BACTERIAL PATHOGENICITY

Blocking of iron uptake by monoclonal antibodies specific for the *Neisseria meningitidis* transferrin-binding protein 2

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The existence of epitopes common to different strains in the *Neisseria meningitidis* transferrin (Tf)-binding protein 2 (TBP2), combined with the ability of polyclonal anti-TBP2 antibodies to inhibit Tf binding and block iron uptake in this species, led to this study on the effect of anti-TBP1+2 monoclonal antibodies (MAbs) to determine the presence of epitopes inside the Tf-binding region. All MAbs used reacted exclusively with the homologous strain when tested by dot-blot of outer membrane vesicles, with the reaction being specific for TBP2 after SDS-PAGE and electroblotting. In contrast, ELISA and iron-uptake blocking assays were also positive with heterologous strains belonging to Rokbi's group II (high mol.wt TBP2). The results confirmed the two group classification proposed by Rokbi and, in contrast to other studies, indicated the existence of epitopes in the Tf-binding region that are common only to strains of Rokbi's group II. These epitopes may become denatured after drying for dot-blot assays or after SDS-PAGE and electroblotting.

Introduction

Neisseria meningitidis is a gram-negative micro-organism able to acquire iron directly from human transferrin (Tf) and lactoferrin [1] as a result of possessing specific receptors for these two proteins, namely the Tf-binding proteins (TBPs) and the lactoferrin-binding proteins (LBPs), synthesised when the bacteria grow in iron-limited media. The importance of the invasive phase in meningococcal meningitis has resulted in the structure, function and immunological properties of the TBPs receiving more attention than those of the LBPs. Interest in including iron-regulated proteins in anti-meningococcal vaccines is growing, and the TBPs are the most promising candidates [2]. TBP2 is a good immunogen, being one of the major outer-membrane antigens in some strains [3–5]. This protein shows genetic, molecular and antigenic heterogeneity between strains [4, 6], allowing the classification of meningococci into two groups that are independent of serogroups, serotypes or strain origin. Group I (type strain B16B6) includes strains with a low mol. wt TBP2, while group II (type strain M982) includes strains with a higher mol. wt TBP2 [6, 7].

Despite the antigenic heterogeneity, the existence of only two TBP2 groups, much fewer than the number

found by any other grouping criteria, supports the possibility of using this protein as an antigen in vaccines. Some authors have reported the existence of antigenic domains common to the TBPs of all *N. meningitidis* strains [8, 9]. Obviously, the TBP2 epitopes with the highest probability of being maintained with minimal or no changes are those corresponding to the Tf-binding site(s). The existence of these common epitopes, and the ability of polyclonal anti-TBP1+2 and anti-TBP2 antibodies to inhibit Tf-binding [10] and block iron uptake by the meningococci [11], led to this study on the effect of anti-TBP2 monoclonal antibodies (MAbs) in an attempt to identify the common epitopes and their relevance in Tf-binding and iron-uptake.

Materials and methods

Bacterial strains and growth conditions

The meningococcal strains used in this study were representative of different serogroups, serotypes and TBP2 groups (Table 1). *N. meningitidis* strain SD was provided by Dr D.A.A. Ala'Aldeen (University of Nottingham). Strains were maintained at –80°C and were then thawed and grown on Choc-Iso Agar plates (GC Medium base plus Biovitex; 1% Biolife, Milan) w/v incubated for 24 h at 37°C in air with CO₂ 5% v/v. One freshly grown isolated colony was used to inoculate flasks containing Mueller-Hinton broth

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Table 1. Characteristics of the *N. meningitidis* strains used in the study

Strain	Serogroup	Serotype	Origin	TBP2 (MDa)	Rokbi's group
P000	B	15:p?	Oropharynx	83.8	II
P391	AA	15:p?	Oropharynx	84.9	II
SD	B	15:p1.16	CSF	78.0	II
V002	B	15:p?	CSF	71.4	I
HG7	C	2b:p1.2	CSF	63.0	I
B16B6	B	2a:p1.2	CSF	63.4	I

AA, autoagglutinable; CSF, cerebrospinal fluid.

(MH) with 39 μM ethylene-diamine-dihydroxy-phenyl acetic acid (MH-EDDA; iron restriction). The flasks were then incubated in a water bath for 14 h at 37°C with constant shaking at 100 rpm.

For iron-uptake blocking experiments, a modified Morse and Bartenstein defined medium deferrated by treatment with Chelex-100 ion-exchange resin (Bio-Rad) was used (mMBDM) [12]. After growth of the bacteria in MH-EDDA for 12 h, 4 ml of the resulting culture were inoculated into 250-ml Erlenmeyer flasks containing 100 ml of mMBDM. The flasks were incubated for 6 h in a water bath at 37°C with constant shaking at 100 rpm to exhaust the intracellular iron pool. The bacteria were recovered by centrifugation at 10 000 *g* for 10 min and resuspended for the assays in fresh mMBDM at a concentration of 10^{10} cells/ml. Viability was estimated to be >95% by comparison of direct microscopy and colony counting.

Outer-membrane protein (OMP) preparations

Excreted outer-membrane vesicles ('blebs') and fragmented bacterial outer membranes were obtained from the cultures by LiCl extraction [13]. Briefly, the cultures were centrifuged at 48 000 *g* for 1 h and the pellets were resuspended in 0.1 M acetate buffer (pH 5.8) with 0.2 M LiCl, incubated at 45°C for 2 h in a water-bath with shaking, passed through a 21-gauge needle, and centrifuged at 10 000 *g* for 15 min. The supernates, containing 'blebs' and membrane fragments, were centrifuged at 200 000 *g* for 10 min. The pellets were then resuspended in H₂O and stored in small volumes at -20°C.

Immune serum and MAbs

A rabbit polyclonal serum against the biologically active TBP1+2 complex of *N. meningitidis* strain SD

was obtained from Dr D.A.A Ala'Aldeen. This serum was raised by inoculating a New Zealand White male rabbit with five doses of the streptavidin-agarose-TBP1+2 complex by subcutaneous injection [14]. All MAbs were specific for the TBPs from *N. meningitidis* strain SD and were produced and characterised as described previously [15, 16]. The characteristics of the MAbs are given in Table 2. A mouse non-immune serum was used as a negative control.

Preparation of [⁵⁵Fe](Fe₂)-Tf

[⁵⁵Fe](Fe₂)-Tf was prepared as described previously [17]. For each mg of apo-Tf (Sigma) dissolved in buffer (Tris 40 mM, pH 7.4; 2 mM sodium bicarbonate), 0.0075 μmol of [⁵⁵Fe]FeCl₃ (1.5 $\mu\text{Ci}/\mu\text{g}$ Fe; Amersham) and 0.075 μmol of sodium citrate were added. This mixture was incubated at room temperature for 30 min, dialysed against the same buffer for 16 h, then against mMBDM for another 6 h, and finally sterilised by filtration through 0.45- μm nitrocellulose filters. Iron saturation of Tf was determined to be 25% after counting the radioactivity associated with Tf and determining the precise Tf concentration.

SDS-PAGE and immunoblotting

OMPs were analysed by SDS-PAGE on acrylamide 10% w/v gels with the discontinuous buffer system of Laemmli [18]. Samples containing 20 μg of protein were either boiled at 100°C for 15 min, heated at 37°C for 20 min, or maintained overnight at room temperature in sample buffer before electrophoresis, depending on the strain [4]. After electrophoresis, the proteins were transferred from the gels to nitrocellulose membranes with a Milliblot-SDE electroblotting system (Millipore) and a low ionic strength discontinuous buffer system comprising two

Table 2. Characteristics of the polyclonal serum and MAbs against the TBP1+2 complexes obtained from *N. meningitidis* strain SD

Serum	Immunoglobulin type	ELISA titres			Bactericidal titre
		TBP1+2	TBP1	TBP2	
Rabbit	Polyclonal	3.08×10^5	...	...	10
MAb8	IgG2a	1.46×10^5	<6	1.26×10^5	<10
MAb11	IgG2a	1.11×10^5	<6	2.70×10^4	<10
MAb103	IgG2a	1.84×10^5	<6	3.18×10^4	<10
MAb109	IgM	2.30×10^3	47	30	<10
MAb110	IgM	1.24×10^4	206	8.03×10^3	<10
MAb112	IgG1	8.30×10^4	39	8.73×10^4	<10

anode buffers (0.3 M Tris plus methanol 10% v/v, pH 10.4, and 25 mM Tris plus methanol 10% v/v, pH 10.4) and one cathode buffer (25 mM Tris, 40 mM 6-amino-hexanoic acid, methanol 20% v/v, pH 9.4), according to the instructions provided by the manufacturer. Electrophoresis was performed for 30 min at 2.5 mA/cm² of gel area.

Transferred proteins were immunoblotted with the rabbit anti-TBP1+2 serum or the MABs. Both serum and antibodies were diluted in TBS-Tween blocking buffer (Tris-buffered saline containing skimmed milk 0.5% w/v, anti-foam A 0.001% v/v and Tween 20 500 g/L) and incubated with the membranes at 37°C for 1.5 h. Bound antibodies were visualised with peroxidase-conjugated goat anti-rabbit or anti-mouse immunoglobulins (Dakopatts, Glostrup, Denmark) and developed with chloronaphthol [19].

Dot-blot assays

Samples (2 µl containing 1 µg total protein) of outer-membrane suspensions were spotted on to nitrocellulose disks placed inside the wells of polystyrene microtitre plates. The disks were then incubated for 1 h with 100 µl of the polyclonal serum or the MABs, diluted in blocking solution and developed as described above for immunoblots.

Liquid-phase whole cell ELISA

This assay was performed as described previously [20] with MultiScreen-HV Filtration Plates and 0.45-µm Durapore membranes (Millipore). Briefly, exponential phase cultures in MH-EDDA were harvested by centrifugation at 10 000 *g* for 10 min. The pellet was washed twice and resuspended to 10⁸ cells/ml in TBS-Tween. Portions (100 µl) of the suspension were added to the same volume of MAb dilutions in bovine serum albumin 1% w/v in TBS-Tween (TBS-Tween-BSA) in the wells of a filtration plate, blocked previously with TBS-Tween-BSA, and the mixtures were incubated overnight at 37°C. The suspensions were then filtered and the bacterial cells were washed once with TBS-Tween before incubation for 5 h at 37°C with 100 µl of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulins diluted in TBS-Tween-BSA. Finally, the cells were washed twice with 200 µl of TBS-Tween and assayed by addition of 100 µl of 5-aminosalicylic acid (1 g/L in 10 mM sodium phosphate, pH 6.0, 100 mM EDTA) for 1 h at room temperature. The reaction was stopped by the addition of 0.3 M NaOH and the absorbance values of the filtrates were read at 450 nm in a microtitre plate reader.

Bactericidal assays

These were performed essentially as described for gonococci by Veale *et al.* [21]. Normal human serum (NHS) was pooled from three donors who were

shown to have low levels of antibodies to meningococcal outer-membrane vesicles. Strain SD was grown in Mueller-Hinton broth containing EDDA 5 µg/ml, washed and resuspended in gonococcal defined medium to *c.* 4 × 10⁴ cells/ml. Serial dilutions of MABs were prepared in a microtitre plate and bacteria were added to a final concentration of 10⁴ cells/ml and NHS to 20% v/v. The percentage of bacteria surviving was compared with a control containing heat-inactivated NHS after incubation for 1 h at 37°C.

Iron uptake blocking assay

The ability of the rabbit immune serum (anti-TBP1+TBP2) and the MABs to block iron uptake from Tf was determined as described previously [11]. Briefly, 100-µl volumes of a 1 in 2 dilution of the inactivated (56°C for 30 min) serum or a 1 in 5 dilution of the MABs in deferrated mMBDM were incubated with 100 µl of bacterial suspension (10⁹ cells) in 1.5-ml polypropylene Eppendorf tubes for 1.5 h at 37°C, after which 100 µl of a ⁵⁵Fe-Tf solution were added and the mixtures were incubated at room temperature for 1 h. Iron uptake was stopped by recovery of the bacteria by centrifugation in a microfuge and washing twice with cold mMBDM. The bacterial pellet was suspended in 200 µl of H₂O, added to 4 ml of scintillation cocktail (Ready-proteinTM; Beckman), and the associated radioactivity was determined by liquid scintillation counting. Controls for non-specific blocking contained normal (non-immune) mouse serum. All experiments were performed with iron-free materials and apparatus.

Statistical analysis

Estimation of the significance of differences was with Student's *t* test [22].

Results

Antigenic heterogeneity and immunogenic epitopes of the TBPs

The anti-TBP1+2 serum and MABs both showed high titres in dot-blot and Western blot assays, with working titres of 1 in 1000 although lower serum dilutions were required for the evaluation of iron-uptake blocking.

Fig. 1 shows the results obtained in Western blot experiments with the polyclonal anti-TBP1+2 (strain SD) serum. A strong cross-reaction was seen with the three strains belonging to Rokbi's group II (including strain SD), but no reaction was seen with the three strains from Rokbi's group I. All MABs reacted exclusively with the homologous strain SD (results not shown). Table 3 summarises the results of dot-blot experiments, confirming the Western blot results.

Fig. 1. Antigenic profiles obtained for *N. meningitidis* strains in Western blot experiments with the polyclonal anti-TBP1+2 rabbit serum derived from strain SD. Strains: a, P000; b, P391; c, SD; d, HG7; e, V002; and f, B16B6.

Liquid-phase whole cell ELISA

Table 4 shows the reactivities of MAb 103 and 110 in a liquid phase whole cell ELISA. MAb103 reacted with the three strains belonging to Rokbi's group II (i.e., strains P000, P391 and SD). A similar, but weaker reaction was obtained with MAb110. Neither MAb reacted with the two Rokbi's group I strains tested.

Inhibition of iron uptake

The immune serum and MAbs were titrated in preliminary experiments against the homologous strain, resulting in working titres of 1 in 2 for the polyclonal anti-TBP1+2 serum and 1 in 5 for the MAbs (some of

Table 4. Liquid-phase whole cell ELISA cross-reactivity of MAb103 and 110 with strains of *N. meningitidis*

Strain	Rokbi's group	Reactivity with	
		MAb103	MAb110
P000	II	+	±
P391	II	+	±
SD	II	++	++
V002	I	—	—
HG7	I	—	—

++, strong; +, good; ±, weak; —, negative.

the MAbs had higher titres of up to 1 in 20, but 1 in 5 was chosen to maintain consistent MAb dilutions). Bacterial suspensions for the experiments were completely iron-exhausted, as demonstrated by their inability to continue growing after passage through the culture plates with Desferal.

Table 5 shows the results obtained in the iron-uptake inhibition experiments. A non-immune mouse serum was used as the 100% uptake (0% blockage) control. The results of statistical tests indicated that only an uptake inhibition of >60% was statistically significant ($p < 0.05$). The polyclonal anti-TBP1+2 rabbit serum blocked iron uptake by the homologous strain only, whereas some MAbs blocked iron uptake, with variable efficacy, by heterologous strains belonging to Rokbi's group II, with MAbs103, 110 and 112 being the most effective.

Discussion

The latest anti-meningococcal outer-membrane based vaccines [23–27] demonstrated the necessity to direct the immune response against epitopes able to specifically induce protective bactericidal antibodies, so avoiding the production of many non-functional antibodies occurring in polyclonal sera. The meningococcal iron-regulated OMPs that form the Tf-receptor complex are highly immunogenic [2–4, 28] and are necessary for Tf-binding and iron internalisation [29–32]. Recent studies have shown the protective and bactericidal effects of TBP2-specific antibodies [2], as well as their ability to inhibit binding of Tf to the receptor complex and iron uptake by the bacteria, thereby stopping bacterial growth [10, 11, 33]. The

Table 3. Dot-blot analysis of the cross-reactivity of the rabbit polyclonal serum and MAbs with strains of *N. meningitidis*

Strain	Rokbi's group	Reactivity of serum and MAbs			
		Polyclonal	MAb103	MAb110	MAb112
P000	II	+	—	—	—
P391	II	+	—	—	—
SD	II	+	+	+	+
V002	I	—	—	—	—
HG7	I	—	—	—	—
B16B6	I	—	—	—	—

Table 5. Iron-uptake blocking produced by the polyclonal anti-TBP1+2 serum and MAbs with strains of *N. meningitidis*

Strain	Rokbi's group	Percentage inhibition of iron uptake by						
		TBP1+2	MAb8	MAb11	MAb103	MAb109	MAb110	MAb112
P000	II	17	18	22	58*	33	34	70*
P391	II	0	66*	66*	57*	72*	61*	61*
SD	II	70*	16	7	70*	0	69*	83*
V002	I	0	0	0	0	0	0	0
HG7	I	0	0	0	15	0	11	15
B16B6	I	0	0	0	0	0	2	33

*Statistically significant ($p < 0.05$).

TBP2 protein is, therefore, considered to be a good candidate for inclusion in anti-meningococcal vaccines.

Despite the existence of two meningococcal TBP2 classes, defined by their molecular mass, antigenic properties and genetic sequence [6], all strains show 47% TBP2 homology [30]. A highly structured and extremely stable TBP2 domain of *c.* 270–290 amino acids is conserved between very divergent polypeptides. This is presumably involved in Tf-binding, suggesting that the Tf-binding epitope could be common to all *N. meningitidis* strains [34]. The main problem presented by many OMPs is that the immune-dominant epitopes exposed to the surface are strain-specific, e.g., the 70-kDa meningococcal protein [35, 36]. The present study analysed the conservation of the TBP2 epitopes for Tf-binding, and their surface accessibility, with MAbs and four different techniques.

Western-blot and dot-blot results showed that all the MAbs tested reacted only with the original homologous strain, whereas the rabbit polyclonal anti-TBP1+2 serum reacted with the TBP2 of all strains from the same Rokbi group as the immunising strain (group II), suggesting that the MAbs could be reacting with strain-specific epitopes. Alternatively, they could be recognising conformational epitopes made non-reactive by electrodenaturation in the Western blot or by drying in the dot-blot techniques. Electrophoretic denaturation of the TBP2 from some strains has been reported [4, 34], and it seems from our study that drying can also affect the TBP2 immune reactivity, as also observed previously [37]. The observed reaction with the homologous strain could be explained by the higher affinity of the MAbs for their corresponding epitope and possibly by a higher molar amount of those specific epitopes.

All denaturation problems were overcome with a liquid-phase ELISA and whole cell suspensions [20], thereby avoiding denaturation of the TBP2 protein and mimicking the in-vivo conditions more realistically. In this assay, all MAbs reacted with both the homologous strain and the heterologous strains belonging to Rokbi's group II. The possible existence of conformational epitopes denatured by electrotransference or drying was also supported by the observation that

MAb109 bound exclusively to TBP1+2 complexes, a fact explicable only on the basis of an interaction with conformational or shared epitopes. Thus it is important to emphasise that solid-phase assays (dot-blot and Western blots) do not provide conclusive results in cross-reactivity studies. These results further support the two-group classification of meningococcal strains proposed by Rokbi and supported by other investigations [3, 4, 6, 38].

It has been reported that rabbit polyclonal anti-TBP1+2 and anti-TBP2 sera inhibited Tf-binding and bacterial growth in the homologous strain [10]. The present study indicated that the rabbit anti-TBP1+2 polyclonal serum blocked iron uptake only in the homologous strain, but previous studies have demonstrated that anti-TBP1+2 and anti-TBP2 mouse polyclonal sera inhibited Tf-binding, blocked iron uptake and impeded bacterial growth in all strains belonging to the same Rokbi group [11, 12]. The liquid-phase ELISA assay used in the present study demonstrated that, of the six MAbs tested, three (MAbs 103, 110 and 112) were able to block iron uptake by all strains from Rokbi's group II, but by none of the strains from Rokbi's group I. This agreed with the results of the MAb binding assays, thereby indicating, in contrast to previous results [34], that the Tf-binding site is not conserved in all *N. meningitidis* strains. Furthermore, cross-blocking of iron uptake by the MAbs, in contrast to the rabbit polyclonal serum, confirmed the diversity in the immune reaction elicited in different animal models and the necessity to choose appropriate models for the study of the different antigens [28, 39].

The polyclonal rabbit anti-TBP1+2 serum showed a bactericidal titre that was 10-fold lower than that of sera raised against whole outer-membrane vesicles, and none of the MAbs showed appreciable bactericidal titres. This contrasts with the fact that some of the MAbs are IgM or IgG2a, well known complement-activating immunoglobulins. Eliciting bactericidal antibodies is an important requirement for antigens which are intended to be included in vaccines [35]. More detailed analysis is required to resolve the conflicts mentioned above and to analyse the specific epitopes to which the MAbs bind.

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References

- Mickelsen PA, Sparling PF. Ability of *Neisseria gonorrhoeae*, *Neisseria meningitidis*, and commensal *Neisseria* species to obtain iron from transferrin and iron compounds. *Infect Immun* 1981; **33**: 555–564.
- Danve B, Lissolo L, Mignon M *et al.* Transferrin-binding proteins isolated from *Neisseria meningitidis* elicit protective and bactericidal antibodies in laboratory animals. *Vaccine* 1993; **11**: 1214–1220.
- Griffiths E, Stevenson P, Ray A. Antigenic and molecular heterogeneity of the transferrin-binding protein of *Neisseria meningitidis*. *FEMS Microbiol Lett* 1990; **69**: 31–36.
- Ferrón L, Ferreirós CM, Criado MT, Pintor M. Immunogenicity and antigenic heterogeneity of a human transferrin-binding protein in *Neisseria meningitidis*. *Infect Immun* 1992; **60**: 2887–2892.
- Ferreirós CM, Ferrón L, Criado MT. *In vivo* human immune response to transferrin-binding protein 2 and other iron-regulated proteins of *Neisseria meningitidis*. *FEMS Immunol Med Microbiol* 1994; **8**: 63–68.
- Rokbi B, Mazarin V, Towner KJ, Williams P. Evidence for in vivo expression of transferrin-binding proteins in *Haemophilus influenzae* type b. *Infect Immun* 1992; **60**: 2986–2991.
- Lissolo L, Maitre-Wilmotte G, Dumas P, Mignon M, Danve B, Quentin-Millet M-J. Evaluation of transferrin-binding protein 2 within the transferrin-binding protein complex as a potential antigen for future meningococcal vaccines. *Infect Immun* 1995; **63**: 884–890.
- Pintor M, Ferrón L, Gómez JA *et al.* Blocking of iron uptake from transferrin by antibodies against the transferrin binding proteins in *Neisseria meningitidis*. *Microb Pathog* (in press).
- Pintor M, Ferreirós CM, Criado MT. Energy-independent binding of iron complexed to small organic chelants by *Neisseria meningitidis*. *J Gen Appl Microbiol* 1994; **40**: 23–34.
- Ferreirós CM, Criado MT, Del Rio MT, Pintor M. Analysis of the expression of outer-membrane proteins in *Neisseria meningitidis* in iron-replete and iron-deficient media. *FEMS Microbiol Lett* 1990; **71**: 49–54.
- Ala'Aldeen DAA, Powell NBL, Wall RA, Borriello SP. Localization of the meningococcal receptors for human transferrin. *Infect Immun* 1993; **61**: 751–759.
- Smith AW, Wilton J, Clark SA, Alpar O, Melling J, Brown MRW. Production and characterization of monoclonal antibodies to outer membrane proteins of *Pseudomonas aeruginosa* grown in iron-depleted media. *J Gen Microbiol* 1991; **137**: 227–236.
- Gorringe AR, Irons LI, Aisen P, Zak O, Robinson A. Purification of *Neisseria meningitidis* transferrin binding proteins and characterization by epitope mapping and iron release studies. In: Evans JS, Yost SE, Maiden MCJ, Feavers IM (eds) The 9th International Pathogenic *Neisseria* Conference, Winchester. 1994: 140–142.
- Pintor M, Ferreirós CM, Criado MT. Characterization of the transferrin-iron uptake system in *Neisseria meningitidis*. *FEMS Microbiol Lett* 1993; **112**: 159–166.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of the bacteriophage T4. *Nature* 1970; **227**: 680–685.
- Wood JN. Solid-phase screening of monoclonal antibodies. In: Walker JM (ed) *Methods in molecular biology*, vol 1. Clifton, NJ, Human Press. 1984: 279–286.
- Pintor M, Ferreirós CM, Criado MT, Ferrón L. Expression levels of human transferrin receptors in *Neisseria* species. *J Microbiol Methods* 1992; **15**: 321–326.
- Veale DR, Penn CW, Smith H. Factors affecting the induction of phenotypically determined serum resistance of *Neisseria gonorrhoeae* grown in media containing serum or its diffusible components. *J Gen Microbiol* 1981; **122**: 235–245.
- Sokal RR, Rohlf FJ. Single-classification analysis of variance. In: Sokal RR, Rohlf FJ (eds) *Biometry*. New York, W.H. Freeman and Co. 1995: 207–271.
- Zollinger W, Boslego J, Moran E *et al.* Meningococcal serogroup B vaccine: protection trial and follow-up studies in Chile. *NIPHS Annals* 1991; **14**: 211–213.
- Fernández de Cossio ME, Ohlin M, Llano M *et al.* Human monoclonal antibodies against an epitope on the class 5c outer membrane protein common to many pathogenic strains of *Neisseria meningitidis*. *J Infect Dis* 1992; **166**: 1322–1328.
- Christodoulides M, McGuinness BT, Heckels JE. Immunization with synthetic peptides containing epitopes of the class 1 outer-membrane protein of *Neisseria meningitidis*: production of bactericidal antibodies on immunization with a cyclic peptide. *J Gen Microbiol* 1993; **139**: 1729–1738.
- Christodoulides M, Heckels JE. Immunization with a multiple antigen peptide containing defined B- and T-cell epitopes: production of bactericidal antibodies against group B *Neisseria meningitidis*. *Microbiology* 1994; **140**: 2951–2960.
- Delvig AA, Wedge E, Caugant DA *et al.* A linear B-cell epitope on the class 3 outer-membrane protein of *Neisseria meningitidis* recognized after vaccination with the Norwegian group B outer-membrane vesicle vaccine. *Microbiology* 1995; **141**: 1593–1600.
- Ala'Aldeen DAA, Stevenson P, Griffiths E *et al.* Immune responses in humans and animals to meningococcal transferrin-binding proteins: implications for vaccine design. *Infect Immun* 1994; **62**: 2984–2990.
- Irwin SW, Averil N, Cheng CY, Schryvers AB. Preparation and analysis of isogenic mutants in the transferrin receptor proteins genes, *tbpA* and *tbpB*, from *Neisseria meningitidis*. *Mol Microbiol* 1993; **8**: 1125–1133.
- Legrain M, Mazarin V, Irwin SW *et al.* Cloning and characterization of *Neisseria meningitidis* genes encoding the transferrin-binding proteins Tbp1 and Tbp2. *Gene* 1993; **130**: 73–80.
- Anderson JE, Sparling PF, Cornelissen CN. Gonococcal transferrin-binding protein 2 facilitates but is not essential for transferrin utilization. *J Bacteriol* 1994; **176**: 3162–3170.
- Petersson A, Maas A, Tommassen J. Identification of iroA gene product of *Neisseria meningitidis* as a lactoferrin receptor. *J Bacteriol* 1994; **176**: 1764–1766.
- Ferreirós CM, Criado MT, Ferrón L, Pintor M. Blocking of iron uptake from transferrin by antibodies against the transferrin-binding system of *Neisseria meningitidis*. The 9th International Pathogenic *Neisseria* Conference, Winchester. 1994: 166–167.
- Vonder Haar RA, Legrain M, Kolbe HVJ, Jacobs E. Characterization of a highly structured domain in Tbp2 from *Neisseria meningitidis* involved in binding to human transferrin. *J Bacteriol* 1994; **176**: 6207–6213.
- Petersson A, Kuipers B, Pelzer M *et al.* Monoclonal antibodies against the 70-kilodalton iron-regulated protein of *Neisseria meningitidis* are bactericidal and strain specific. *Infect Immun* 1990; **58**: 3036–3041.
- Ala'Aldeen DAA, Davies HA, Borriello SP. Vaccine potential of meningococcal FrpB: studies on surface exposure and functional attributes of common epitopes. *Vaccine* 1994; **12**: 535–541.
- Cornelissen CN, Sparling PF. Iron piracy: acquisition of transferrin-bound iron by bacterial pathogens. *Mol Microbiol* 1994; **14**: 843–850.
- Ferreirós CM, Criado MT, Pintor M, Ferrón L. Analysis of the molecular weight heterogeneity of the transferrin receptor in *Neisseria meningitidis* and commensal *Neisseria*. *FEMS Microbiol Lett* 1991; **83**: 247–254.
- Ferrón L, Ferreirós CM, Criado MT, Pintor M. Reliability of laboratory models in the analysis of Tbp2 and other meningococcal antigens. *FEMS Immunol Med Microbiol* 1994; **9**: 299–305.