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Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: immunology of glycoconjugates with high carbohydrate loading

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Abstract We investigated the immune responses of rabbits that were immunised with lipopolysaccharide (LPS)-based glycoconjugates by measuring the reactivity of the derived sera to a panel of selected wild-type and mutant strains of *Neisseria meningitidis*. In all cases, high titers of antibodies capable of recognising LPS elaborating the identical structure as presented on the immunising glycoconjugate were obtained, and in most cases the derived sera also recognised heterologous strains including wild-type, but at lower titers. However, although serum bactericidal antibodies were consistently obtained against strains elaborating the same LPS structure as the immunising antigen, this functional response was not observed against wild-type strains. We identified several potentially competing neo-epitopes that had been introduced via our conjugation strategies, which might compete with the conserved inner core oligosaccharide target region, thus reducing the antibody titers to epitopes which could facilitate bactericidal killing. This study has therefore identified key factors that are crucial to control in order to increase the likelihood of obtaining bactericidal antibodies to wild-type meningococcal cells with LPS-derived glycoconjugates. Glycoconjugates utilised in this study, have been found to contain epitopes that do not contribute to the derivation of antibodies that may facilitate bactericidal killing of wild-type strains and must be avoided in future LPS-based glycoconjugate preparations.

Keywords *Neisseria meningitidis* · Conjugate vaccine · LPS

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Introduction

We have been examining the candidacy of relatively conserved regions of inner core lipopolysaccharide (LPS) as a vaccine antigen to combat meningococcal disease [1, 2]. Proof of concept has been established based on the bactericidal activity of a monoclonal antibody (L3B5) that is specific for an inner core epitope of *N. meningitidis* [3], passive protection of animals (infant rats) by L3B5 [3] and the induction of bactericidal antibodies by conjugates made from O-deacylated LPS (LPS-OH) derived from an immunotype L3 *galE* mutant [4]. Conjugation methodology developed with LPS-OH had a number of problems including aggregation and solubility, which reflected the amphiphilic nature of the LPS-OH molecule and precluded the reproducible preparation of glycoconjugates with high carbohydrate loading. We recently reported a significant improvement in the chemistry of glycoconjugate production, which has resulted in the development of a robust and reproducible methodology that results in the production of glycoconjugates with high carbohydrate loading [5]. In this

study we have prepared glycoconjugates from four meningococcal mutant strains, each with a different glycosyltransferase responsible for outer core LPS extension mutated, in order to evaluate their immunogenicity in the rabbit model as a potential vaccine candidate.

Experimental

Growth of bacteria and preparation of purified LPS

Neisseria meningitidis mutant strains *icsB*, *galE*, *lgtA* and *lgtB* in the MC58 background were grown and the LPS isolated as described previously [5]. The mutant strains elaborate LPS with outer core truncations as illustrated in Fig. 1.

Preparation of conjugates from purified LPS

Conjugates were prepared and characterised as described previously [5].

Immunisation

New Zealand white rabbits were immunised subcutaneously with the glycoconjugates. Each rabbit received 25 or 50 μg of conjugated carbohydrate (CRM_{197} as the protein carrier) as 2×0.5 ml per immunisation with Freunds adjuvant for the prime injection and incomplete Freunds adjuvant for the boosts. Rabbits were boosted on day 28 and 56 and sera recovered following terminal heart puncture on day 70. Rabbits also received control immunisations, which consisted of the *Dictyostelium discoideum* deacylated LPS-derived carbohydrate (50 μg) admixed with the appropriate amount of protein (CRM_{197}) and adjuvant.

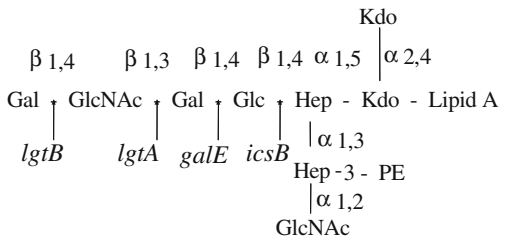

Fig. 1 Representation of the structure of *N. meningitidis* L7 immunotype LPS. Genes encoding the glycosyltransferases responsible for outer core extension (or in the case of *galE* an epimerase that precludes the production of galactose) are indicated as *icsB* etc. Residues are as follows; Kdo is 2-keto-3-deoxy octulosonic acid, Hep is L-glycero-D-manno heptose, Glc is glucose, Gal is galactose, GlcNAc is 2-acetamido-2-deoxy-glucose, PE is phosphoethanolamine. Linkages of sugar residues are indicated beside each bond

ELISA

Purified and well-characterized wild-type and mutant LPS were used in solid-phase indirect ELISA as described previously [4]. For whole cell ELISA formalin killed cells were washed 3x in PBS and then diluted to 10^7–10^8 cells/ml and loaded (100 μl /well) into Maxisorp plates and incubated to dryness, uncovered at 37°C overnight. Subsequent steps were performed as for LPS ELISA.

Bactericidal assay

Serum bactericidal activity with rabbit sera was evaluated as previously described [6].

Results and discussion

The four conjugates utilised in this study, each with a different glycosyltransferase responsible for outer core LPS extension mutated (Fig. 1) were prepared and characterised as described previously [5]. The number of carbohydrate molecules attached per carrier protein for each conjugate was determined by MALDI-TOF MS and revealed that the *icsB*, *galE*, *lgtA* and *lgtB* conjugates contained, on average, 9, 17, 16 and 16 oligosaccharide units respectively (data not shown). Rabbits were immunised in a regimen consisting of a prime (D0) and two boosts (D28 & D56), following which post-immune sera (D70) were obtained and titrated against LPS and O-deacylated LPS (LPS-OH) with the same structure as elaborated on the immunising antigen. The end-point titers of the sera ranged from 1:2,000 to 1:250,000 (data not shown). This enabled appropriate dilutions of sera to be used in order to determine the recognition of LPS and LPS-OH from the range of mutants and wild-type strains using ELISA (Table 1). To facilitate interpretation, we also assayed titers to an irrelevant protein, human serum albumin (HSA), with and without the maleimide linker used in the glycoconjugates attached, to ascertain the level of immune response attributed to the maleimide and hydrazido functionalities. In most cases, the ELISA data revealed high and sometimes the highest titers to the LPS-OH molecules and high titers to the linker molecules. In some cases, apart from the immunising antigen, only the LPS-OH, but not the corresponding LPS molecules, were recognised. This suggested that the inner core 2-keto-3-deoxy octulosonic acid (Kdo) disaccharide along with the mono-acylated β-glucosamine residue of the derivatized lipid A region dominated the immune response. This was confirmed when LPS-OH, but not LPS, from *Moraxella catarrhalis* was recognised (data not shown). Although *M. catarrhalis* elaborates a very different core oligosaccharide than *N. meningitidis*, it does share the same

Table 1 ELISA[1] determination of recognition of LPS and LPS-OH from *N. meningitidis* MC58 background strains (as indicated) and an irrelevant protein HSA with maleimide linker utilised in glycoconjugates with post-immune rabbit sera (D70) following immunisations of rabbits with glycoconjugates (as indicated)

Conjugate Sera / Strain	*icsB* LPS	*icsB* LPS-OH	*galE* LPS	*galE* LPS-OH	*lgtA* LPS	*lgtA* LPS-OH	*lgtB* LPS	*lgtB* LPS-OH	wt LPS	wt LPS-OH	HSA-mal[2]
lgtA conjugate											
Rabbit 1 (1:400)[3]	nd[4]	nd	0.5	1.0	2.2	2.8	1.0	1.2	0.4	0.5	2.3
Rabbit 2 (1:400)	nd	nd	0.2	1.2	2.5	2.8	0.8	1.3	0.6	0.7	1.5
Rabbit 3 (1:400)	nd	nd	0.2	0.8	1.7	2.2	0.6	1.2	0.4	0.5	1.5
galE conjugate											
Rabbit 1 (1:100)	2.3	nd	1.8	3.3	3.7	3.0	4.0	3.6	3.3	3.3	3.8
Rabbit 4 (1:100)	0.9	nd	1.6	2.7	2.8	2.2	3.2	2.6	2.5	2.3	3.4
Rabbit 6 (1:100)	0.5	nd	1.5	2.9	2.7	2.5	3.6	3.0	2.6	2.0	2.7
lgtB conjugate											
Rabbit 1 (1:1600)	0	0.6	0	0.8	0	0.4	1.4	2.2	0	0.2	1.2
Rabbit 2 (1:1600)	0	2.7	0	3.0	0	2.3	2.6	3.7	0	0.8	2.1
Rabbit 3 (1:1600)	0.8	3.5	0.2	3.6	0.7	3.3	4.2	4.3	0.7	1.7	2.5
Rabbit 4 (1:1600)	0.2	2.6	0	2.5	1.5	2.7	1.3	3.0	0.8	1.5	2.6
Rabbit 5 (1:1600)	0	3.0	0	3.2	0	2.7	4.0	4.3	0.7	2.5	2.6
icsB conjugate											
Rabbit 1 (1:3200)	1.0	3.3	0	2.5	0	2.7	0	2.5	0.7	0.3	3.4
Rabbit 2 (1:3200)	4.2	4.3	0	3.3	0	3.5	0	3.7	1.5	1.0	3.5
Rabbit 3 (1:400)	1.7	4.0	0	4.0	0	4.1	0.2	4.2	3.5	2.3	4.0

[1] ELISA values after 60 min. at OD_{405nm} are detailed; [2] HSA protein alone was not recognised by any sera; [3] Dilutions are shown after each serum in parentheses; [4] nd, not determined.

Kdo disaccharide and lipid A region [7–9]. This suggests that the presence of only one N-linked fatty acid on the lipid A β-glucosamine is an important requirement for immune recognition, because when this region of the molecule is fully acylated (i.e. LPS), immune reactivity is often abolished. This preference for epitopes only elaborated by the LPS-OH molecules was most noticeable in the sera derived from immunisation with the *icsB* and *lgtB* conjugates, where apart from the homologous antigen, LPS molecules were hardly recognised. Closer examination of cross-reactivity to the *lgtA* conjugate-derived sera identified that the terminal galactose "tip" residue was somewhat immunodominant, as when the galactose, as a terminal residue, was absent, as is the case in the *galE* and *lgtB* mutant backgrounds, immune reactivity was reduced. There was equivalent immune reactivity of sera from rabbits immunised with the *galE* conjugate to both extended and truncated LPS and LPS-OH molecules, suggesting that in this background the Kdo disaccharide / lipid A region is not immunodominant. Nevertheless, a significant immune response to the linkers was observed. Titers obtained to the *galE* conjugate were relatively low, but a contributing factor to this result was likely because we observed degradation of this conjugate during the weeks when the rabbit immunisations were performed.

Rabbit sera were examined for their ability to recognise formalin killed whole cells of each of the meningococcal strains (Table 2). In each case the greatest immune reactivity was to homologous strains. However, we note the modest reactivity with non-meningococcal *M. catarrhalis* strains, illustrating some specificity of the sera to the Kdo disaccharide / lipid A region. The *galE* derived sera were the most broadly cross-reactive, albeit at low titers. The ability of the *lgtB* and *icsB* derived sera to recognise heterologous whole cells was surprising in the light of the LPS cross-reactivity studies. This might be explained by the variations in presentation of epitopes on ELISA plates reflecting differences in the source of the antigen (whole cells *vs.* LPS).

Nevertheless, the key experiments were to examine whether the derived sera were capable of facilitating bactericidal killing of wild-type meningococci. The sera from rabbits immunised with conjugates of the different LPS mutants were bactericidal to the homologous mutant strains, as evidenced by a consistent increase in bactericidal titers from pre- to post-immune sera (Table 3). However, because the *lgtB* mutant was susceptible to killing by complement alone, the ability of antibodies to facilitate killing of this strain could not be determined. We note that in the case of the complement resistant *icsB* strain, bactericidal activity was observed with

Table 2 ELISA[1] determination of recognition of whole cells[2] from *N. meningitidis* MC58 mutant and wild-type (as indicated) strains and a *M. catarrhalis lgt2* mutant strain with post-immune rabbit sera (D70) following immunisations of rabbits with glycoconjugates (as indicated)

Conjugate Sera / Strain	*galE*	*lgtA*	*lgtB*	MC58	H44/76	*M. catarrhalis lgt2*
lgtA conjugate						
Rabbit 1 (1:400)[3]	0.3	1.4	nd[4]	0.5	nd	0.1
Rabbit 2 (1:400)	0.5	1.2	nd	0.2	nd	0.2
Rabbit 3 (1:400)	0.6	1.0	nd	0.4	nd	0.1
galE conjugate						
Rabbit 1 (1:100)	2.7	2.6	2.8	2.5	nd	0.4
Rabbit 4 (1:100)	2.3	2.3	2.4	1.8	nd	0.7
Rabbit 6 (1:100)	2.7	2.2	2.4	1.4	nd	0.5
lgtB conjugate						
Rabbit 1 (1:1600)	0.5	0.5	1.7	1.2	0.7	0.4
Rabbit 2 (1:1600)	0.5	0.5	2.8	2.1	1.0	0.4
Rabbit 3 (1:1600)	0.7	0.6	4.2	3.7	2.6	0.5
Rabbit 4 (1:1600)	2.3	2.1	2.4	2.0	1.0	0.3
Rabbit 5 (1:1600)	0.5	0.5	3.8	3.0	2.0	0.4
icsB conjugate						
Rabbit 1 (1:3200)	1.0	0.4	0.3	0.3	nd	0.1
Rabbit 2 (1:3200)	3.7	1.7	1.8	1.6	nd	0.2
Rabbit 3 (1:400)	2.3	1.7	1.5	1.6	nd	0.5

[1] ELISA values after 60 min. at OD_{405nm} are detailed; [2] Cells were killed with formalin, washed with water and resuspended at the same OD prior to plating; [3] Dilutions are shown after each serum in parentheses; [4] nd, not determined.

sera from three of the five rabbit's that were immunised with the *lgtB* conjugate.

Crucially, limited evidence of killing of wild type strains was observed. Post-immune sera from rabbits 1 and 2 that had been immunised with the *lgtA* conjugate exhibited a 3-fold and 4-fold increase respectively in bactericidal titers over pre-immune sera when tested against the H44/76 wild-type strain. However, it was observed that the post-immune sera from immunisation of control rabbits from the *lgtA* study, which received non-conjugated but simply admixed carrier protein and LPS-OH from the *lgtA* mutant strain, also killed the H44/76 strain at titers of 1:512 (data not shown). Post-immune sera from rabbit 1 that had been immunised with the *lgtB* conjugate exhibited a 4-fold increase in bactericidal titer over pre-immune sera when tested against the H44/76 wild-type strain.

This conjugation strategy therefore facilitates the killing of homologous strains but not that of wild type strains. Several contributory factors may be responsible for this. We have observed a consistent immune response to the linkers which, although not completely immunodominant, does diminish the efficiency of the immune response to the inner core LPS target region. Bartoloni *et al* have previously observed the immunodominance of the adipic acid dihydrazide (ADH) linker [10]. In their studies ADH was used to link the capsular polysaccharide of *N. meningitidis* serogroup B to the carrier protein CRM_{197}. It was observed that the majority of the derived immune response was to the ADH linker, though this was not the case when they used the same conjugation strategy with the *N. meningitidis* serogroup C capsular polysaccharide, suggesting that the relative immunogenicities of the carbohydrate antigens are important factors in deriving the desired immune response. The immunodominance of maleimide containing linkers had also been examined previously [11]. Studies by Peeters *et al* found that flexible non-aromatic linkers did not induce linker specific antibodies, whereas more constrained linkers did induce high levels of linker specific antibodies. We suspect that since the N-(β-maleimidopropionic acid) hydrazide trifluoroacetic acid salt (BMPH) maleimide containing linker used in this study is somewhat flexible and does not contain aromatic residues it is the hydrazido functionality of this linker that is causing the undesirable immune response. This has been supported by our observations that sera from a conjugate prepared with ADH as the only linker was capable of recognising the irrelevant protein human serum albumin (HSA) with the linker BMPH attached (data not shown). The utility of certain maleimide (but not hydrazido) containing linkers is supported by the fact that commercial vaccines have been produced utilising similar maleimide linkers as in this study, though without the hydrazido functionality, such as the synthetic *Haemophilus influenz*ae capsular polysaccharide serotype B vaccine, developed by Cuban scientists [12]. An immune response to the maleimide functionality could be anticipated with the Cuban vaccine if indeed the maleimide functionality was immunodominant, although this has not been reported, and the Cuban vaccine has proven to have an excellent safety profile and provokes *Haemophilus influenzae* serotype B specific antibody

Table 3 Bactericidal titers[1] of pre- (D0) and post- (D70) immunisation rabbit sera against *N. meningitidis* MC58 mutant and wild-type (as indicated) strains

Conjugate Sera / Strain	Pre / Post	*icsB*	*galE*	*lgtA*	*lgtB*	H44/76	MC58
lgtA conjugate							
Rabbit 1	Pre	nd[2]	nd	4	nd	32	16
	Post	nd	nd	**1024**	nd	256[3]	16
Rabbit 2	Pre	nd	nd	4	nd	64	16
	Post	nd	nd	**2048**	nd	1024[3]	16
Rabbit 3	Pre	nd	nd	4	nd	32	16
	Post	nd	nd	**1024**	nd	128	16
galE conjugate							
Rabbit 1	Pre	nd	4	nd	nd	16	2
	Post	nd	**256**	nd	nd	32	4
Rabbit 4	Pre	nd	4	nd	nd	32	4
	Post	nd	**128**	nd	nd	64	4
Rabbit 6	Pre	nd	32	nd	nd	16	4
	Post	nd	128	nd	nd	64	16
lgtB conjugate							
Rabbit 1	Pre	4	nd	nd	Tox[4]	32	4
	Post	8	nd	nd	Tox	32	4
Rabbit 2	Pre	16	nd	nd	Tox	32	4
	Post	**2048**	nd	nd	Tox	64	4
Rabbit 3	Pre	8	nd	nd	Tox	32	4
	Post	**64**	nd	nd	Tox	**512**	4
Rabbit 4	Pre	4	nd	nd	Tox	64	4
	Post	**128**	nd	nd	Tox	128	4
Rabbit 5	Pre	4	nd	nd	Tox	16	4
	Post	4	nd	nd	Tox	16	4
icsB conjugate							
Rabbit 1	Pre	4	nd	nd	nd	32	16
	Post	**2048**	nd	nd	nd	32	16
Rabbit 2	Pre	16	nd	nd	nd	32	16
	Post	**8192**	nd	nd	nd	64	32
Rabbit 3	Pre	8	nd	nd	nd	32	16
	Post	**2048**	nd	nd	nd	32	16

[1] Bactericidal titers expressed as the reciprocal of the serum dilution yielding >=50% killing when compared to the corresponding pre-immune sera; [2] nd, not determined; [3] Final bleed control sera also killed H44/76 cells at dilutions of 1:512; [4] Complement alone killed *lgtB* mutant strain.

responses similar to the non-synthetic carbohydrate containing vaccine [13].

A potentially critical issue in understanding the lack of bactericidal activity of rabbit sera to wild-type meningococci, concerns the importance of the presentation of the different terminal LPS sugar structures that are characteristic of the mutants used in the candidate vaccine immunogens. For example, the exposure of a terminal galactose in the *lgtA* mutant or the 2-acetamido-2-deoxy-glucose in the *lgtB* mutant, that are not exposed in the wild-type. These "tip epitopes" appear to be an important source of irrelevant antibodies when considering activity against wild-type strains of meningococci. Sera raised to *lgtA* LPS derived conjugates showed significantly reduced recognition to the *galE* antigen, which is lacking only the terminal galactose residue when compared to *lgtA*. Similarly, the terminal β-2-acetamido-2-deoxy-glucose residue of the *lgtB* antigen, absent in *lgtA* and *galE*, seemed crucial to afford recognition with *lgtB* conjugate derived sera. Most intriguingly, the Kdo disaccharide-β-glucosamine region of the immunising antigens seemed to be especially immunodominant based on the finding that most LPS-OH molecules were well recognised irrespective of the immunising antigen. Furthermore, the *M. catarrhalis* LPS-OH, which only shares this region as common with meningococcal LPS-OH, was also recognised and together these data suggest that this Kdo disaccharide-β-glucosamine region of the conjugate is also somewhat immunodominant and should be avoided in order to "focus" the immune response on the target region.

This study has shown that it is possible to raise high titers of antibodies that are bactericidal against the homologous mutant. We conclude that immune responses to unwanted

neoepitopes, such as the terminal residues (tip epitopes), the Kdo disaccharide-O-deacylated lipid A region and linker molecules, diminish the efficiency (amount and affinity) of the immune response to the inner core LPS epitopes of wild-type strains. The production of new glycoconjugates avoiding these unwanted neoepitopes, enabling higher titers to the target inner core epitopes, will be the subject of future studies.

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