Opsonisation and phagocytosis of group B meningococci by polymorphonuclear leucocytes: comparison of sulphonamide sensitive and resistant strains

K N WARD,* A FLEER,† J VERHOEF,† D M JONES*

From the *Public Health Laboratory, Withington Hospital, Manchester, England; and the †Laboratory of Microbiology, Utrecht, The Netherlands

SUMMARY A large proportion of disease caused by sulphonamide resistant strains of group B type 15 meningococci affects patients 10–24 years. In contrast, disease caused by sulphonamide sensitive strains conforms to the usual pattern, and most infection occurs in early childhood. In an attempt to explain this phenomenon possible differences in susceptibility of resistant and sensitive strains to phagocytosis by polymorphonuclear leucocytes were investigated, using radioactively labelled bacteria.

In initial experiments a group B resistant strain required higher concentrations of normal human serum and longer opsonisation times for phagocytosis than an ungroupable non-pathogenic meningococcus. Comparison of sulphonamide resistant and sensitive group B meningococci showed that with either heat inactivated serum or agammaglobulinaemic serum, phagocytosis did not occur with any of the strains, whereas if these two sera were used together, phagocytosis was restored to the level seen with normal human serum. Thus both antibody and complement are required for phagocytosis. Furthermore, opsonisation depended on an intact classical pathway of complement for each group B strain. In all the experiments there was no significant difference between the phagocytosis of sulphonamide sensitive and resistant group B strains neither with regard to the efficiency of opsonisation by normal human serum nor the exact requirements for antibody and complement.

Disease caused by *Neisseria meningitidis* is endemic throughout the world and still presents a serious problem both in developing and industrialised countries despite the availability of antibiotics. Meningococcal meningitis and meningococcaemia are primarily diseases of early childhood. Over half of all cases occur in children less than 5 years of age, with the peak incidence at 6–8 months of life.¹ In England and Wales during the period 1966–83 meningococcal infections caused 70–80 deaths per year predominantly in children under 5 years old.²

Group B strains of *N meningitidis* account for about 60% of infections in England and Wales, and this proportion has not varied since 1966, but if these strains are further subdivided into serotypes then it becomes evident that the relative proportions of the different group B serotypes have changed. In 1974–5 type 2 strains were the most common (60%), but since

that time there has been a steady decrease in the prevalence of this serotype so that in 1983 only 13% of strains were type 2. On the other hand, serotype 15 strains have been increasing in prevalence and so has the proportion of strains that are resistant to sulphonamide.² By 1985 56% of all group B strains isolated were type 15 and 60% of these were resistant to sulphonamide.³ These type 15 resistant meningococci have been implicated in recent local outbreaks in England,² and in Norway they have been causing a high incidence of meningococcal disease since 1974–5.³ Other north European countries including The Netherlands have also experienced an increase in the prevalence of type 15 and a decrease in type 2 group B strains.³

If the infections caused by the different serotypes of group B meningococci in England and Wales are analysed with respect to the age of the patient, all except type 15 follow the usual pattern, with most of the infections being in the first few years of life. With

type 15 strains, however, there are more infections in the 10–24 year age group than in those under 4 years of age.² Moreover, it is only the type 15 strains resistant to sulphonamide that predominantly affect teenagers and young adults, whereas infections due to the sensitive type 15 strains occur mainly in early childhood.⁴ Thus it is the resistance to sulphonamide rather than the serotype of the group B meningococcus that is associated with a change in the age of the susceptible population.

Immunity to meningococcal disease depends on the presence of bactericidal antibody,⁵ and it may be assumed that the increased incidence of group B serotype 15 infections in England and Wales is somehow associated with lack of antibody to the type 15 strain in the population. This would not explain, however, the changed age distribution of infections caused by the sulphonamide resistant strain, and other aspects of immunity were therefore considered. As phagocytosis is of major importance in host defence against other encapsulated bacteria, and meningococci are phagocytosed by polymorphonuclear leucocytes,^{6,7} it might be that the changed pattern of disease caused by the resistant meningococci is related to defective phagocytosis resulting from a fundamental change in the invasive properties of the organism. This paper reports a study of the factors necessary for the opsonisation and phagocytosis of group B meningococci by polymorphonuclear leucocytes, and compares strains resistant and sensitive to sulphonamide.

Material and methods

SERUM

Normal human serum was obtained by venepuncture from 10 healthy medical students; the samples were pooled and stored in aliquots at -70°C and thawed immediately before use. Where necessary, heat inactivated serum was prepared by incubation at 56°C for 30 minutes, or alternatively, the classical pathway was blocked by chelation with 8mM Ethyleneglycol-bis-(β -aminoethyl ether)— $\text{N,N,N,N}^1\text{—tetraacetic acid}$ in the presence of 2.5 mM Mg^{2+} (Mg-EGTA).

Agammaglobulinaemic serum was prepared from the blood of a patient with X-linked hypogammaglobulinaemia, which was kindly provided by Dr J Roord of the Wilhelmina Kinderziekenhuis, Utrecht. The original specimen contained no detectable IgM or IgA but a significant amount of IgG (5 g/l); after absorption of the serum with Sepharose-Protein A (Pharmacia, Uppsala, Sweden) a negligible amount of IgG remained, and both the classical and alternative pathways of complement still had activity within the normal range.

BACTERIA

Strains of *N meningitidis* were grouped by the method

of Olcén, Danielsson, and Kjellander⁸ and typed using monoclonal antibodies.⁹ If the minimal inhibitory concentration (MIC) of sulphonamide for the strain was ≥ 20 mg/l the strain was defined as sulphonamide resistant, whereas if the MIC was ≤ 3.2 mg/l it was defined as sulphonamide sensitive. All the group B strains were isolates from the blood or cerebrospinal fluid of patients with meningitis; two were sulphonamide resistant, F55 (type 15 subtype P1-16) and F906 (not typable), and two were sulphonamide sensitive F900 (type 2b) and F994 (type 15). F957, an ungroupable, untypable strain sensitive to sulphonamide, was isolated from a post-nasal swab taken from a healthy carrier.

CULTURE AND RADIOACTIVE LABELLING OF MENINGOCOCCI

All strains were freeze dried or stored at -70°C in 10% glycerol broth. Before use the bacteria were cultured overnight at 37°C on blood agar plates in candle extinction jars. The next day 5 ml of Mueller Hinton broth (Difco Laboratories, Detroit, USA) containing $10 \mu\text{Ci}$ of [^3H] glucosamine (specific activity 5.8 Ci/mmol; The Radiochemical Centre, Amersham, UK) was inoculated with 50–100 colonies, and the culture was incubated in a 37°C waterbath with continuous shaking at about 20 cycles/minute. After two hours the meningococci were harvested in the logarithmic growth phase, centrifuged at $1200 \times g$ for 15 minutes, and washed three times in phosphate buffered saline (PBS). A standard suspension of meningococci was made by resuspending the bacteria in PBS so that the optical density was 0.214 at 660 nm.

The above protocol for labelling the meningococci regularly resulted in counts of about 10^6 /ml of final suspension, and if the radioactively labelled bacteria were then incubated at 37°C for 30 minutes, centrifuged, and washed as above, there was no noticeable loss of radioactivity, indicating that the label was stable.

ISOLATION OF POLYMORPHONUCLEAR LEUCOCYTES

A healthy adult blood donor not receiving medication gave 500 ml of venous blood which was then heparinised (10 u/ml), and the erythrocytes were sedimented by gravity for one hour in 6% dextran (molecular weight 70 000, Fluka, Buchs, Switzerland) in normal saline at room temperature using 150 ml dextran per 500 ml of whole blood. The leucocyte rich plasma was then centrifuged at $160 \times g$ for five minutes and the pellet resuspended in Hanks's balanced salt solution containing 0.1% gelatin (HBSS-Gel). Thirty ml of this suspension was layered on to 15 ml of Ficoll-Paque (Pharmacia, Uppsala, Sweden) and centri-

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fused at $160 \times g$ for 35 minutes. The supernatant was discarded, and any remaining erythrocytes in the pellet were lysed using hypotonic saline (0.2%) for one minute after which time enough hypertonic saline was added for the suspension to become isotonic. The cells were subsequently centrifuged and washed twice in HBSS-Gel. The final cell suspension always contained at least 98% polymorphonuclear leucocytes, as determined by differential counts.

OPSONISATION AND PHAGOCYTOSIS ASSAY

Bacterial opsonisation and phagocytosis were assessed by a previously described quantitative assay,¹⁰ using radioactively labelled meningococci and human polymorphonuclear leucocytes. The method was modified by substituting [³H] glucosamine for [³H] thymidine because meningococci could not be labelled with the latter compound despite extensive variation of the culture conditions.

Opsonisation was performed by incubating 0.1 ml of the standard suspension of labelled bacteria at 37°C in a shaking water bath, together with normal human serum which was diluted either in HBSS-Gel or in Mg-EGTA veronal buffer to a concentration which varied according to the experiment. The opsonisation was stopped at a predetermined time by adding ice cold PBS, and the bacteria were then centrifuged at $1200 \times g$ for 15 minutes and resuspended in 0.5 ml of HBSS-Gel. Phagocytosis was assessed in duplicate phials as follows: 0.1 ml of the opsonised meningococci were incubated for 12 minutes together with 0.1 ml of polymorphonuclear leucocytes at a concentration of 5×10^6 /ml in a 37°C shaking water bath (150 cycles/minute). After this, radioactively labelled bacteria which were not associated with the polymorphonuclear leucocytes were separated from the phagocytes by three cycles of differential centrifugation at $160 \times g$ for five minutes and the radioactivity in the pellet was determined in a liquid scintillation counter (MK II, Nuclear Chicago, Chicago, USA). The percentage of bacteria taken up by the polymorphonuclear leucocytes was calculated from the ratio of the uptake of radioactivity by the phagocytes to the total added radioactivity counted in a separate phial, and this figure was taken as a measure of bacterial opsonisation and phagocytosis.

Results

Before comparisons could be made of differences between sulphonamide sensitive and resistant group B meningococci it was necessary to define the conditions under which opsonisation and phagocytosis of the bacteria might occur. F906, a group B strain, was opsonised for 30 minutes with concentrations of pooled normal human serum which varied from

0–40% and then incubated with polymorphonuclear leucocytes. Fig 1 shows that the percentage uptake of the radioactively labelled bacteria by polymorphonuclear leucocytes depends on the concentration of serum used in the opsonisation step of the assay. It is clear that 5% serum is inadequate for opsonisation and phagocytosis, whereas significant uptake occurs at higher concentrations of serum, with maximal values being achieved above 20%. Fig 2 shows that if the same strain of meningococcus is opsonised with 20% normal human serum for one minute then uptake by polymorphonuclear leucocytes does not occur. Longer opsonisation times result in a clear uptake of radioactively labelled bacteria, and this uptake approaches a maximum when meningococci opsonised for 30 minutes are used.

Thus F906, a group B meningococcus, is resistant to opsonisation and to phagocytosis, requiring both a high concentration of serum and a long time for opsonisation. In strong contrast, phagocytosis approaching the maximum was seen after an ungroupable meningococcus (F957) had been opsonised for only one minute with a low concentration (5%) of pooled normal human serum (fig 3). Moreover, when F957 was opsonised using 10% heat inactivated serum for five minutes, the uptake of radioactivity by polymorphonuclear leucocytes was $31 \pm 2.4\%$ compared with $73\% \pm 36\%$ for whole,

Fig 1 Phagocytosis of F906, a group B, untypable, sulphonamide resistant meningococcus, after opsonisation for 30 minutes with different concentrations of normal human serum (●). Each value is expressed as mean ± SEM ($n = 4$).

Fig 2 Phagocytosis of F906, a group B, untypable, sulphonamide resistant meningococcus, after opsonisation for different lengths of time with 20% normal human serum (●) and in absence of serum (○). Each value is expressed as mean $\pm$ SEM ($n = 5$).

unheated serum (mean $\pm$ the standard error of the mean where $n = 3$ in both cases). These results show that an ungroupable meningococcus can be opsonised and phagocytosed in the absence of complement, albeit less efficiently.

Having established the criteria for the opsonisation and phagocytosis of one strain of group B meningococci, a comparison was made between four different group B meningococci. Two were sulphonamide sensitive, a type 2b strain (F900) and a type 15 strain (F994), and two were sulphonamide resistant, an untypable strain (F906) and a type 15 subtype P1-16 strain (F55). Fig 4 shows the results observed when the bacteria were opsonised for either five or 30 minutes with 20% pooled normal human serum. It is clear that there is no appreciable difference in uptake by the polymorphonuclear leucocytes between the two sensitive and the two resistant strains. Fig 4 also shows the result of using heat inactivated serum for opsonisation of the meningococci; in the absence of complement there is a pronounced reduction in the uptake of radioactivity by the polymorphonuclear leucocytes; three strains show no significant opsonisation and the fourth, F55, shows a much reduced uptake.

The role of antibody in the phagocytosis of group B meningococci was investigated using

agammaglobulinaemic serum for opsonisation. Table 1 shows the results obtained for the four strains. In each case neither 20% heat inactivated normal human serum alone nor 20% agammaglobulinaemic serum alone was sufficient to opsonise the bacteria, but when the heat inactivated and the agammaglobulinaemic serum are added together then the uptake of radioactivity by polymorphonuclear leucocytes is not significantly different from that obtained using 20% normal human serum. Thus both complement from the agammaglobulinaemic serum and antibody from the heat inactivated serum are required for optimal opsonisation and phagocytosis of all group B strains.

Although there was no difference between the four group B strains with respect to their absolute dependence on complement for opsonisation and phagocytosis, subtle differences in the mechanism of

Fig 3 Phagocytosis of F957, an ungroupable, untypable meningococcus, after opsonisation for different lengths of time with 5% (●) and 2.5% (▲) normal human serum and in absence of serum (○). Each value is expressed as mean $\pm$ SEM ($n = 3$).

Fig 4 Phagocytosis of four different group B meningococci after opsonisation for five and 30 minutes with 20% normal human serum (●) and 20% heat inactivated normal human serum (○). Each value is expressed as mean $\pm$ SEM (n is given on figure for each strain).

Table 1 Phagocytosis of four different strains of group B meningococci after opsonisation for 30 minutes with agammaglobulinaemic serum or heat inactivated serum, or both (each value is expressed as mean ($\pm$ SEM) (n = 2))

Opsonising serum	% uptake of radioactivity by polymorphonuclear leucocytes*			
	Sulphonamide sensitive strains		Sulphonamide resistant strains	
	F900	F994	F906	F55
20% normal human serum	26 (11)	49 (6.4)	48 (0.7)	59 (2.5)
20% heat inactivated normal human serum	2.1 (0.9)	2.9 (1.0)	13 (3.3)	17 (5.4)
20% agammaglobulinaemic serum	2.0 (0.6)	1.7 (0.7)	6.8 (2.1)	9.1 (0.3)
20% heat inactivated normal human serum together with 20% agammaglobulinaemic serum	36 (4.2)	53 (0.1)	51 (4.0)	56 (2.7)

Table 2 Role of classical pathway of complement in opsonisation and phagocytosis of four different group B meningococci

Opsonising serum†	% uptake of radioactivity by polymorphonuclear leucocytes*			
	Sulphonamide sensitive strains		Sulphonamide resistant strains	
	F900 (n = 4)	F994 (n = 4)	F906 (n = 3)	F55 (n = 5)
20% normal human serum	36 (1.5)	51 (2.9)	40 (6.5)	51 (2.6)
20% normal human serum with Mg-EGTA	2.6 (0.3)	5.7 (1.7)	12 (0.4)	21 (3.2)
20% heat inactivated normal human serum	2.3 (0.4)	1.6 (0.3)	2.3 (0.4)	10 (5.1)

†Incubated for 30 minutes.

*Each result is expressed as mean $\pm$ SEM (n is given in the table for each strain)

opsonisation might have been shown between resistant and sensitive meningococci if the classical sulphonamide pathway of complement were blocked using Mg-EGTA during the opsonisation step of the assay. Table 2 shows the results of such experiments; for all four strains tested, opsonisation and phagocytosis were largely abolished in the presence of Mg-EGTA with the level of uptake not significantly different from that observed with heat inactivated serum. Thus activation of the classical pathway is necessary for opsonisation and phagocytosis of both sulphonamide resistant and sulphonamide sensitive strains.

Discussion

In these experiments a modification of a quantitative assay using radioactively labelled bacteria¹⁰ was used to study the opsonisation and phagocytosis of meningococci by human polymorphonuclear leucocytes. The conditions were defined for optimal and reproducible uptake of a group B meningococcus (F906) that had been isolated from a case of meningitis. In contrast, an ungroupable, non-pathogenic meningococcus isolated from a throat swab (F957) was more efficiently phagocytosed, requiring a lower concentration of pooled normal human serum and a much shorter opsonisation time. The same result was obtained when four extra individual sera (additional to those included in the normal human serum pool) were used separately to opsonise F957 (unpublished data). This implies that the more efficient uptake seen for this ungroupable meningococcus was due to a uniformly high titre of opsonins for F957 in the blood of all the donors to the pool, and was not the result of very high titres in the blood of only one or two donors to the pool. Ungroupable meningococci have also been shown to be more serum sensitive than group B meningococci.¹¹ This relative susceptibility of the ungroupable meningococcus to both phagocytosis by polymorphonuclear leucocytes and killing by serum is presumably reflected in the rarity of disease caused by these organisms.

Other workers have studied the phagocytosis of meningococci using different assays from the one used here. Roberts^{6,7} assessed uptake indirectly by measuring the percentage decrease in the number of viable extracellular bacteria, whereas Lehman and Solberg¹² used chemiluminescence. In both these systems phagocytosis of meningococci depended on both thermolabile and thermostable factors. Our method is a simple and precise method for measuring phagocytosis of meningococci and confirms and extends the above findings. All four group B meningococci tested were highly dependent on complement for opsonisation, as shown by the results obtained using heat inactivated serum; this requirement for complement was further confirmed using C3 deficient serum for opsonisation of all four strains when there was no significant uptake by polymorphonuclear leucocytes (unpublished data). Opsonically active C3 fragments deposited on the surface of the bacterium could have resulted from activation of either the classical or the alternative pathway of complement.¹³⁻¹⁵ In the case of group B meningococci chelation of normal serum with Mg-EGTA considerably reduced opsonisation, indicating dependence on the classical pathway. Ingestion of bacteria, particularly encapsulated bacteria, by polymorphonuclear leucocytes requires the cooperation of both antibody and complement,^{16,17} and evidence was obtained confirming this for all the group B meningococci tested. Agammaglobulinaemic serum alone was not sufficient for opsonisation but if it was added together with heat inactivated serum then phagocytosis was restored to the level obtained using normal human serum.

In contrast to the above described absolute dependence of phagocytosis of group B meningococci on both antibody and complement, reduced but significant opsonisation and phagocytosis of the ungroupable meningococcus (F957) was seen using heat inactivated serum. This difference may reflect a higher titre of opsonising antibody against F957 in the pooled human serum compared with titres of opsonising antibody against group B meningococci, such that phagocytosis may proceed in the absence of

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complement, albeit less efficiently. Roberts⁷ reported this phenomenon for groups A and C meningococci where opsonic activity in low titre antisera depended on both heat stable and heat labile factors (complement), whereas phagocytosis was observed in the presence of heat inactivated high titre antisera alone.

With regard to the factors necessary for opsonisation and phagocytosis of the different strains of group B meningococci by polymorphonuclear leucocytes, the only difference detected between the sulphonamide sensitive and resistant strains was that the resistant strains routinely showed a tendency to a slightly higher uptake (5–20%) after opsonisation with heat inactivated serum than that seen with sensitive strains (5%). The explanation for this phenomenon is unclear. It seems unlikely that the pooled human serum had a higher titre of antibody against the sulphonamide resistant organisms compared with the sensitive organisms, but the slightly raised level of uptake may possibly have reflected increased hydrophobicity of the sulphonamide resistant organisms. It is clear, however, that this small discrepancy does not support the hypothesis that sulphonamide resistant group B meningococci, in particular, type 15, are more resistant to phagocytosis than other closely related strains. Neither does the observation that the efficiency and kinetics of opsonisation by normal human serum is very similar for all the strains. Moreover, there are no differences in the requirement for antibody and complement between the strains, although others have shown variable opsonic requirements among serotypes within the same bacterial species.^{18 19} In the case of meningococci it may be that both group and type specific antibodies have a role in opsonisation and phagocytosis²⁰; but there is no evidence from our results to suggest that type specific antibodies are important as all the group B strains tested were equally opsonised by the normal human serum pool.

Finally, as the results of other studies^{21 22} indicate that opsonising antibodies to meningococci may have a part to play in protection against meningococcal disease, our technique for the measurement of the opsonisation and phagocytosis of group B meningococci should prove a useful tool in the study of the immune response to meningococci either during the course of natural infections or as part of the assessment of vaccine responses.

We are grateful for the excellent technical help of Helga Timmermans and Nomdo Westerdal.

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Requests for reprints to: Dr KN Ward, Clinical Virology, Level 6, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2QW.