins was seen with serotypes Ib and II, however, as shown by a lack of phagocytosis and a diminished chemiluminescence response (Fig. 5).

Effect of enzyme and enzyme plus inhibitor treatment on opsonisation and subsequent phagocytosis of group B streptococci serotypes

Opsonisation of all the serotypes with pooled nor-

Fig. 6 Effect of trypsin treatment on opsonisation and phagocytosis of serotypes Ia, Ib, II, and III of group B streptococci.

Fig. 7 Effect on phagocytosis and luminol enhanced chemiluminescence response of unopsonised group B streptococci III and group B streptococci III opsonised with MgEGTA chelated serum, serum heated to 50°C for 20 min, and 56°C for 30 min. PMNS = polymorphonuclear leucocytes.

mal human serum following incubation in Todd-Hewitt broth supplemented with neuraminidase had no effect on phagocytosis. Opsonisation following incubation in Todd-Hewitt broth supplemented with 0.5% trypsin, however, resulted in enhanced phagocytosis of all the serotypes and an increased chemiluminescence response, although resistant serotypes (Ib and II) were not rendered fully sensitive to opsonophagocytosis, as shown by a lower chemiluminescence response of these serotypes compared with the sensitive serotypes (Fig. 6). None of the unopsonised serotypes treated with trypsin was phagocytosed to any appreciable extent and produced only a slight chemiluminescence response (Fig. 6). Serotypes Ib and II cultured in Todd-Hewitt broth supplemented with trypsin plus inhibitor (at all the concentrations used) were not opsonised to any appreciable extent and normal phagocytes challenged with these organisms failed to elicit a chemiluminescence response.

Effect on phagocytosis and luminol enhanced chemiluminescence response of group B streptococci III opsonised with MgEGTA chelated serum and serum heated to 50°C for 20 min and 56°C for 30 min

Fig. 7 shows the results of the experiments to determine the relative contributions of classical and alternative pathways of C3 activation to opsonisation of group B streptococci type III. Phagocytosis

of group B streptococci III after opsonisation with serum chelated with MgEGTA was little affected, with no appreciable inhibition of chemiluminescence response. In contrast, phagocytosis after opsonisation with serum heated to 50°C for 20 min, leading to inactivation of factor B, resulted in reduced opsonisation with impaired phagocytosis and a diminished chemiluminescence response.

Discussion

The resistance to opsonophagocytosis by serotypes Ib and II may be due to the fact that the antigenic determinants present in these serotypes are not immunologically "recognised" by serum opsonins and/or that they are incapable of activating the classical or alternative pathway of complement for effective opsonisation. Resistance to opsonisation may well correlate closely with virulence. In this study trypsin treatment followed by opsonisation reversed the resistance of serotypes Ib and II to opsonophagocytosis. Trypsin may expose certain protein antigens on the cell walls of these resistant serotypes, thus enabling activation of the alternative pathway to occur, or it may provide the site for immunological recognition by serum opsonins, resulting in more efficient opsonisation and phagocytosis. Results of culture of resistant serotypes in the presence of trypsin and inhibitor tend to support this concept since there was little opsonisation of serotypes Ib and II after inactivation of trypsin. It is unlikely that trypsin acted as an opsonin itself since unopsonised bacteria treated with trypsin were not phagocytosed to any appreciable extent and failed to elicit any significant chemiluminescence response.

Virulence is ascribed to sialic acid, which is considered to act as a virulence factor in one of two ways: either by directly enhancing the invasiveness of the organism or by acting as an antiphagocytic factor. Type III opsonin resistant strains have a higher sialic acid content.⁷ Our studies show, however, that organisms opsonised after culture with neuraminidase type V had no effect on opsonophagocytosis, a paradoxical finding in view of the fact that sialic acid, a major immunodeterminant of group B streptococci, seemed to be unaffected by the sialidase. This suggests that while sialic acid may be an important virulence factor in some strains of group B streptococci, virulence may also be associated with protein components of the cell wall which may act as antiphagocytic factors by resisting opsonisation.

Epidemiological observations on the distribution of group B streptococci isolates from early onset type septicaemia without meningitis (mortality greater than 50%) from neonates and asymptomatic

mothers have shown that 26% of the strains were of serotypes Ia, Ib, or Ic; 38% were of serotype II; and 36% were of serotype III.⁸ Early onset type infection with meningitis and late onset type meningitis (beyond the 10th day of life) with significantly lower mortality rates have a striking predominance of type III isolates.⁸⁻¹⁰ In view of these observations, we believe that studies of the kind presented here may offer some explanation in the understanding of virulence factors and may be of use in determining immunological adjuncts to antibiotic treatment, particularly as vaccines based on polysaccharide components of the bacterial cell wall are advocated for the induction of immunity in women to prevent neonatal infection.¹¹

The fact that inactivation of factor B led to diminished opsonisation for serotype III of group B streptococci suggests an essential role for alternative pathway of complement in opsonophagocytosis of group B streptococci and excludes the possibility of opsonophagocytosis being mediated by interaction of antigen-antibody with neutrophil Fc receptors. The essential ligand is presumably C3b, which is the most important opsonic ligand generated during complement activation.¹² Potentially the fixation of C3b by the alternative pathway is a major factor in natural host defence because activation of this pathway can occur in the absence of antibody.⁵ It has been shown, however, that the presence of specific anticapsular antibody—that is, acquired immunity—will permit alternative complement pathway activation by the organism in its fully sialated state.¹³ The exact mechanism by which alternative pathway is activated by the specific antibody remains to be elucidated. It is suggested,¹³ however, that the antibody may be functioning in one of two ways: either antibody binds and neutralises sialic acid residues leading to impaired β 1H binding of C3b compared with B, thus allowing the formation of a deregulated C3bBb convertase; or, alternatively, the carbohydrate groups of immunoglobulin provide a protected site for C3b deposition. In the present report, studies of specific antibody were not carried out. From other studies,¹³ however, it would appear that the serum used in this study contained a critical concentration of antibody with specificity for sialic acid containing immunodeterminant for alternative pathway activation to occur. Further experiments are in progress to study opsonophagocytic

variations of clinically derived strains with particular reference to strains Ib and II.

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