Detection of antibodies to common antigens of pathogenic and commensal *Neisseria* species

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Summary. Sera from 29 children and six adults were used to investigate the nature of antigenic cross-reactivity between *Neisseria polysaccharea*, *N. lactamica* and *N. meningitidis* B,15P1.16 by immunoblotting. Major common antigens of 68–70 Kda, 60–65 Kda and 15–20 Kda were detected. Antibody directed against them uniformly decreased after absorption of the sera with the three different *Neisseria* species. Antigens of 55 Kda and 35 Kda specific to *N. meningitidis*, and one of 43 Kda specific to *N. lactamica*, were also demonstrated. Antibody against all antigens was more prevalent in bactericidal than in non-bactericidal sera, although these differences were statistically not significant. Differences in antibody prevalence between carriers of *Neisseria* spp. and non-carriers of these organisms were even less marked. Examination of sera by whole-cell enzyme-linked immunosorbent assay against *N. meningitidis* B,15P1.16 and *N. lactamica* gave an absorbance ratio of 1:1. Only four sera from children showed no reactivity against the meningococcal strain. These common antigens are likely to be important in vaccine development.

Introduction

Over the last 3 years, in England and Wales, there has been a wave of increased incidence of meningococcal disease. Group B strains still predominate but the proportion of disease due to group C strains has increased (Jones, 1988). Group B type 15 strains were initially the most prevalent but these are now superseded by nontypable strains.

The group B capsule is poorly immunogenic (Wyle *et al.*, 1972) and cross-reacts with human embryonic brain tissue (Finne *et al.*, 1983). The search for suitable antigens for use as vaccines against disease due to group B meningococci has led to the development of some prototype vaccines utilising the serotype epitopes of their outer-membrane proteins (OMP) and lipopolysaccharide (LPS) (Zollinger *et al.*, 1978; Jennings *et al.*, 1984; Rosenqvist *et al.*, 1988). The diversity of OMP serotypes and LPS immunotypes of pathogenic meningococcal strains (Zollinger and Mandrell, 1977), together with the current prevalence of nontypable strains, are factors which limit the usefulness of these vaccines. Recently described common neisserial antigens (Jennings *et al.*, 1980; Newhall *et al.*, 1980; Cannon *et al.*, 1984; Koomey and Falkow, 1984; Rothbard *et al.*, 1984; Martin *et al.*,

1986) are attractive agents for use in vaccines because most of them have been shown to be immunogenic. However, the protective role of the antibodies to these antigens is not clear.

Protective immunity to *N. meningitidis* depends upon the presence of bactericidal antibody (Goldschneider *et al.*, 1969) which can be formed through nasopharyngeal carriage of meningococcal strains (Reller *et al.*, 1973). Carriage rates of meningococci have been found to be only 2–5% in endemic areas, with a prolonged duration of carriage and slow transmission (Cann *et al.*, 1987), yet “adult” levels of immunity are reached by ten years of age (Frasch, 1977) and the incidence of invasive disease falls markedly over the 1–5-year age group. It is likely, therefore, that immunity is also acquired by carriage of other organisms. The cross-reactive nature of the *Escherichia coli* K1 capsule with group B meningococci has long been recognised (Grados and Ewing, 1970; Robbins *et al.*, 1972) but, more recently, cross-reactivity between meningococci, commensal *Neisseria* spp. and *N. gonorrhoeae* has been reported. Both *N. lactamica*, a common commensal of the nasopharynx in young children, and *N. polysaccharea* also cross-react with meningococcal antisera (Gold *et al.*, 1978; Bouquete *et al.*, 1986). Similarly, mice immunised with meningococci develop bactericidal antibodies to gonococci (Cremieux *et al.*, 1984). A 70-Kda iron-regulated protein, first de-

scribed by West and Sparling (1985), has been demonstrated in pathogenic and most non-pathogenic *Neisseria* spp. with hyperimmune mouse and human carrier sera (Aoun *et al.*, 1988a). A protective function has been postulated for antibody to this antigen because of its immunogenicity during the course of infection and carriage and the observation that significantly fewer patients developing gonococcal infection possess anti-70 Kda antibodies (Aoun *et al.*, 1988b).

The aim of this study was to investigate the nature of the cross-reactivity between *N. meningitidis*, *N. lactamica* and *N. polysaccharaea* by immunoblotting children's and adult sera. The children's blot patterns have been analysed according to carriage and non-carriage of these organisms, strain specific bactericidal activity and whole cell ELISA activity, in an attempt to identify patterns of antibody prevalence commonly associated with these factors.

Materials and methods

Organisms and sera

Organisms. The *N. meningitidis*, *N. lactamica* and *N. polysaccharaea* strains used were those obtained in a previous study of nasopharyngeal meningococcal carriage in primary school children (Cann *et al.*, 1987; Cann and Rogers, 1989). Additional meningococcal strains were isolated from the nasopharynx of adults attending the Genitourinary Medicine Clinic, Westminster Hospital, London SW1. Lysates of *N. gonorrhoeae*, Kellogg strain F62 and strain G7 (Johnston *et al.*, 1976), were provided by Dr C. Ison.

Sera. Sera from 72 children, also obtained from our previous study and for which strain specific bactericidal activity titres had been determined, were examined by whole-cell ELISA, 29 of them, of which 10 were from carriers of *Neisseria* spp. (1 *N. polysaccharaea*, 2 *N. lactamica*, 1 ungroupable *N. meningitidis* and 6 *N. meningitidis* group B) were further examined by immunoblotting. Five sera were from adult meningococcal carriers attending the Genitourinary Medicine Clinic and one was from a convalescent adult.

Preparation of antigen

Strains of *Neisseria* spp. were incubated overnight in 50 ml of Tryptone Soya Broth (Oxoid; CM129) at 36°C on a gyratory shaker at 150 rpm. The cultures were centrifuged, the cell pellets were washed in saline, and, after further centrifugation, the cells were resuspended in saline. The protein concentration of the suspensions was measured by the method of Lowry *et al.* (1951) and adjusted with saline to 2 µg/µl. Such standardised cell suspensions were then diluted with an equal volume of

sample buffer (SDS 4%, mercaptoethanol 10%, glycerol 20% in 0.125 M Tris-HCl, pH 6.8).

Gel electrophoresis

The diluted cell suspensions were boiled for 2 min and 10 µl (10 µg of protein) was loaded on to 12.5% polyacrylamide gels and electrophoresed for 1 h in a discontinuous buffer system (Laemmli, 1970). The following proteins as mol. wt markers (Kda) were also electrophoresed: bovine serum albumin (66); ovalbumin (45); pepsin (34.7); trypsinogen (24); β lactoglobulin (18.4); and lysozyme (14.3).

Immunoblots

The separated proteins were transferred electrophoretically to nitro-cellulose membranes (BA85, 0.45 µm; Schleicher and Schuell, Dassell, Federal Republic of Germany) in 25 mM Tris, 190 mM glycine buffer, pH 8.3, with methanol 20%, by the method of Towbin *et al.* (1979) in a Bio-Rad Trans Blot cell (Bio-Rad, Watford, England) at room temperature at 0.25A for 5 h. Protein-free sites were saturated by incubation with milk powder 4% in buffered saline (NaCl 0.9%, 10 mM Tris pH 7.4) at 36°C for 1 h. The membranes were probed with human sera overnight on a gyratory shaker at 36°C. After washing (3 × 15 min) in buffered saline and milk powder 0.1%, the membranes were incubated on a gyratory shaker at 36°C for 5 h with alkaline phosphatase-conjugated anti-human γ chain (D336; Dakopatts, DK-2600 Glostrup, Denmark) diluted 1 in 7500. After further washing (3 × 15 min), the membranes were incubated for 10 min in fresh substrate solution: 0.1 M ethanolamine-HCl (44 ml), 1 M MgCl₂ (0.2 ml), nitroblue tetrazolium 1 mg/ml (N 6876 Sigma Chemical Co., St Louis, MO, USA) (5 ml) and 5-bromo-4-chloro-3-indolylphosphate 4 mg/ml (B 8503, Sigma) (0.75 ml) in methanol:acetone 2:1. All dilutions were made in buffered saline with milk powder 0.1%.

Sera from non-carriers were used at an arbitrary antibody titre of 1 in 200 (to correspond with the dilution used for the whole-cell ELISA). Antibody titres in sera from carriers (adults and children) were measured against the homologous organism by a dot blot method in which 100-µl samples of a standardised suspension (E₅₄₀ 0.05) of organisms were applied to a nitro-cellulose membrane by means of a Bio-Rad Bio-Dot apparatus. Strips of membrane were incubated with serial dilutions of sera and developed as described above. These sera were then blotted at their end point antibody titre. The conjugate antibody was also titred by this method.

The children's sera were blotted against *N. polysaccharaea*, *N. lactamica* and *N. meningitidis* B,15P1.16. The adult sera were blotted against several strains of *Neisseria* spp. The adult convalescent serum was absorbed with *N. meningitidis* B,15P1.16, *N. lactamica* and *N. polysaccharaea* and then blotted to look for differential absorption of antibody activity.

Determination of strain specific bactericidal antibody titres

This was done at the Meningococcal Reference Laboratory, Withington Hospital, Manchester against *N. meningitidis* B,15P1.16 as described previously (Cann *et al.*, 1987).

Whole-cell enzyme-linked immunosorbent assay (ELISA)

A modification of the method described by Abdillahi and Poolman (1987) was used. *N. meningitidis* B,15P1.16 and *N. lactamica* were cultured overnight in Tryptone Soya Broth (Oxoid, CM129) at 36°C, on a gyratory shaker, before being heat-inactivated (56°C for 1 h) and coated on to microtitration plates (M129A Dynatech Laboratories Ltd, Billingshurst). Plates were coated in large batches to minimise variations through antigen preparation and in coating. The coated plates were washed thrice in Tween 20 0.05% w/v in phosphate-buffered saline (0.16 M NaCl, 8 mM NaP₂HPO₄, 1 mM KH₂PO₄, 3 mM KCl), pH 7.4 (PBS-Tween), and then blocked with bovine serum albumin (A7030 Sigma) 1% in PBS-Tween for 1 h at 36°C. The plates were washed thrice and 100 µl of test sera diluted 1 in 200 was added for 1 h at 36°C. After a further three washings, 100 µl of alkaline phosphatase conjugate at a dilution of 1 in 1000 was added and incubated for 1 h at 36°C. Anti-human γ , μ and α chain conjugates (D336, D337, D338; Dakopatts) were tested against the meningococcal antigen; only γ chain was tested against *N. lactamica*. After a further three washings, 100 µl of phosphatase substrate (104-0; Sigma) diluted to 1 mg/ml in 0.1 M glycine, 65 mM NaOH, 1 mM ZnCl₂ and 1 mM MgCl₂ buffer, pH 10.4, was added. After incubation for 45 min at 36°C, the absorbance at 450 nm was read with a Titertek Multiscan spectrophotometer. All washes and dilutions were made with PBS-Tween. To reduce positive reactions in the antigen control wells, the conjugates were absorbed, before dilution, with two meningococcal strains (B,15P1.16 and C untypable) and a *N. lactamica* strain. Tests were performed in duplicate with an antigen control for each conjugate and a blank well for each serum. Two control sera, one strongly and the other weakly reactive, were included in each batch of tests. The dilution at which the serum was used was decided by titrating the two control sera to obtain the maximum difference in absorbance between them.

Statistics

The χ^2 and Fischer's exact (2 tail) tests were used to determine the significance of the numbers of sera with positive reactions for different mol.-wt antigens in: carrier and non-carrier sera; bactericidal and non-bactericidal sera; and weakly, moderately and strongly reactive sera by whole-cell ELISA.

Results

Immunoblots

Children's sera. Examples of immunoblot patterns seen amongst the 29 sera examined are shown in fig. 1. Bands in the 68–70-, 60–65- and 15–20-Kda regions were often demonstrated in all three *Neisseria* spp. Bands in the 55-Kda and 35-Kda regions, specific to *N. meningitidis*, and one at 43 Kda specific to *N. lactamica*, were also frequently seen.

The patterns of reactivity of antibodies in sera from carriers and non-carriers to antigens of three species of *Neisseria* are shown in fig. 2A and B. Generally, there was no significant difference between these two groups in the prevalence of antibodies to common antigens. The greatest difference was seen in the prevalence of antibody to the *N. meningitidis* B,15P1.16 35-Kda antigen which was more frequently present in sera from carriers than from non-carriers ($p=0.1$).

Fig. 2C and D shows a comparison of the reactivity of antibodies in bactericidal and non-bactericidal sera to antigens of three species of *Neisseria*. Antibodies to all antigens were more prevalent in bactericidal than in non-bactericidal sera. The greatest differences were seen in the greater prevalence of antibody to the 62–65-, 55- and 15–20-Kda antigens in bactericidal than in non-bactericidal sera ($p=0.1$ in each case).

The pattern of reactivity of antibodies to different neisserial antigens in relation to ELISA results for *N. meningitidis* B,15P1.16 is given in fig. 3. Most sera showed some ELISA activity at the 1 in 200 dilution and only four sera were weakly reactive (absorbance <0.1). Anti-neisserial antibody could still be demonstrated in two of these sera by immunoblotting. There were no significant differences in the prevalences of antibodies between moderately (absorbance >0.1 <0.25) and strongly (absorbance >0.25) reactive sera. The greatest differences in prevalence of antibody between these reactive sera were seen in that to the 55-Kda antigen ($p=0.06$) and the 46-Kda antigen ($p=0.17$). The latter antigen also appeared to be specific to *N. meningitidis* B,15P1.16 although it was detected less frequently than the 55-Kda antigen. The small number of sera which were weakly reactive were not included in the statistical analysis.

Adult convalescent serum. Common bands were demonstrated in the region of 68–70 Kda, 60–65 Kda and 15–20 Kda (fig. 4). The reactivity of these bands uniformly decreased following absorption of the serum with either *N. meningitidis* B,15P1.16, *N. lactamica* or *N. polysaccharaea*.

Fig. 1. Immunoblots of children's sera (1 in 200). Lane 1, *N. meningitidis* B,15P1.16, lane 2, *N. lactamica*; lane 3, *N. polysaccharea* antigens. (A) serum with a bactericidal activity of 1 in 8 and a moderate ELISA titre from a non-carrier. (B) serum with a bactericidal activity of <1 in 2 and a strong ELISA titre from a non-carrier.

Adult meningococcal carrier sera. These five sera (dilution range 1 in 250–1 in 500), and also the adult convalescent serum were blotted against 18 *N. meningitidis* (12 groupable and six ungroupable), 10 *N. lactamica*, six *N. polysaccharea* and two *N. gonorrhoeae* strains. The table shows the number of strains in which neisserial common and apparently species-specific antigens were detected. The 65- and 20-Kda antigens were found in all but one of the strains studied. The 70-Kda antigen was also very common. The 55- and 35-Kda antigens, which appeared to be specific to *N. meningitidis*, were found in approximately 55–75% of strains.

Whole-cell ELISA

The ratio of γ -chain activity against *N. meningitidis* B,15P1.16 and *N. lactamica* was 1:1. The ratio of γ -chain: μ -chain: α -chain activity against *N.*

meningitidis B,15P1.16 was 5:2:5:1. In two sera from carriers of *N. polysaccharea* and *N. meningitidis* (ungroupable, nontypable), a relatively high level of reactivity with α chain, which was in excess of that with the μ chain, was detected. Interestingly, when these sera were blotted with an α -chain conjugate, this activity was directed against the 52–55- and 33–36-Kda antigens specific to *N. meningitidis* B,15P1.16. Only four of the sera were weakly reactive by this method, two of them failing to exhibit any anti-neisserial antibody on blotting at the dilution used. No correlation was found between ELISA activity and bactericidal antibody titre.

Discussion

A close relationship has been shown to exist between *N. gonorrhoeae*, *N. meningitidis*, *N. lactamica* and *N. polysaccharea* by DNA hybridisation

Fig. 2. Frequency of occurrence of antibody to different *Neisseria* antigens in sera: (A) from non-carrier children (19); (B) from carrier children (10); (C) non-bactericidal for *N. meningitidis* B,15P1.16 (12); (D) with bactericidal activity against *N. meningitidis* B,15P1.16 (17). ■ *N. meningitidis* B,15P1.16; ▨ *N. lactamica*; ▩ *N. polysacchara*.

Fig. 3. Frequency of occurrence of antibody to different *Neisseria* antigens in children's sera in relation to ELISA activity of the sera: (A) strong activity (≥ 0.25) (10 sera); (B) moderate activity ($\geq 0.1 < 0.25$) (15 sera); (C) weak activity (< 0.1) (4 sera). ■ *N. meningitidis* B,15P1.16; ▨ *N. lactamica*; □ *N. polysaccharaea*.

studies and whole-cell SDS-PAGE profiles (Guibourdenche *et al.*, 1986; Cann and Rogers, 1989). This study has identified three antigens to which cross-reactive antibody is directed. The absorption studies with different *Neisseria* spp. demonstrate that these distinct sites are common to each species rather than being different epitopes on the same protein. The table shows that these common antigens were present in the majority of the strains examined, confirming the findings of Aoun *et al.* (1988a) with regard to the 70-Kda band but expanding the role of the 65-Kda band which Aoun *et al.* (1988a) found only infrequently. We found

antibody to the 65-Kda antigen to be slightly more prevalent than that to the 70-Kda antigen in both children's and adults' sera. The 15-20-Kda antigen gave a hazy appearance on the nitro-cellulose membrane. It is probably pilus protein. Lipopolysaccharide (LPS) was visualised as the typical "smeared" appearance distal to this band. All meningococcal LPS, except that of group A strains, has been shown to consist of the same tetrasaccharide backbone bound to 2-keto-3-deoxyoctonate (KDO) (Jennings *et al.*, 1973), making immunological cross-reactivity possible at this site. The structure of LPS of commensal *Neisseria* spp. has

Table. Frequency of detection of common and species-specific antigens of different *Neisseria* spp. with six adult meningococcal carrier sera

Antigen (Kda)	Number of strains in which antigen was detected				
	<i>N. meningitidis</i>		<i>N. lactamica</i> (10)	<i>N. polysaccharaea</i> (6)	<i>N. gonorrhoeae</i> (2)
	groupable (12)	ungroupable (6)			
70	8	5	8	6	2
65	12	6	10	6	2
20	12	5	10	6	2
55	8	5	0	0	0
43	0	0	4	0	0
35	7	3	0	0	0

Fig. 4. Immunoblot of an adult convalescent serum (1 in 250) against different *Neisseria* spp. (A) lanes 1-3 *N. polysaccharea*; lanes 4-7 *N. meningitidis* (ungroupable, nontypable). (B) lanes 1-3 *N. meningitidis* B, nontypable; lanes 4-7 *N. lactamica*.

not been investigated. The H8 antigen described by Cannon *et al.* (1984) should have appeared in the 30-Kda region under the experimental conditions used in this study. Various bands were seen between 20 and 30 Kda but were relatively infrequent at the dilution used (1 in 200).

The initial study of the human antibody response to the 70-Kda antigen (Aoun *et al.*, 1988a) showed that 76% of sera from carriers of *Neisseria* spp. had antibody to it. We found that 50–60% of carriers had antibody to it and that non-carriers had this antibody as frequently. Our blots were performed with sera more dilute than those used by Aoun and her colleagues (1 in 200 *vs* 1 in 50). This may have contributed to the slightly lower frequency of detection of antibody. The similarity in antibody prevalence between carriers and non-carriers applied to all major antigens detected. The presence of antibody in non-carriers is probably the outcome of previous or undetected carriage of meningococci or commensal *Neisseria* spp. Carriage may boost production of antibody to common epitopes and induce strain or species specific antibody production. Indeed, the carriers in this study had detectable antibody to the *N. meningitidis* 35-Kda antigen more frequently than did non-carriers, but there was no significant difference in the frequency of antibody to the 55-Kda antigen in these two groups.

The strain-specific bactericidal activity assay is a relatively crude test of biological activity whereas immunoblotting is a non-functional antibody assay. Our findings indicate that antibody to the same antigens is present in both bactericidal and non-bactericidal sera. This suggests that the lack of bactericidal activity does not necessarily imply susceptibility to infection, or that the protective antigen site does not blot. Conversely, the correlation of bactericidal activity with protective efficacy has been questioned by Saukkonen *et al.* (1988). The differences in prevalence of antibody between bactericidal and non-bactericidal sera were more marked than those between carriers and non-carriers. Half of the bactericidal sera tested were from non-carriers. Again, the bactericidal activity probably arises from previous or undetected carriage. Three sera tested from carriers of commensal *Neisseria* spp. were bactericidal.

Different antigens may be exposed in the whole-cell ELISA and immunoblot due to methods of

antigen preparation. Moreover, immunoblotting is only a semi-quantitative technique, as bands are either present or absent at the serum dilution used. Therefore, the equal prevalence of antibody to common epitopes between sera moderately and strongly reactive by ELISA may reflect the greater ability of ELISA to detect difference in antibody titre. The more frequent prevalence of antibody to the *N. meningitidis*-specific 55-Kda antigen may have contributed to the strong ELISA reaction of some sera.

Sera tested by the whole-cell ELISA method were equally reactive against *N. meningitidis* B,15P1.16 and *N. lactamica*. Antibodies to the cross-reactive antigens were visualised by blotting. The presence of these common epitopes means that the whole-cell ELISA method is not specific to one antigen and also explains the lack of correlation of the ELISA with bactericidal activity. However, the whole cell ELISA is a sensitive measure of overall anti-neisserial antibody even though it is not species or serotype specific. These common antigens are components of the outer membrane and would, therefore, contribute to any serum reactivity where outer-membrane complexes are used as antigens for an ELISA (Rosenqvist *et al.*, 1983, 1988). Kristiansen *et al.* (1988) demonstrated a low level of anti-meningococcal antibody in children under 12 years of age, using a whole cell ELISA method. We also have demonstrated that antibody is present in children 8–11 years, by ELISA and immunoblotting.

This study confirms that the human, humoral immune response to *N. meningitidis* is to several antigens, rather than to a single one. Immunity may depend upon the presence of a variety of specific antibodies at a critical concentration; the relative levels of these different antibodies may also influence the clinical spectrum of meningococcal disease that occurs. Incorporation of one or more of these common neisserial antigens into a vaccine may reduce the mortality of meningococcal disease and possibly the incidence also.

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