BACTERIAL PATHOGENICITY AND CHARACTERISATION

Analysis of TbpA and TbpB functionality in defective mutants of *Neisseria meningitidis*

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Iron uptake analysis suggested that the *Neisseria meningitidis* transferrin (Tf) binding proteins, TbpA and TbpB, form only one type of receptor complex. Mutants defective in the synthesis of either TbpA or TbpB, but not defective in both proteins, can bind Tf, suggesting that both proteins are surface exposed and function in Tf binding. Also, iron uptake from Tf into the meningococci did not require the presence of both Tbps. The TbpB-defective mutant incorporated *c.* 37% of the iron taken up by the wild-type strain, but this was insufficient for bacterial growth. The TbpA-defective mutant incorporated *c.* 50% of the iron taken up by the wild-type strain and was able to grow with Tf as the only iron source. Mouse antibodies specific for TbpA were able to block *c.* 70% of the iron uptake from Tf in the wild-type strain, whereas they blocked only 22% of iron uptake in the TbpB-defective mutant and did not block uptake in the TbpA-defective strain. These results emphasise that TbpA should be considered in future vaccine trials in which iron-restricted proteins are to be included in the vaccine formulation.

Introduction

The poor efficacy of polysaccharide anti-meningococcal vaccines has stimulated a search for alternative protective antigens. Some outer-membrane proteins (OMPs) are antigenic both *in vivo* and *in vitro* and show inter-strain cross-reactivity, the transferrin (Tf) binding proteins (Tbps: TbpA and TbpB) being some of the most relevant [1, 2]. These particular proteins are of interest because anti-TbpB antibodies are bactericidal and protective and can block iron uptake from Tf, thus interfering with meningococcal growth [3]. The Tf-receptor complex (and its component proteins, TbpA and TbpB) has been identified and purified [4], and studies on its structural, biochemical, functional and antigenic characteristics have been done [1, 2, 4–6]. However, some uncertainties such as the TbpA:TbpB ratio, the necessity of both proteins for iron uptake, their antigenic heterogeneity and the efficacy of specific antibodies still persist. In gonococci, TbpA-defective mutants are incapable of Tf-iron internalisation and growth [6], but mutants lacking TbpB can grow, although their iron-uptake efficiency is lowered [7]. In contrast, data obtained with meningo-

cocci suggest that both types of mutants are unable to grow with Tf as the only iron source and both proteins seem to be necessary in this species [8]. This study tried to define more precisely the role of each Tbp in Tf binding and iron uptake in *N. meningitidis*.

Materials and methods

N. meningitidis mutants obtained and described by Irwin *et al.* [8] were kindly provided by Professor P. Borriello (Department of Microbiology, Queen's Medical Centre, Nottingham). The wild-type strain was *N. meningitidis* B16B6 and mutants were N16T2K (TbpA⁺TbpB⁻), N16T1E (TbpA⁻TbpB⁺) and N16T12EK (TbpA⁻TbpB⁻). The strains were maintained at -80°C and cultured in Mueller-Hinton medium (MH; iron sufficient), MH-EDDA (MH with 39 µM ethylene diaminedihydroxyacetic acid; iron restricted), or in an iron-restricted defined medium (mMBDM; used for uptake experiments) as described previously [9]. Binding of human transferrin was determined by a modified ELISA [10]. Kinetic assays and determinations of affinity and receptor copy numbers were done with ¹²⁵I-(Fe₂)Tf and KCN-killed bacterial suspensions [3, 10]. Uptake of ⁵⁵Fe was measured in live bacterial suspensions by ⁵⁵Fe₂-Tf (25% saturation) [5] or ⁵⁵Fe-complexes with organic acids [9]. The ability of mouse

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anti-TbpA serum (obtained by adsorption of anti-TbpAB serum [3] with N16T1E cells) to block iron uptake from Tf was also determined in kinetic assays. Growth with human Tf as the only iron source was monitored in mMBDM medium containing 30 μ M Fe₂-Tf [11].

Results and discussion

The characteristics of the binding of Tf by all strains are shown in Fig. 1. The kinetics are compatible with the presence of specific receptors, except for the double mutant. The wild-type strain bound almost twice as much [¹²⁵I]-Tf as the TbpA or TbpB mutants, which bound similar amounts of Tf (c. 1.6 pmoles/10⁹ bacteria). The double mutant showed a very low binding, considered to be non-specific, and was not included in subsequent binding experiments. Binding data were analysed to estimate the number of receptor copies and their affinity constants (K_a). Scatchard plots were linear and adjusted to a model of a single, simple and homogeneous population (Hill's coefficients close to 1.0) with high correlation coefficients ($C \geq 0.95$), both in the wild-type and in the isogenic mutants. Table 1 summarises the data on bound Tf, Tf receptor

Fig. 1. Saturation kinetics of the binding of human transferrin to killed *N. meningitidis* strain B16B6 (wild-type, ●) and its isogenic Tbp-defective mutants (TbpA[−]/TbpB⁺, ▼; TbpA⁺/TbpB[−], ■; TbpA[−]/TbpB[−], ☆) in a radiolabelled receptor assay with [¹²⁵I]-transferrin.

numbers/cell and the corresponding affinity constants (K_a) of the strains. The uptake kinetics of ⁵⁵Fe from Tf into the strains showed saturation at 15–20 min (data not shown). After 120 min, the double mutant had not incorporated iron, and the TbpB and TbpA mutants had incorporated, respectively, 37% and 50% of the iron taken up by the wild-type strain. Iron uptake from low mol. wt ferric chelates (citrate, isocitrate, pyruvate, lactate and EDDA) was not affected by the mutations (data not shown). The effect of mouse anti-TbpA antibodies on the uptake of iron is also shown in Table 1. Only the blocking effect in the wild-type strain (70%) was statistically significant (Mann-Whitney test; $p < 0.05$). Results of the growth assays done to study the effect of the mutations on the ability of *N. meningitidis* strains to use human Tf as the only source of iron are shown in Fig. 2. The TbpB and the double knock-out mutants showed almost negligible growth, but the TbpA mutant grew up to an optical density only slightly lower than that of the wild-type strain. All strains were unable to grow in the medium lacking an external iron source.

The quantification of the binding of human Tf and the determination of the number and affinity of the receptors for this molecule in each mutant allowed the differentiation of the affinity of the TbpA and

Fig. 2. Growth kinetics of *N. meningitidis* strain B16B6 (wild-type, ●) and its isogenic Tbp-defective mutant strains (TbpA[−]/TbpB⁺, ▼; TbpA⁺/TbpB[−], ■; TbpA[−]/TbpB[−], ☆) in defined medium with iron-loaded human transferrin as the only iron source.

Table 1. Characteristics of the transferrin receptor system in *N. meningitidis* strain B16B6 (wild-type) and its isogenic TbpA and TbpB defective mutants

Strain no.	Phenotype	Bound transferrin (pmol/10 ⁹ bacteria)	K_a (L/mol)	Receptors/cell	Iron uptake* (%)	Blocking of uptake† (%)
B16B6	TbpA ⁺ TbpB ⁺	3.56	4.30×10^7	1044 ± 160	100	70
N16T2K	TbpA ⁺ TbpB [−]	1.91	2.61×10^8	602 ± 45	37	22
N16T1E	TbpA [−] TbpB ⁺	1.95	1.62×10^7	814 ± 42	50	0

*Expressed as a percentage of that of the wild-type strain.

†Expressed as the percentage inhibition of uptake by anti-TbpA serum relative to the uptake in the same strain in the presence of non-immune serum.

TbpB molecules for human Tf. The analyses show single and homogeneous receptor populations in all strains, in disagreement with the results of Cornelissen *et al.* [6] that suggested the existence of two different receptor populations in wild-type strains. The results of the present study also conflict with recent data by Powell *et al.* [12] which suggested that the two Tbps do not form a single Tf receptor. If either of these hypotheses were correct, the different affinities of the two receptor types (or the two Tbps) should have resulted in the detection of two differentiated receptor populations, but this was not the case in the present and previous studies [5].

If the mutants have not altered their TbpA and TbpB expression, TbpA has an affinity for Tf six times higher than the TbpAB complex and 16 times greater than TbpB. Although this greater affinity is widely accepted, Powell *et al.* [12], with the same mutants, found that the TbpB molecule was more effective in binding Tf than TbpA. Perhaps this discrepancy could be caused by the different methodologies employed and the fact that these authors obtained their data from electron microscopic examination of bacteria labelled with gold-coupled Tf. In our opinion, this method is less precise than radioligand binding assays, in which total binding to the bacteria is evaluated in conditions that minimise stress to the bacterial cells. The data also suggest that the TbpAB complex is formed by single copies of TbpA and TbpB, agreeing with Powell *et al.* [12], but in disagreement with other results that suggest that the complex would be formed by two TbpA molecules and one TbpB molecule [13], or with studies in *N. gonorrhoeae* in which a complex formed by several TbpB molecules and one molecule of TbpA is postulated [14].

The results with regard to iron uptake and growth demonstrate that both the TbpA and TbpB defective mutants are capable of incorporating iron, but only the TbpA mutant gets sufficient iron for growth. This suggests that TbpB could have greater importance than TbpA for iron uptake although, until now, TbpA has been assumed to be the most important. Alternatively, the binding of Tf to TbpB in the TbpA mutant could alter the Tf molecule enough to induce iron release, which would then be internalised by other non-specific mechanisms [9].

Anti-TbpB antibodies are known to be bactericidal, protective to a variable degree, and capable of inhibiting meningococcal iron uptake and growth both *in vivo* and *in vitro* [3, 11, 15]. Ala'Aldeen and Borriello [16] suggested that only anti-TbpA antibodies were bactericidal, whereas Rokbi *et al.* [17] found that specific anti-TbpA antibodies did not bind to live meningococcal cells. Thus, the role of anti-TbpA antibodies is not clear. In the present study, specific anti-TbpA antibodies bound to the wild-type strain and to the TbpB mutant and this binding was

able to block iron uptake from Tf. The blockage was much lower in the TbpB mutant, probably due to the higher affinity for Tf in the mutant, which could cause a displacement of the antibodies by a competition phenomenon, or to the existence of conformational epitopes unrevealed in the absence of the TbpB molecule. The results of the present study, supported by data obtained with specific anti-TbpA antibodies, that show an almost complete cross-reactivity with 28 *N. meningitidis* strains from different serogroups, serotypes and TbpB isotypes (unpublished observations), confirm that TbpA must not be discarded *a priori* in future vaccine formulations that incorporate the iron-regulated proteins.

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