Immunogenicity and cross-reactivity of the 70-Kda iron-regulated protein of *Neisseria meningitidis* in man and animals

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Summary. The immune response to different serogroups and serotypes of *N. meningitidis* has been examined in acute and convalescent sera from patients with meningococcal diseases. The focus of the study was the *c.* 70-Kda iron-regulated outer-membrane protein (FeRP-70). FeRP-70 was demonstrated on all strains of different serogroups and serotypes examined by sodium dodecylsulphate-polyacrylamide gel electrophoresis or Western blots of outer-membrane proteins (OMPs). Immunoblotting experiments demonstrated the presence of considerable amounts of anti-FeRP-70 IgG antibodies in the acute and convalescent sera of six patients; the antibodies reacted with homologous and heterologous strains. However, sera from two patients who died of severe meningococcal septicaemia had no antibodies against FeRP-70 or any other OMPs demonstrable by immunoblotting. Absorbed rabbit hyperimmune sera reacted with FeRP-70 of their homologous strains, but, unlike human sera, with only a few of the heterologous strains. We believe that FeRP-70 is strongly immunogenic *in vivo*, cross-reactive amongst different strains, and that man and animals differ considerably in their response to similar meningococcal antigens. The functional attribution of human antibody response against this protein requires further exploration.

Introduction

Despite extensive studies, the mechanisms responsible for the development of natural immunity to *Neisseria meningitidis* infection remain unclear. At least 13 serogroups (A, B, C, D, X, Y, Z, 29E, H, I, K, L and W135) of *N. meningitidis* have been described and polysaccharide vaccines offer good protection against infection with groups A, C, Y and W135. However, this protection is group-specific and is not effective in children under 2 years old, the age group most susceptible to the disease.¹ Furthermore, the capsular polysaccharide of serogroup B meningococci, responsible for the majority of cases in the recent outbreak in Europe, is poorly immunogenic.² Since second episodes of invasive meningococcal disease are rare in the absence of immune deficiency, it is possible that antibodies against non-capsular antigens could offer good protection against both homologous and heterologous serogroups.

It has been reported that meningococci are able

to use even partially saturated transferrin (TF) and lactoferrin (LF) as sole sources of iron for growth *in vitro*.^{3,4} Schryvers and Morris^{5,6} recently reported the identification of two iron-regulated proteins (FeRP) on the cell surface of *N. meningitidis*, that are highly specific for human TF and LF.

Black *et al.*⁷ demonstrated the occurrence in convalescent sera of patients of IgG and IgM antibodies against many FeRPs of homologous and heterologous strains, including the proposed transferrin-binding protein described by Schryvers and Morris,⁵ but they were unable to find them in acute sera.

In this study we have examined the immune response in acute and convalescent sera from patients of different ages, who were infected with meningococci of different serogroups and serotypes, and who presented with different clinical features. Concurrently, the immune response in rabbits to live meningococci was examined and its correlation with the human response is discussed. Our attention was focused principally on a *c.* 70-Kda iron-regulated major outer-membrane protein.

Materials and methods

Bacterial strains and growth conditions

The *N. meningitidis* strains used in this study were clinical isolates from the blood, CSF, nasopharynx or joints of patients who presented with different clinical features. The serogroups and serotypes of the isolates, their isolation site and the age and clinical features of the patients are shown in the table. Meningococci were subcultured on Mueller Hinton Agar (MHA) or in Mueller Hinton Broth (MHB) (Oxoid), and grown for 24 h in CO₂ 5% in air. Iron limitation was achieved by the addition of 10, 25, 50 or 100 µM desferrioxamine (Desferal; Ciba) to MHA and MHB after autoclaving—MHA-D and MHB-D respectively; also, 5 µM desferrioxamine was used with MHB.

Outer-membrane protein (OMP) preparation

Meningococci were harvested from MHA and MHA-D after incubation for 24 h. Crude OMPs were prepared by the Lithium acetate chloride (LAC) extraction method.⁸ Sarkosyl-extracted OMPs were prepared according to the method described by Filip *et al.*⁹ Protein contents were estimated with the BioRad protein estimation reagent, based on the method of Bradford,¹⁰ and preparations were diluted to a protein concentration of 1 mg/ml.

Sera

Sera were available from eight patients (Nos. 3, 4, 5, 8, 10, 11, 12 and 17) from whom isolates were also obtained. Acute sera were taken within 24 h of the onset of symptoms. Convalescent sera were obtained from 4 weeks after treatment.

Rabbit hyperimmune sera (RHIS) were raised against strain SD, EB and AS (serogroups B, 29E and A respectively). New Zealand White rabbits were primed with weekly intravenous injections of 10⁸ live organisms, grown under iron-depleted conditions (MHA-D), suspended in normal saline.

Partially purified human and rabbit antibodies against iron regulated proteins were prepared from the patient's sera and RHIS by suspending 10⁸ homologous bacteria that had been grown on Columbia blood agar, in 5 ml of diluted (1 in 50) and decomplexed sera, and incubating the mixture overnight at room temperature. Antigen-antibody complexes were separated by centrifugation at 1200 *g* for 20 min at 4°C and filtering (0.2-µm membrane filters; Millipore). The aim was to exclude antibodies other than those recognising FeRPs that would be expressed predominantly under iron-limited conditions.

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was performed in linear acrylamide 10%

Table. Serogroup, serotypes and site of isolation of *N. meningitidis* strains, and the clinical syndrome and age of the patients

Patient no.	Strain	Group/type	Site of isolation	Clinical syndrome	Age (years)
1	AR	A4 P1.9	CSF	Meningitis	50
2	AS	A4 P1.7	CSF	Meningitis	12
3	GN	C NT	Blood	Acute meningococcaemia	15
4	OR	C2a P1.2	Blood	Acute meningococcaemia	17
5	BM	W135	Joint	Acute arthritis	60
6	EB	29E	Throat	Carrier	15
7	Bb	B 2b	Blood	Chronic meningococcaemia	25
8	SD	B15 P1.16	CSF	Meningitis	25
9	582	B15 P1.16	CSF	Meningitis	34
10	SH	B15 P1.16	Throat	Septicaemia	17
11	JB	B NT	Blood	Meningitis	50
12	Rs	B NT	CSF	Chronic/acute meningococcaemia	19
13	J3	B NT P1.15	NA	NA	NA
14	J49	B NT P1.15	NA	NA	NA
15	J51	B NT P1.15	NA	NA	NA
16	J72	B NT P1.15	NA	NA	NA
17	YA	B NT	Blood	Septicaemia	20
18	LV	C NT	Blood	Septicaemia	20
19	Rt	W135	Blood	Meningococcal pneumonia	20

NA, details not available.

gels by the method of Laemmli.¹¹ OMPs were mixed with equal volumes of sample buffer (0.0625 M Tris-HCl, pH 6.8, SDS 2%, 2-mercaptoethanol 5%, sucrose 10% and Bromophenol blue 0.002%) and the mixture was boiled for 5 min. Electrophoresis was at 30 mAmps, and the gels were stained with Coomassie Brilliant Blue in methanol: water: acetate (5:5:2) and destained with water: methanol: acetate (5:3:2). The following mol. wt (Kda) markers (BioRad) were used: Myosin, 200; *Escherichia coli* galactosidase, 116.25; Phosphorylase b, 97.4; BSA, 66.2; ovalbumin, 42.7; carbonic anhydrase, 31; trypsin inhibitor, 21.5; and lysozyme, 14.4.

Western blotting

After SDS-PAGE, separated proteins of the crude preparations of OMPs were transferred on to nitrocellulose membranes (Schleicher & Schuell, membrane filter BA85, 0.45 μ m), in 25 mM Tris, pH 8.3, 190 mM glycine with methanol 15%, in a BioRad Transblot system. Transfer was performed at 30V at room temperature, for 12 h.

Transferred proteins were immunoblotted with the homologous and heterologous absorbed acute and convalescent sera from patients diluted 1 in 200, and with absorbed RHIS diluted 1 in 1000. All antibodies were diluted in a blocking buffer (PBS'A, skimmed milk 3% and Tween-20 0.1%). Antigen-antibody complexes were

visualised by probing with peroxidase-conjugated rabbit anti-human IgG or IgA, or with goat anti-rabbit sera.

Results

Comparative growth rates (fig. 1) demonstrate the inhibition of *N. meningitidis* B15 P1.16 (SD) by addition of increasing amounts of the iron-chelating agent desferrioxamine to MHB. Significant inhibition of growth was observed when concentrations of desferrioxamine in the broth exceeded 10 μ M. At concentrations higher than 100 μ M, there was no growth. When desferrioxamine was added to MHA plates, a similar pattern of growth inhibition was seen. Also, the profiles of the OMPs of bacteria grown in broth and on agar were identical on SDS-PAGE (not shown).

FeRPs begin to be expressed at low concentration of desferrioxamine, FeRP-70 being well established at concentrations of 10 μ M. The 70-Kda protein was the most prominently exhibited FeRP in all the strains. An example of FeRPs is shown in fig. 2a for strain GN grown in the presence of 25 μ M desferrioxamine. This protein is sarkosyl-insoluble, as shown in fig. 2b, indicating that it is bound to the outer membrane.

Fig. 1. Growth of *N. meningitidis* (strain SD, B15 P1.16) at different concentrations of desferrioxamine. Mueller Hinton broth (---) with 5 (●), 10 (△), 25 (▲), 50 (□) or 100 (■) μ M desferrioxamine.

Fig. 2. Coomassie Brilliant Blue-stained SDS-PAGE of OMPs of strain GN (CNT), grown on MHA with (+) or without (-) 25 μ M desferrioxamine. (a) Crude LAC extracts; (b) Sarkosyl-treated extracts. *FeRP-70.

Immunoblotting of absorbed acute sera of patients nos. 3, 4, 5, 8, 11 and 12 against homologous isolates demonstrated the presence of considerable amounts of IgG antibodies directed predominantly against FeRP-70. Patients nos. 5 and 11 are shown as typical examples (fig. 3a and b). However, acute sera of patients nos. 10 and 17, both of whom died of meningococcal septicaemia, demonstrated no antibody reaction with any meningococcal proteins, including FeRPs, when immunoblotted against homologous isolates. Absorbed convalescent sera of patients nos. 3, 4, 5, 8, 11 and 12 also had large amounts of IgG antibodies predominantly against the FeRP-70s of homologous strains, patients nos. 5 and 11 being typical examples (fig. 4a and b).

At dilutions of 1 in 200 or 1 in 100, there were no detectable amounts of IgA against any FeRPs in non-absorbed acute and convalescent sera from patients. As so few proteins were recognised it was unnecessary to use absorbed sera to make this observation. Immunoblotting of absorbed acute and convalescent sera of patients nos. 3, 4, 5, 8, 11, and 12 against heterologous isolates demonstrated cross-reaction with FeRP-70 from all the other

Fig. 3. Absorbed acute sera of patients nos. 5 (a) and 11 (b) immunoblotted against OMPs of their homologous (BM, and JB) isolates grown with (+) or without (-) desferrioxamine; *FeRP-70.

meningococcal isolates, irrespective of the clinical features or age of the patient, or the site from which strains were isolated, and irrespective of the serotype and the serogroup of the heterologous strain. Typical examples are shown in fig. 5a and b, in which the predominant recognition of FeRP-70 is shown. There is also faint recognition under these conditions of an additional protein in strains EB and SD.

To examine the extent of the similarity between human and animal immune responses after exposure to similar stimuli, three rabbits were immunised with three different isolates (including SD for which human anti-serum was available). Absorbed rabbit hyperimmune sera were immunoblotted against homologous and heterologous meningococci that were grown under iron-limited and iron-sufficient conditions. Fig. 6 shows that the absorbed RHIS raised initially against strain SD (B15 P1.16) clearly recognised FeRP-70 bands of both the homologous strain and heterologous strains J3 (B

Fig. 4. Absorbed convalescent sera of patients nos. 5 (a) and 11 (b) immunoblotted against OMPs of their homologous (BM and JB) isolates grown with (+) or without (–) desferrioxamine; *FeRP-70.

NT P1.15) and J49 (B NT P1.15), but, unlike human serum, did not recognise FeRP-70 of strain J51 and J72 (both B NT P1.15), or seven other strains (582, B15 P1.16; Bb, B2b; Rs, B NT; JB, B NT; AR, A4 P1.9; AS, A4 P1.7; EB, 29E) examined (data not shown).

Similar absorptions were performed with RHIS which were raised against strains EB (29E) and AS (A4 P1.7). Immunoblotting showed that anti-AS RHIS recognised the FeRP-70 of strains SD (B15 P1.16) and EB (29E), as well as that of its homologous strain. However, neither absorbed anti-SD nor absorbed anti-EB RHIS recognised FeRP-70 of the AS strain, although they recognised the homologous strains.

Discussion

When grown under iron-restricted conditions, *N. meningitidis* expresses several new proteins.^{7,12}

While not all of these are strictly iron derepressible, some being induced by other nutrient limitation,¹³ the protein of c. 70 Kda appears to be a major iron-regulated protein. We have studied the expression and antigenicity of this protein in various meningococcal isolates. FeRP-70 was induced in some strains at concentrations of desferrioxamine (10 μ M) insufficient to decrease growth rates. At 25 μ M these strains expressed a c. 70-Kda protein which was not further enhanced by increasing concentrations of desferrioxamine. In keeping with other reports,⁷ we have shown that FeRP-70 is Sarkosyl-insoluble (i.e., bound to the outer membrane) and, using human sera, that FeRP-70 is expressed and highly immunogenic *in vivo*. These observations indicate that FeRP-70 could be surface-exposed. The use of absorbed sera has enabled us to differentiate between recognition of FeRP-70 and a co-migrating protein expressed under iron sufficient conditions which is also recognised by human sera.¹⁴

Results obtained with acute and convalescent sera of six patients, immunoblotted against homologous and heterologous isolates, demonstrated that FeRP-70 is conserved amongst *N. meningitidis* strains of all serogroups and serotypes; all FeRP-70s are cross-reactive and the human immune system recognises those epitopes that are conserved in all strains. This might have important implications in terms of the candidacy of this FeRP for vaccine production.

In contrast, our studies with rabbit hyperimmune sera showed incomplete cross-reaction between FeRP-70s of different strains (fig. 6). This may indicate that FeRP-70 may have some epitopes that are shared by a range of isolates and others that are strain-specific. Alternatively it could indicate that man and animals respond to the same stimulus in a completely different manner. This is exemplified by the fact that strain SD gave rise in patient no. 8 to fully cross-reactive anti-FeRP-70 antibodies but failed to do so in rabbits. Therefore, it is possible that the lack of complete cross-reactivity against other meningococcal antigens, i.e., human transferrin-binding proteins detected with animal sera,¹⁵ may not necessarily reflect the situation in man. However, rabbit antibodies raised to purified FeRP-70 are fully cross-reactive.⁸

Others⁷ have shown that FeRP-70 is immunogenic in sera from patients recovering from meningococcal disease; by contrast, we have shown, additionally, immunoreactivity in some acute sera obtained within 24 h of onset of symptoms. Cann and Rogers¹⁴ have shown that antibodies can be produced against the common antigens of *N. meningitidis*, although in variable amounts, in both

Fig. 5. Absorbed convalescent sera of patients nos. 5 (a) and 11 (b), from whom strain BM and JB were isolated respectively, immunoblotted against OMPs from the homologous and eight heterologous strains—JB (B NT), BM (W135), SD (B15 P1.16), J3 (B NT P1.15), GN (C NT), EB (29E), AS (A4 P1.7), Rt (W135) and Lv (CNT). All strains were grown in the presence of desferrioxamine.

bactericidal and non-bactericidal sera, and in carriers and non-carriers. However, the response to a neisserial common 70-Kda protein was noted in organisms grown under iron sufficient conditions and represents a protein that co-migrates with FeRP-70. Our findings of antibodies to FeRP-70 in acute sera could indicate that pre-existing antibodies, which may or may not serve a protective function, were produced either in response to a period of mucosal colonisation preceding invasion, or by rapid recruitment of previously primed cells in response to invasion. Such rapid recruitment could serve to modify disease expression. In this

context, it might be noteworthy that intense reactivity was seen in the acute serum of patient no. 5 who had septic arthritis (and negative blood cultures), whereas no reactivity was seen in the sera of patients nos. 10 and 17, both of whom died of fulminant meningococcaemia. Exposure to poorly virulent strains, such as 29E, which are only rarely associated with invasive disease, could be a potential source of such prior priming as indicated in this work by the expression of a cross-reactive FeRP-70. Alternatively, or additionally, exposure to non-pathogenic *Neisseria* spp. such as *N. polysaccharea* or *N. lactamica* could be the source of such prior

Fig. 6. Absorbed rabbit hyperimmune serum raised against strain SD (B15 P1.16) and absorbed with homologous iron replete cells, western blotted against OMPs from homologous and heterologous (all B NT P1.15) strains, grown in the presence (+) or absence (–) of desferrioxamine. Strain J51 and J72 were not recognised with the serum.

priming.¹⁴

These findings indicate that the 70-Kda iron-regulated outer-membrane protein could be a candidate for an effective vaccine and that the

function of antibodies directed against this protein should be explored further.

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Note added in proof

Since submission of this paper, we have found that immunoblotting overnight with serum, diluted 1 in 100, from a patient with meningococcal infection and the patient's homologous strain showed a good IgA response to FeRP-70. Therefore, it is possible that some of the patients reported here may have produced an IgA response which would not have been detected by the methods used.

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