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Secreted proteins of *Neisseria meningitidis* protect mice against infection

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ABSTRACT

We addressed the hypothesis that meningococcal secreted proteins (MSPs) can elicit protective immunity against meningococcal disease. Endotoxin-depleted MSP preparations were used to immunise a group of 15 six-week-old BALB/c mice (25 μg MSPs/dose mixed with Freund's complete adjuvant) on days 0, 14 and 21. Mice were challenged 2 weeks later with 10^7 colony forming units of live *Neisseria meningitidis* strain MC58 (serogroup B, ET-5). Negative and positive control groups of 15 mice each were injected with adjuvant only, or a live attenuated strain of MC58, respectively. Seven out of 15 mice (47%) from the negative control group died after 72 h of challenge, whereas none of test or positive control group died. Protection afforded by the anti-MSP immune response can be at least partly attributed to complement-mediated bacterial lysis, detectable *in vitro* using the serum of immunised mice. Murine anti-MC58 MSP sera were bactericidal against homologous and five unrelated ET-5 serogroup B strains. However, failed to kill strains from other hypervirulent clonal lineages belonging to the same or different serogroups, despite the presence of cross-reactive antibodies detectable by immunoblotting. Similar sera raised against MSPs from an isolate belonging to the ET-37 electropherotype lineage were bactericidal against all tested isolates of this lineage and, in addition, against some but not all isolates belonging to the ET-5 lineage. FACS analysis of intact bacteria treated with anti-MSPs confirmed surface-binding of antibodies.

1. Introduction

Neisseria meningitidis (the meningococcus) is an encapsulated Gram-negative diplococcus that inhabits the nasopharynx of humans. Carriage rates in the general population are usually around 10% and in overcrowded populations can reach up to 30% or more [1,2]. The complex host–pathogen relationship is usually of a commensal nature. Occasionally, however, meningococcal carriage leads to invasive disease: a phenomenon usually associated with strains belonging to a small number of hypervirulent clonal lineages [3,4]. *N. meningitidis* is the leading cause of pyogenic meningitis worldwide and is the only bacterium capable of generating outbreaks of meningitis and septicaemia. Attack rates vary between 1 and 3 per 10^5 population, depending on the endemic or epidemic prevalence of disease in a given geographical location. Despite the organism being sensitive to several front line antibiotics, a significant number of patients diagnosed with meningococcal infection die of overwhelming disease and survivors may suffer serious complications. Mortality rates vary from 2 to 3% in cases of uncomplicated meningitis to 50% or more in cases of septic shock [5]. Thus, there is a pressing need to develop new therapeutic and preventative strategies to reduce the incidence, mortality and morbidity of invasive meningococcal disease.

There are currently vaccines available against four of the five serogroups commonly associated with human disease (serogroups A, C, Y and W-135) [6]. Development of vaccines effective against serogroup B, which is responsible for the majority of meningococcal disease in many developed countries, is hampered by the fact that the serogroup B capsular polysaccharide is poorly immunogenic. Approaches to developing vaccines against this serogroup have focused on non-capsular antigens including lipooligosaccharide (LOS) and outer membrane proteins (OMP) either individually or in complex preparations such as outer membrane vesicles (OMV). Several OMV vaccines have undergone extensive clinical trials in Scandinavia and South America [7–9] but results have been largely disappointing, particularly among children under the age of four, the most vulnerable age group. The antigenically hypervariable nature of dominant antigens such as PorA may account for these failures [10]. More recently, a multivalent vaccine based on antigens discovered by 'reverse vaccinology' has been developed and has been shown to provide protection against a wide range of isolates [11].

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Table 1
Serum bactericidal activity of sera from mice immunised with MSPs from MC58 or Z4262 against a range of meningococcal strains. Results are reported as the reciprocal of the dilution of serum needed to achieve >50% bacterial killing. Assays were performed in duplicate. *Key*: N.D. = not done; > and < = titres higher or lower than stated but further serial dilutions not tested.

Strain (alternative designation)	Origin	Serogroup	Year of isolation	Clonal complex	Clinical description	Reference	Serum bactericidal activity				
							Immunising strain				
							MC58				Z4262
							R1	R2	R3	R4	Pool
MC58	UK	B	1985	ET-5/ST-32	Invasive	[28]	>2,048	>2,048	>2,048	>2,048	<8
H44/76	Norway	B	1976	ET-5/ST-32	Invasive	[29,30]	8,192	4,096	8,192	2,048	<8
Z4672 (BZ169)	Netherlands	B	1985	ET-5/ST-32	Invasive	[3]	2,048	>2,048	8,192	4,096	2,048
BZ83	Netherlands	B	1984	ET-5/ST-32	Invasive	[29–31]	16,384	16,383	16,384	16,384	2,048
C311	Unknown	B	Unknown	ET-5	Invasive	[32]	16,384	>2,048	16,384	8,192	4,096
NG6/88	Netherlands	B	1963	ST-41,44/lineage 3	Invasive	[29,30]	<32	<32	<32	<32	4,096
2996	Japan	B	1979	ST-41,44/lineage 3	Carrier	[33]	<32	<32	<32	<32	<8
SWZ107	Switzerland	B	1985	ST-35	Invasive	[29,30]	<32	<32	<32	<32	<8
NGE28	Norway	B	1988	Unknown	Carrier	[29,30]	<32	<32	<32	<32	<8
BZ232	Netherlands	B	1964	ET-76/ST-38	Invasive	[29,30]	<32	<32	<32	<32	4,096
Z4711 (1000)	USSR	B	1988	ST-18	Invasive	[29,30]	<32	<32	<32	<32	<8
Z4262	USA	B	1964	ET-37/ST-11	Unknown	[30]	N.D.	N.D.	N.D.	N.D.	4,096
NGP165	Norway	B	1974	ET-37	Unknown	[29,30]	<32	<32	<32	<32	<8
FAM18	USA	C	1980s	ET-37/ST-11	Invasive	[30]	<32	<32	<32	<32	4,096
Z4631 (90/18311)	UK	C	1990	ET-37/ST-11	Unknown	[29]	<32	<32	<32	<32	1,024
Z2491	Gambia	A	1983	IV-1/ST-4	Invasive	[34]	<32	<32	<32	<32	512
Z6432 (A22)	Norway	W-135	1986	IV-1	Carrier	[29]	<32	<32	<32	<32	<8

In this study, we address the hypothesis that MSPs contribute to protective immunity against meningococcal disease. Using a murine challenge model we demonstrate the ability of MSPs to generate cross-protective but clonal lineage-restricted immunity against meningococcal infections.

2. Materials and methods

2.1. Bacterial strains, growth conditions and secreted protein preparation

Meningococcal strains used in this study are described in Table 1. In all experiments, with the exception of MSP preparation, *N. meningitidis* was grown on Brain Heart Infusion (BHI) agar with Levanthal's supplement in 5% CO_2 at 37 °C overnight. Meningococcal secreted proteins (MSPs) were prepared as described previously [12] with some minor modifications designed to minimise cell lysis. Meningococci were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) for 12 h at 37 °C in an atmosphere of 5% CO_2, before centrifugation at 20,000 × *g* for 20 min at 4 °C. Supernatants were passed through 0.2 μm filters (Sartorius) and concentrated approximately 50-fold by centrifugation at 2000 × *g* using centrifugal concentrators with a 30-kDa molecular weight cut-off (VivaScience). Lipooligosaccharide was selectively removed by mixing the concentrated proteins with an equal volume of polymyxin B-conjugated agarose slurry (Sigma–Aldrich), incubating for 1 h at 4 °C with end-over-end agitation, followed by centrifugation to remove agarose beads. Residual LOS content was measured using an E-TOXATE ® kit (Sigma) according to the manufacturer's instructions. Protein concentration of the samples was measured using a BIO-RAD Protein Assay Kit according to the manufacturer's instructions. The protein content of MSP preparations was analysed by immunoblotting as described previously [12]. Blots were probed with a rabbit polyclonal antiserum raised against MSPs. An anti-PorA monoclonal antibody (NIBSC) was used to check for contaminating outer membrane proteins [12]. Anti-rabbit and anti-mouse IgG-peroxidase conjugates (Sigma) were applied as appropriate, before developing the blots with a chromogenic 4-chloro-1-naphthol substrate solution (Sigma).

2.2. Immunisation of mice with MSPs

For active immunisation studies, 15 female adult mice (6-week-old, Balb/C) received 25 μg of MSPs by subcutaneous (s.c.) injection on days 1, 21 and 28 [13]. Antigens in a 200-μl total volume were emulsified with Freund's complete adjuvant (1:1, v/v) prior to injection. At the same time, 15 mice received adjuvant only and another 15 mice were immunised with live attenuated strain 23A10 (1×10^6 cfu) by intraperitoneal route as positive control. On day 42, animals were challenged with 10^7 colony forming units (cfu) of live wild-type MC58 strain by the intraperitoneal (i.p.) route. Sera were collected from four individual mice on day 35 by cardiac puncture. Bacteria for the inoculum were grown overnight on solid media and re-suspended in 400 μl of PBS. The number of cfu was estimated by measuring the O.D. A_{260} of a lysate of the suspension in 1% SDS/0.1 M NaOH; results were confirmed by plating bacteria on solid media. Bacteria were re-suspended in BHI/0.5% iron dextran (Sigma, Poole, United Kingdom) prior to administration. Survival of animals was compared using a one-tailed Student's *t*-test. All animal experimental protocols were reviewed and approved by the Home Office, UK.

2.3. Immunologic assays

Serum bactericidal assays (SBA) were performed according to CDC protocol using baby rabbit sera as a complement source (Pelfreeze). Approximately 1000 cfu of each strain in a final volume of 100 μl were incubated at 37 °C for 1 h in the wells of a microtitre dish with complement and serial dilutions of serum. The number of viable bacteria before and after exposure to serum was measured by plating on solid media. Assays were performed in duplicate. Results presented are calculated as the reciprocal of the dilution of serum that gave >50% bacterial killing. Negative controls include pre-immune sera from homologous mice and sera from mice injected with adjuvant only.

Whole-cell ELISA was used to detect *Neisseria*-specific antibodies in sera as previously described [14].

For fluorescent-activated cell sorting (FACS) analysis, bacteria were harvested after overnight growth, re-suspended in PBS, and washed before fixation with paraformaldehyde (4%).