Glycoconj J (2014) 31:25–39
DOI 10.1007/s10719-013-9500-z

Investigating the candidacy of LPS-based glycoconjugates to prevent invasive meningococcal disease: conjugates based on core oligosaccharides

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Received: 15 July 2013 / Revised: 16 August 2013 / Accepted: 20 August 2013 / Published online: 7 September 2013

Abstract In this study we have prepared glycoconjugates with core oligosaccharides (OS) from the lipopolysaccharide (LPS) of *Neisseria meningitidis*, thus avoiding the neo-epitopes of the deacylated lipid A region of the derived LPS molecule identified in our previous studies. A comprehensive investigation was performed with glycoconjugates prepared from the most extended to the most truncated core OS still maintaining the conserved inner core epitope. As previously, we have established reproducible bactericidal killing of the homologous antigen elaborating strain, but a failure to kill wild-type strains. In these studies it was evident that the linker molecules used in the conjugation methodologies were dominating the immune response. However, when *galE* core OS based conjugates were prepared without utilizing linkers, *via* direct reductive amination, we failed to generate an immune response to even the homologous antigen. We also identified that immunisation with the *galE* antigen *via* linker methodologies provoked an immune response that was dependent upon key residues of the conserved inner core OS structure, whereas the immune responses to *lgtB* and *lgtA* antigens did not involve the inner core OS. This comprehensive study has, despite our best efforts, cast significant doubt as to the utility of the conserved inner core region of the meningococcal LPS as a potential vaccine antigen.

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Keywords *Neisseria meningitidis* · Conjugate vaccine · LPS · Core oligosaccharide

Introduction

Several groups have been examining the potential of lipopolysaccharide (LPS) antigens as vaccine candidates to combat disease caused by *Neisseria meningitidis*. We have shown that naturally occurring human serum antibodies to inner core epitopes protect against invasive meningococcal disease [1] and Schmiel *et al*. have shown that antibodies to LPS are important in natural and vaccine induced serum bactericidal activity against *N. meningitidis* [2]. Weynants *et al*. showed that LPS can confer a broad cross-bactericidal response [3], however in healthy young adults an experimental detoxified LPS-based vaccine was safe but with low immunogenicity [4]. The low immunogenicity could perhaps be attributed to the host-like structures elaborated on the vaccine, but Cheng *et al*. have established that human derived IgG antibodies that prevent meningococcal disease recognised the host-like outer core lacto-N-neotetraose structure [5]. We have been examining the candidacy of relatively conserved regions of inner core LPS as a vaccine antigen to combat meningococcal disease [6, 7]. Proof of concept has been established based on the bactericidal activity of a monoclonal antibody (mAb B5) that is specific for an inner core epitope of *N. meningitidis* [8], passive protection of infant rats by mAb B5 [8] and the induction of bactericidal antibodies by conjugates made from O-deacylated LPS (LPS-OH) derived from an immunotype

L3 *galE* mutant [9]. 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 [10]. 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 [11]. We identified several potentially competing neo-epitopes that had been introduced *via* our conjugation strategies, which diluted the response to the conserved inner core oligosaccharide (OS) target region, thus reducing the antibody titers to epitopes which could facilitate bactericidal killing. In this study we have prepared glycoconjugates from the most truncated to the most extended meningococcal core OS (Fig. 1), still maintaining the conserved inner core epitope, thus avoiding the neo-epitopes of the deacylated lipid A region of the derived LPS molecule and describe here their preparation and immunology.

Materials and methods

Growth of bacteria and preparation of purified LPS

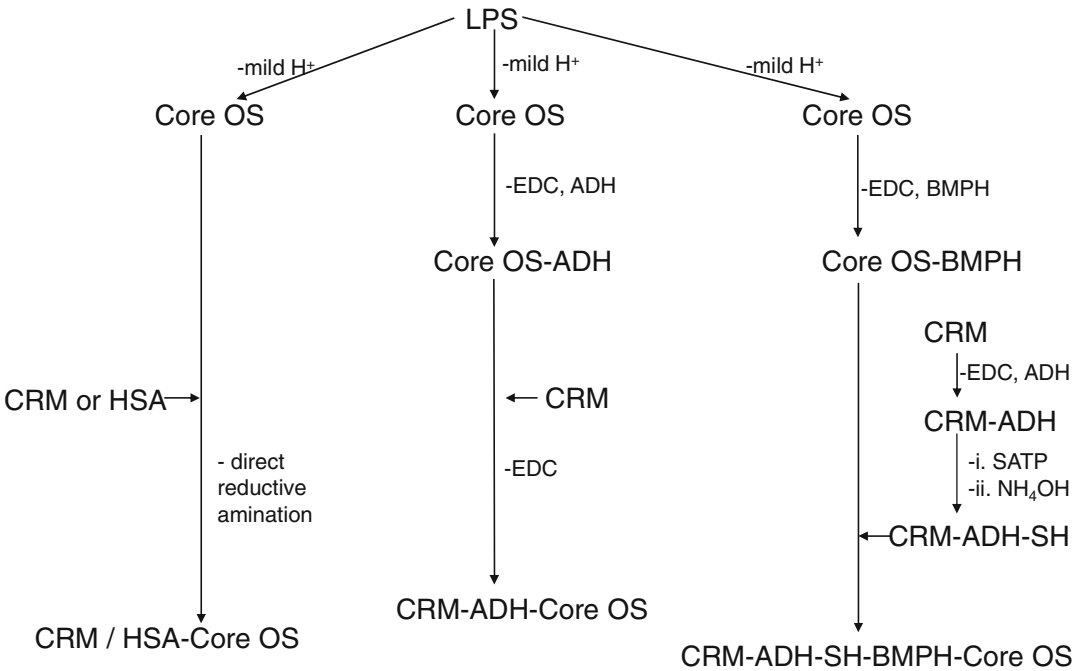
Neisseria meningitidis mutant strains *icsA*, *icsB*, *galE*, *lgtA*, *lgtB* and wild-type all in the MC58 background and (where appropriate) their corresponding *lpt3* mutants were grown and the LPS isolated as described previously [10].

Preparation of O-deacylated LPS (LPS-OH) and core oligosaccharides

Purified LPS from the meningococcal *galE, icsB and icsA* mutant strains were treated with anhydrous hydrazine as described previously to prepare O-deacylated LPS (LPS-OH) [6]. The composition of the LPS-OH was confirmed by ES-MS analysis. Core oligosaccharides (OS) were isolated by treating the LPS with 2 % acetic acid (12.5 mg/mL, 100 °C, 1.5 h) with subsequent removal of the insoluble lipid A by centrifugation (5,000× *g*). The lyophilised OS samples were subsequently further purified on a Sephadex G-25 column (Pharmacia).

Glycoconjugate production

Glycoconjugates were prepared from core oligosaccharides as described below and illustrated in the following scheme:



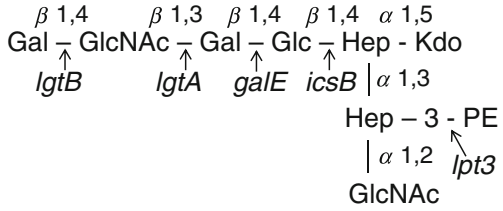


Fig. 1 Structures of the core oligosaccharide of *N. meningitidis*. Residues are as follows; *Kdo* is 3-deoxy-D-*manno*-oct-2-ulosonic acid, *Hep* is L-*glycero*-D-*manno*-heptose, *Glc* is D-glucose, *Gal* is D-galactose, *GlcNAc* is 2-acetamido-2-deoxy-D-glucose and *PE* is phosphoethanolamine. Linkages of sugar residues and transferases responsible for the addition of the glycose and phosphoethanolamine residues are as indicated

Purification and activation of protein carrier CRM_{197}

CRM_{197} was purified by an ultra-15 spin column 10 KDa cut-off (Amicon) as described previously [10]. The purified protein was activated by targeting the carboxyl groups to elaborate thiol linkers by the sequential incorporation of adipic dihydrazide hydrazide, (ADH, Pierce) and *N*-succinimidyl-S-acetylthiopropionate (SATP, Pierce) as described previously [10]. Human serum albumin (HSA) was activated similarly to CRM in order to prepare controls for the ELISA.

Incorporation of linker molecules to the core oligosaccharides

Core oligosaccharides were modified with either adipic dihydrazide hydrazide, (ADH, Pierce) or *N*-(β-Maleimidopropionic acid) hydrazide (BMPH), in 100 mM 2-(N-morpholino) ethanesulphonic acid (MES, Aldrich) at pH 4.8. ADH (100× molar excess) modification was performed in the presence of a 30× molar excess of 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Pierce) and 3× molar excess of *N*-hydroxysulphosuccinimide (sulpho-NHS, Pierce) at 22 °C for 3 h, ensuring the pH was maintained at 4.8. The product was purified on a 1 kDa cut off spin column (Pall Life Sciences, MI, USA), washed six times and lyophilised. Activated core OS following ADH activation was characterised by NMR. BMPH (10× molar excess) modification was performed with a 50× molar excess of EDC and 3× molar excess of sulpho-NHS for 3 h at 22 °C, maintaining pH at 4.8. The product was purified on a Sephadex G-25 column (Pharmacia) and subsequently lyophilised and characterised by CE-ES-MS.

Conjugation

For maleimide (BMPH) modified carbohydrate to thiol (ADH-SATP) modified protein conjugation reactions, the appropriately activated carbohydrates and protein were left to react at 22 °C for 5 h at pH 6.8 in 100 mM sodium phosphate with a second addition of carbohydrate provided after 1 h so that a 50× molar excess of carbohydrate was used in total. The reaction was allowed to continue overnight at 4 °C. For direct (ADH) conjugation reactions the ADH activated carbohydrates were reacted with CRM_{197} overnight at a molar ratio of ~50× carbohydrate to protein in 100 mM 2-(*N*-morpholino) ethanesulphonic acid (MES, Aldrich) at pH 5.2, by reaction with an 1,800× molar excess of EDC at 4 °C. For direct reductive amination conjugation reactions a 175× molar excess of carbohydrate was added to CRM (2 mg/ml) with 5 mg/mL $NaCNBH_3$ in 0.1 M sodium phosphate buffer at pH 8.0. The reaction was filter sterilized and left at 37 °C for 72 h. Conjugation products were concentrated to ~1 mL on an ultra-15 spin column with 10 KDa cut-off (Amicon) as described previously [10]. The concentrates were washed and concentrated a further four times using Dulbecco's PBS (Gibco) containing 10 mM sodium citrate (Sigma). The final concentrate was stored at 4 °C. The glycoconjugates were characterised by MALDI-MS, SDS-PAGE (7.5 or 10 %) and Western blotting as described below.

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× 0.25 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 a trial bleed from the middle ear artery on day 28 and *via* terminal heart puncture on day 70. Rabbits also received control immunisations, which consisted of core OS (50 μg) admixed with the appropriate amount of protein and adjuvant.

ELISA

Purified and well-characterized wild-type and mutant LPS were used in solid-phase indirect ELISA as described previously [11]. Whole cell ELISA was performed as described previously [11].

Bactericidal assay

Serum bactericidal activity with rabbit sera was evaluated as previously described [12].

Analytical methods

Sugars were determined as their alditol acetate derivatives by GLC-MS as described previously [13].

Mass spectrometry and nuclear magnetic resonance spectroscopy

Capillary electrophoresis electrospray mass spectrometry (CE-ES-MS), and NMR spectroscopy were performed as described previously [10]. Matrix-assisted laser desorption ionization-time

of flight (MALDI-TOF) mass spectra were obtained as described previously [10].

SDS-PAGE and western blotting

The conjugates were separated on 10 % Tris–HCl pre-cast gels under reducing conditions and trans-blotted to polyvinylidene fluoride (PVDF) membranes as described previously [10]. Membranes were immuno-stained with carbohydrate specific mAb B5 [6], as described previously [11].

Results

The number of carbohydrate molecules attached per carrier protein for each conjugate was determined by MALDI-TOF MS (Table 1), examples of MALDI spectra for the *galE* conjugates are shown in Fig. 2. Conjugates prepared utilising maleimide-thiol chemistry resulted in loadings ranging from 8 to 30 carbohydrate molecules per carrier protein, and we were able to somewhat control the loading by varying the molar ratios of carbohydrate to protein in the conjugation reaction. For conjugates prepared *via* direct linkage of protein to carbohydrate

Table 1 Details of core conjugates prepared

Conjugate/details	Loading[a]	Rabbits immunised	Titer giving OD=1 vs. homologous LPS[b]
icsB conjugates			
icsB -BMPH-SATP-ADH-CRM	30	3 @ 50 μg RIBV1-3	D42: 400, –, 6,400 D70: 3,200, 200, 25,600
icsB -ADH-CRM	15	3 @ 50 μg RIAV4-6	D42: –, –, – D70: –, 800, –
galE conjugates			
galE-BMPH-SATP-ADH-CRM (High)	12	3 @ 50 μg RG50V4-6	D42: –, –, – D70: 1,600, 100, 800
galE-BMPH-SATP-ADH-CRM (Low)	9	3 @ 50 μg RG15V1-3	D42: 50, 50, – D70: 800, 400, 1,600
galE-ADH-CRM	7	3 @ 50 μg RGCV1-3	D42: 50, 50, – D70: 640, 320, 40
galE-CRM	5	6 @ 50 μg RE48V1-3 RE96V1-3	D42: –, –, –, –, –, – D70: –, –, –, –, –, –
galE-HSA	10	3 @ 50 μg REGV1-3	D42: –, –, – D70: –, –, –
lgtA conjugates			
lgtA-BMPH-SATP-ADH-CRM (High)	18	3 @ 25 μg RNAV1–3	D42: 25,600, 3,200, 3,200 D70: 25,600, 6,400, 12,800
		3 @ 50 μg RNAV4–6	D42: 3,200, 3,200, 25,600 D70: 25,600, 6,400, 102,400
lgtA-BMPH-SATP-ADH-CRM (Low)	11	3 @ 50 μg RABV1–3	D42: 150, 600, 800 D70: 300, 1,000, 10,000
lgtB conjugates			
lgtB-BMPH-SATP-ADH-CRM (High)	10	3 @ 50 μg RBBV4–6	D42: 6,400, 3,200, 25,600 D70: 6,400, 6,400, 25,600
lgtB -BMPH-SATP-ADH-CRM (Low)	8	2 @ 50 μg RBBV7–8	D42: 200, 400 D70: 1,600, 3,200
lgtB -ADH-CRM	7	3 @ 50 μg RBAV1–3	D42: 6,400, 1,600, 12,800 D70: 12,800, 3,200, 12,800
wt conjugates			
wt-BMPH-SATP-ADH-CRM	8	3 @ 50 μg RWCV4–6	D42: –, 400, – D70: –, 6,400, –
wt-ADH-CRM	9	3 @ 50 μg RWCV1–3	D42: –, 100, 50 D70: 400, 100, 50

[a] Oligosaccharide molecules per CRM carrier protein as determined by MALDI-MS

[b] Titers for D42 & D70 rabbit sera vs. homologous LPS antigen after 60 min. at $OD_{405\ nm}$ are detailed

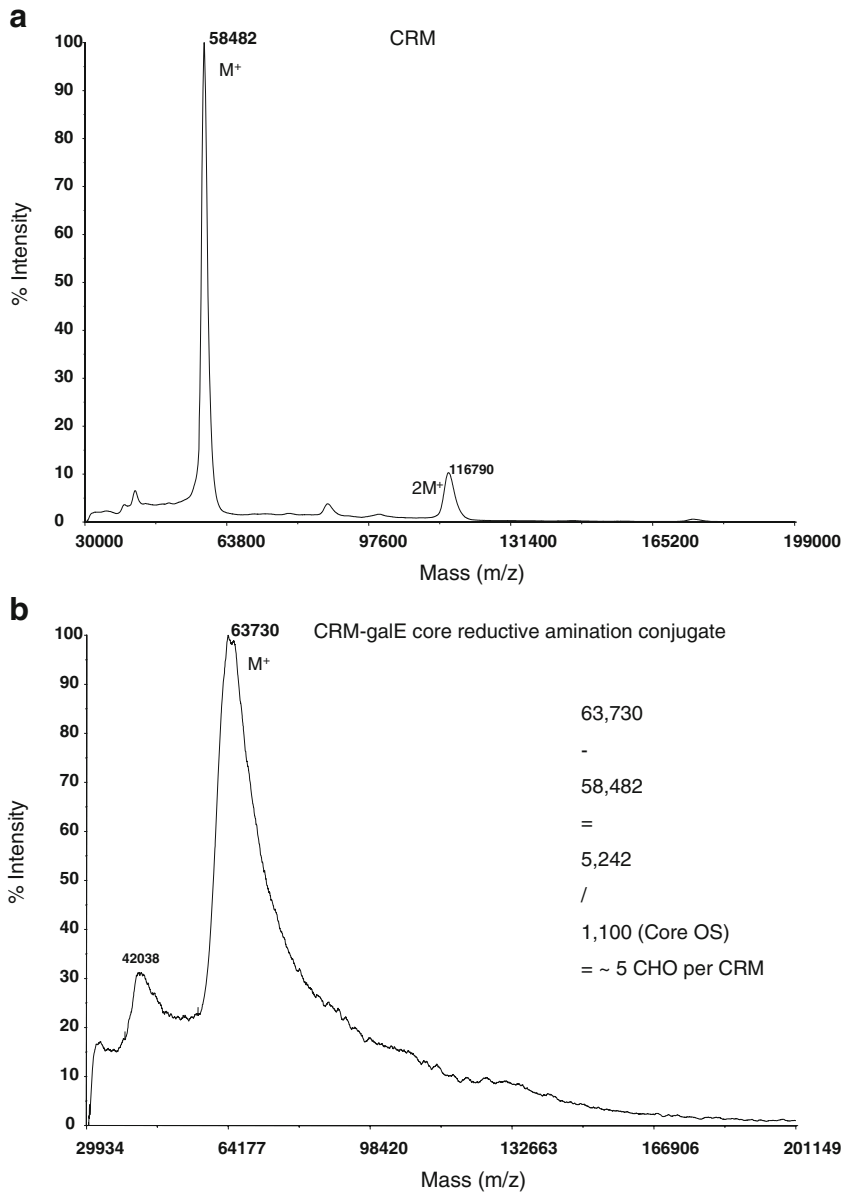


Fig. 2 MALDI-MS analyses of **a** CRM, **b** CRM-*galE* reductive amination conjugate, **c** HSA, **d** HSA-*galE* reductive amination conjugate, **e** CRM-ADH-*galE* conjugate, **f** ADH activated CRM, **g** ADH-SH activated CRM, **h** CRM-BMPH-ADH-SH-*galE* conjugate (high loading)

with adipic acid dihydrazide linker we achieved loadings ranging from 7 to 15 carbohydrate molecules per carrier protein, and for direct reductive amination we achieved 5 and 10 carbohydrate molecules attached for CRM and HSA respectively. Rabbits were immunised in a regimen consisting of a prime (D0) and two boosts (D28 & D56), following which post-immune sera (D42 & D70) were obtained and titrated against LPS with the same structure as elaborated on the immunising antigen. The end-point titers of the sera ranged from zero to 1:100,000 (Table 1). Generally titers obtained with sera from rabbits that had received immunisations with the ADH style conjugates were significantly lower than for the same antigen presented at higher loadings *via* the maleimide-thiol chemistry style loadings (Table 1). The direct reductive amination style conjugates resulted in no immune response to the carbohydrate antigen. For the most part *lgtA* and *lgtB* conjugates provoked higher titers to their homologous antigens than the *icsB*, *galE* and wt conjugates (Table 1). Appropriate dilutions of sera based on the homologous antigen titrations were used in order to determine the recognition of LPS from the range of mutants and wild-type strains by ELISA. Recognition of an irrelevant protein, human serum albumin (HSA), with and without the linkers used in glycoconjugate production attached, was also determined in order to ascertain the level of immune response attributed to the maleimide and hydrazido functionalities of the linkers. In most cases, the ELISA data revealed high titers to the immunising antigen, but also to the linkers and carrier protein, CRM (Figs. 3a and 4a). The specificity of the immune response

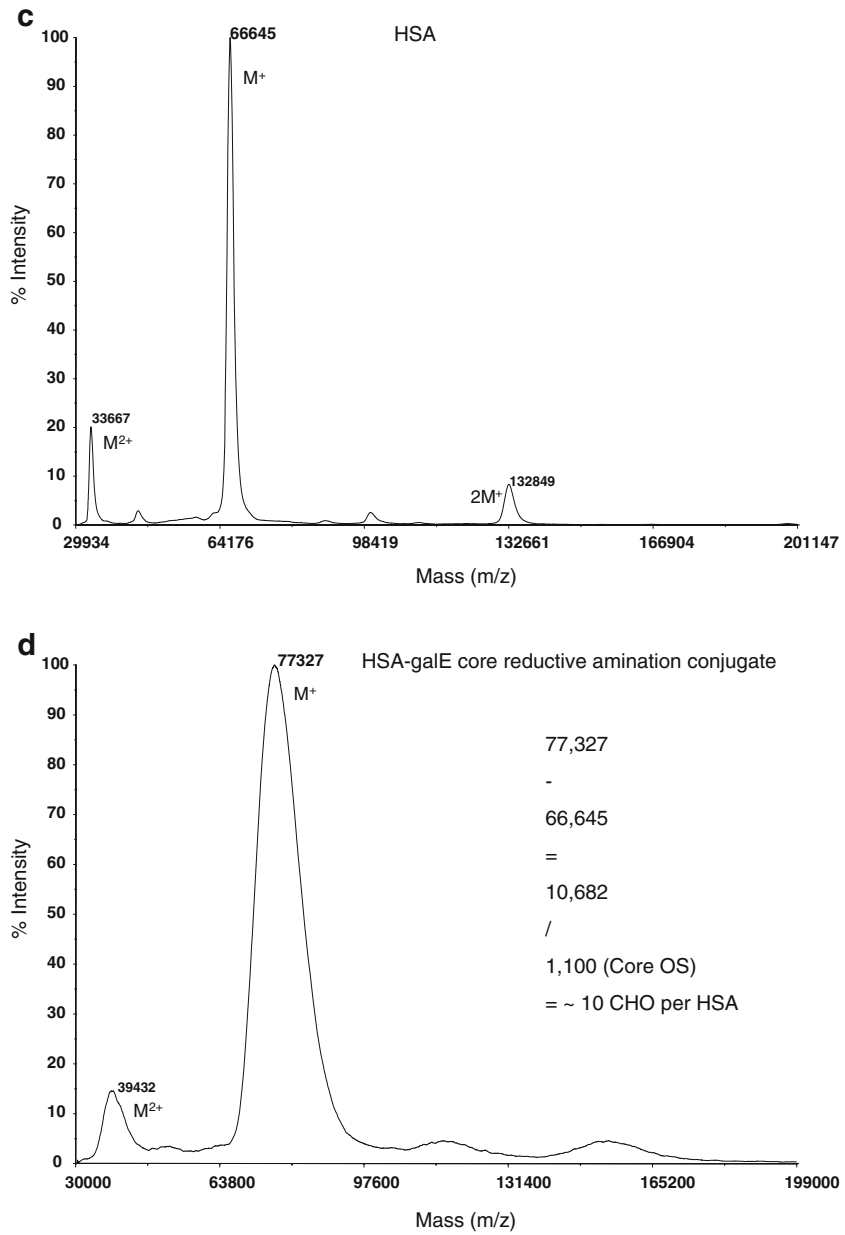


Fig. 2 (continued)

was indicated by a failure to recognise non-meningococcal LPS from *Moraxella catarrhalis* and also an inability to recognise the irrelevant protein HSA. However, as it was clear that HSA was not recognised by any of the rabbit sera, it was disappointing to observe high recognition of the linker molecules, suggesting that they were relatively immunogenic as presented to the host in the context of the conjugates. The breadth of cross-reactivity to other meningococcal LPS antigens was low, with the majority of sera obtained being quite specific for the immunising antigens. Sera derived from *galE* conjugates was reasonably cross-reactive, albeit at low titers (Fig. 3a), and we also observed two rabbits (RIBV3 & RIAV5) from the *icsB* conjugates and three rabbits (RNAV1, RNAV6 & RABV3) from the *lgtA* conjugates to be broadly cross-reactive at high titers (data not shown). We also examined the importance of the inner core residues to antibody recognition, by examining the sera derived from the *galE* and *lgtB* conjugates for their ability to recognise their homologous LPS with and without the phosphoethanolamine (PEtn) residue present (Figs. 3b and 4b). This revealed that the inner core was an important epitope of the *galE* immunogen, but was less significant for the *lgtB* derived immunogen, as recognition was significantly reduced for the majority of the *lpt3* mutants, lacking PEtn, when compared to the corresponding PEtn residue-containing mutant when sera derived from rabbits following immunisation with the *galE* conjugate was examined (Fig. 3b). This was not the case when rabbit sera derived from immunisation with *lgtB* conjugates was examined, as the absence of PEtn did not

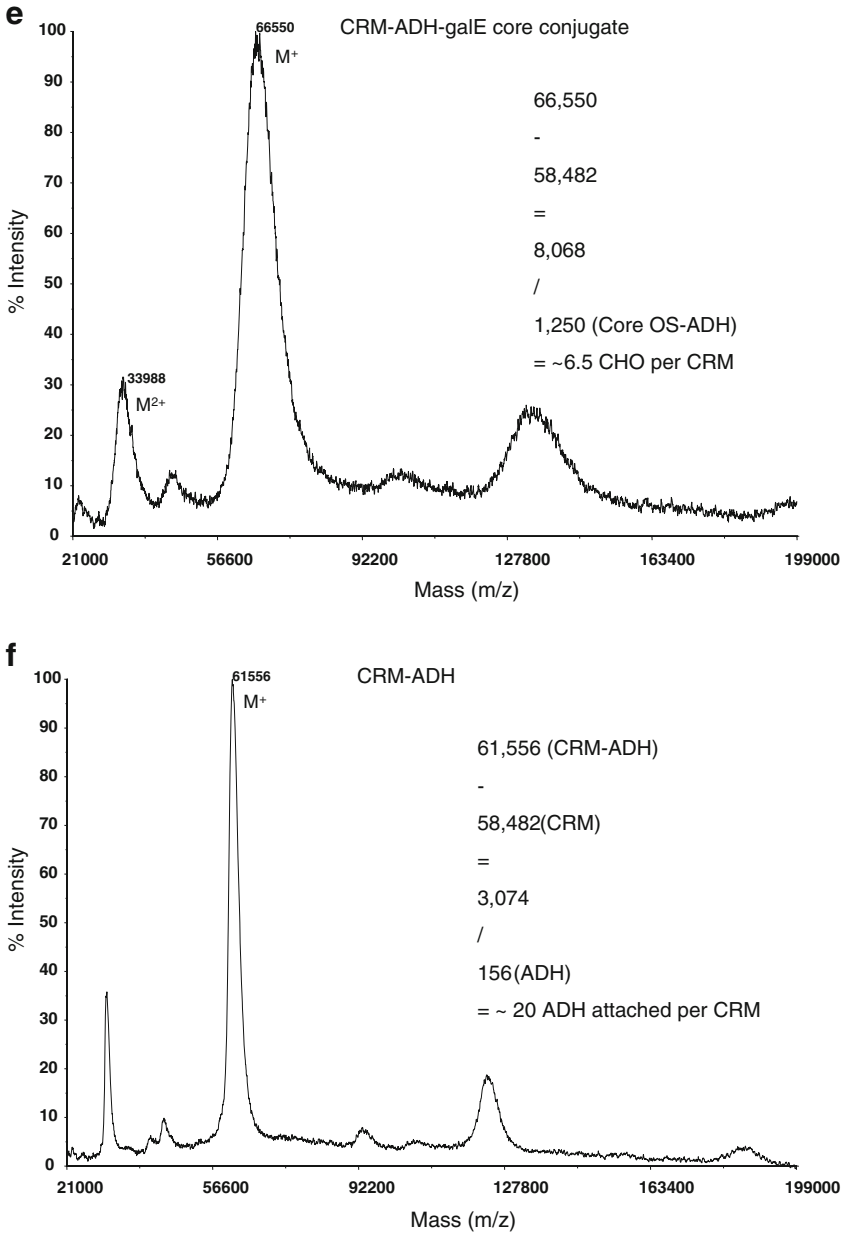


Fig. 2 (continued)

make any significant difference to recognition apart from in one rabbit (RBBV6) (Fig. 4b). We also examined the importance of other residues of the inner core oligosaccharide to facilitate recognition with sera derived from rabbits following immunisation with the *galE* conjugate. This was achieved by examining recognition of O-deacylated LPS (LPS-OH) derived from *icsB* mutants (lacking the inner core glucose residue) and *icsA* mutants (lacking both the inner core glucose and *N*-acetyl glucosamine residues) which revealed that the glucose residue was somewhat important for recognition, but absence of the *N*-acetyl glucosamine residue completely precluded recognition (Fig. 3c). Rabbit sera were subsequently 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 the homologous strains and recognition of wild-type strains was for the most part somewhat lower. The sera from rabbits immunised with conjugates of the different LPS mutants were then examined for their ability to facilitate bactericidal activity, and killing of the homologous strains was observed, as evidenced by a consistent increase in bactericidal titers from pre- to post-immune sera (Table 3). Crucially, limited evidence of killing of wild-type strains was observed, although the sera from rabbit RWCV5 that had received a conjugate with the wild-type core

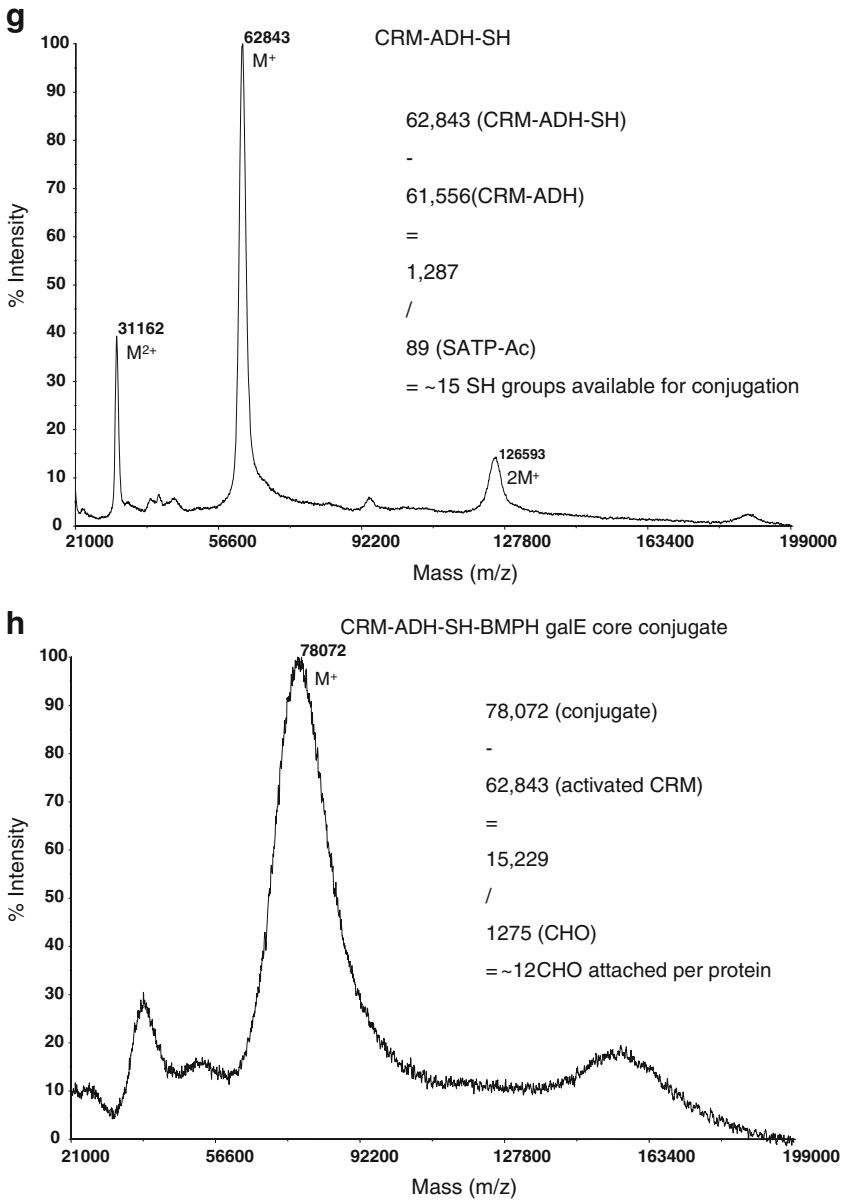


Fig. 2 (continued)

OS as the carbohydrate antigen did kill the wild-type strains MC58, H44/76 and NZ 98/254 tested. There was also some evidence of killing of strain H44/76 with sera from rabbits RBBV5 and RBBV6 that had received the *lgtB* conjugate and rabbit RNAV6 that had received the *lgtA* conjugate though no killing of the MC58 wild type strain was observed (Table 3).

Discussion

This study has illustrated a reliable strategy to prepare glycoconjugates with a high loading of LPS derived carbohydrates. In this study we have utilised core OS as the carbohydrate antigen in an effort to avoid the neo-epitopes of the deacylated lipid A region that were encountered in our previous strategies [9–11]. We continued to use maleimide-thiol chemistry to achieve this high loading and compared this methodology to utilising adipic acid di-hydrazide to bridge the carbohydrate to the protein carrier and finally to a non-linker direct reductive amination strategy. Although higher loading (8 to 30 carbohydrate molecules per carrier protein) was achieved *via* maleimide-thiol chemistries, respectable loadings ranging from 7 to 15 carbohydrate molecules per carrier protein were obtained *via* the single linker molecule and even without linkers, reasonable loadings of 5 to 10 carbohydrate molecules was achieved. There was a possible correlation

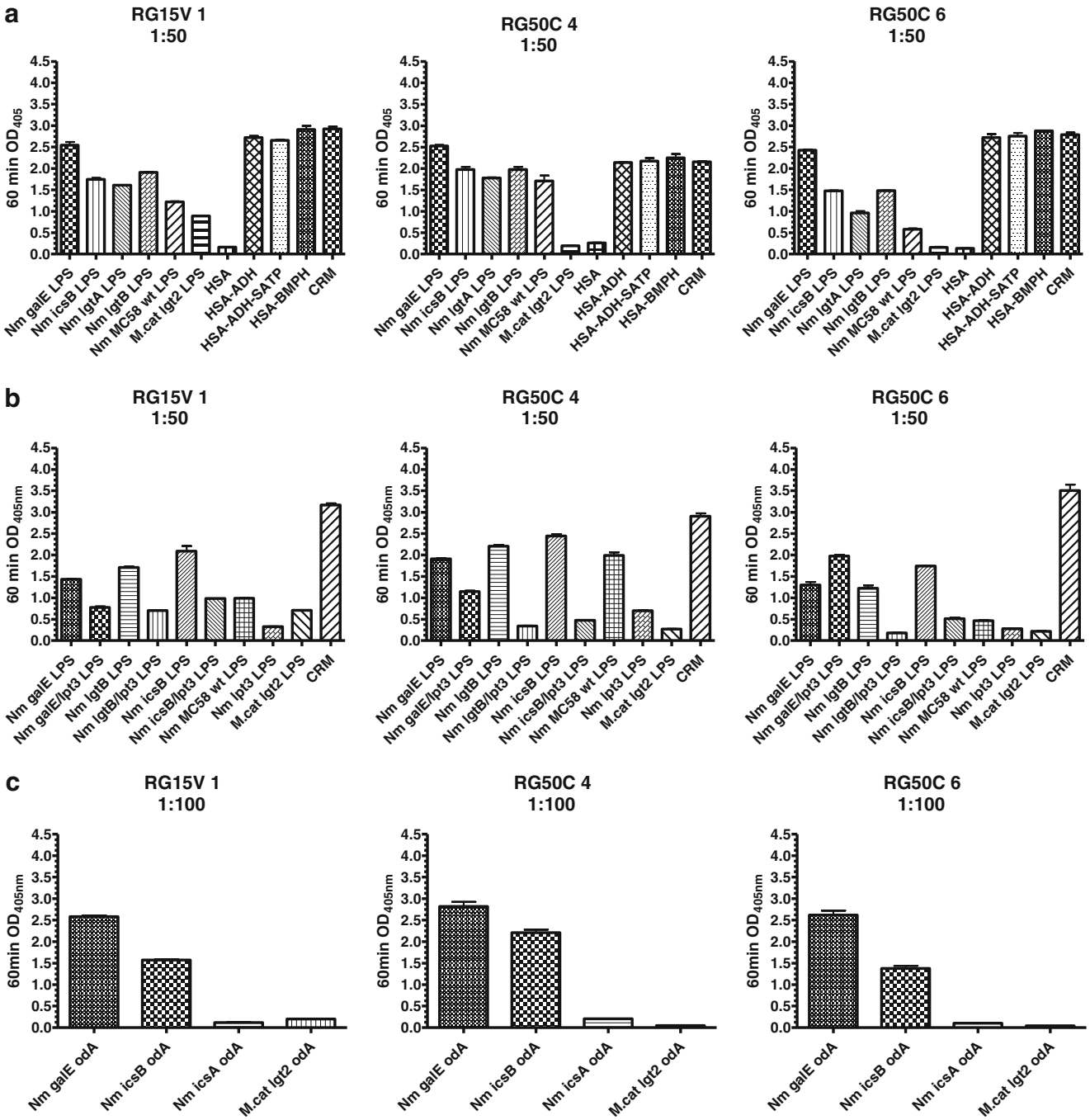


Fig. 3 Cross-reactivity and specificity of rabbit sera (as detailed) derived from immunisation with CRM-BMPH-ADH-SH-galE conjugate; **a** Recognition of purified LPS and proteins with and without linkers as indicated; **b** Recognition of purified LPS with and without (*lpt3* mutants) phosphoethanolamine as indicated; **c** Recognition of purified O-deacylated LPS with and without glucose reside (*icsB* mutant) and with and without both glucose and N-acetyl glucosamine residues (*icsA* mutants) when compared to *galE* immunogen. Dilution of each individual serum is shown in *parentheses*

between size of carbohydrate antigen and conjugate loading as the smallest carbohydrate antigen achieved the highest loadings, perhaps consistent with steric accessibility to some conjugation sites, although this can also be affected by the molar ratios employed in the conjugation reaction. Generally titers obtained with sera from rabbits that had received immunisations with the ADH style conjugates were significantly lower than for the same antigen presented at higher loadings *via* the maleimide-thiol chemistry style loadings, perhaps indicating that a threshold of loading was required to facilitate a strong immune response to the carbohydrate antigen. It was also perplexing and disappointing that no immune response to the carbohydrate antigen was

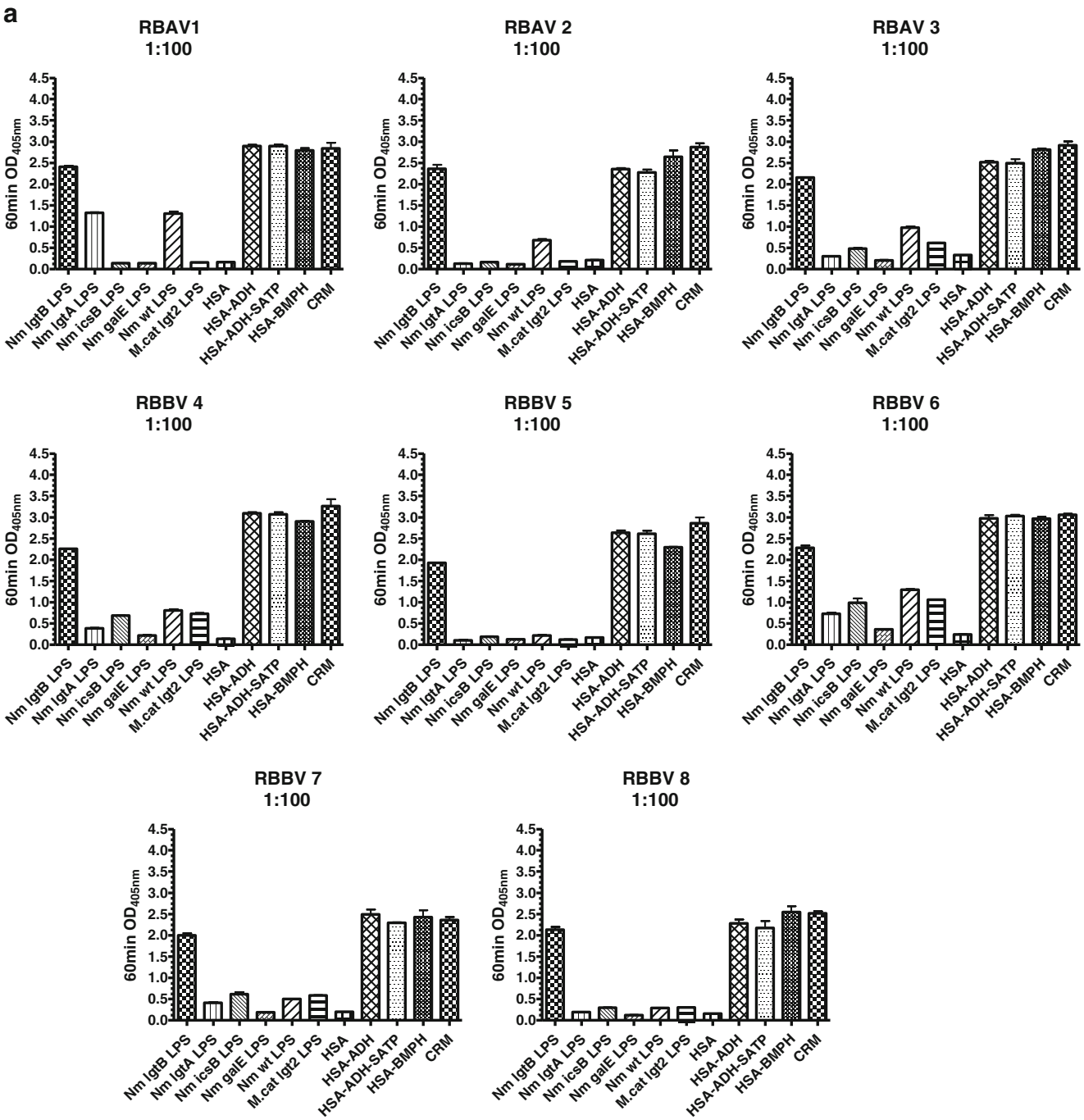
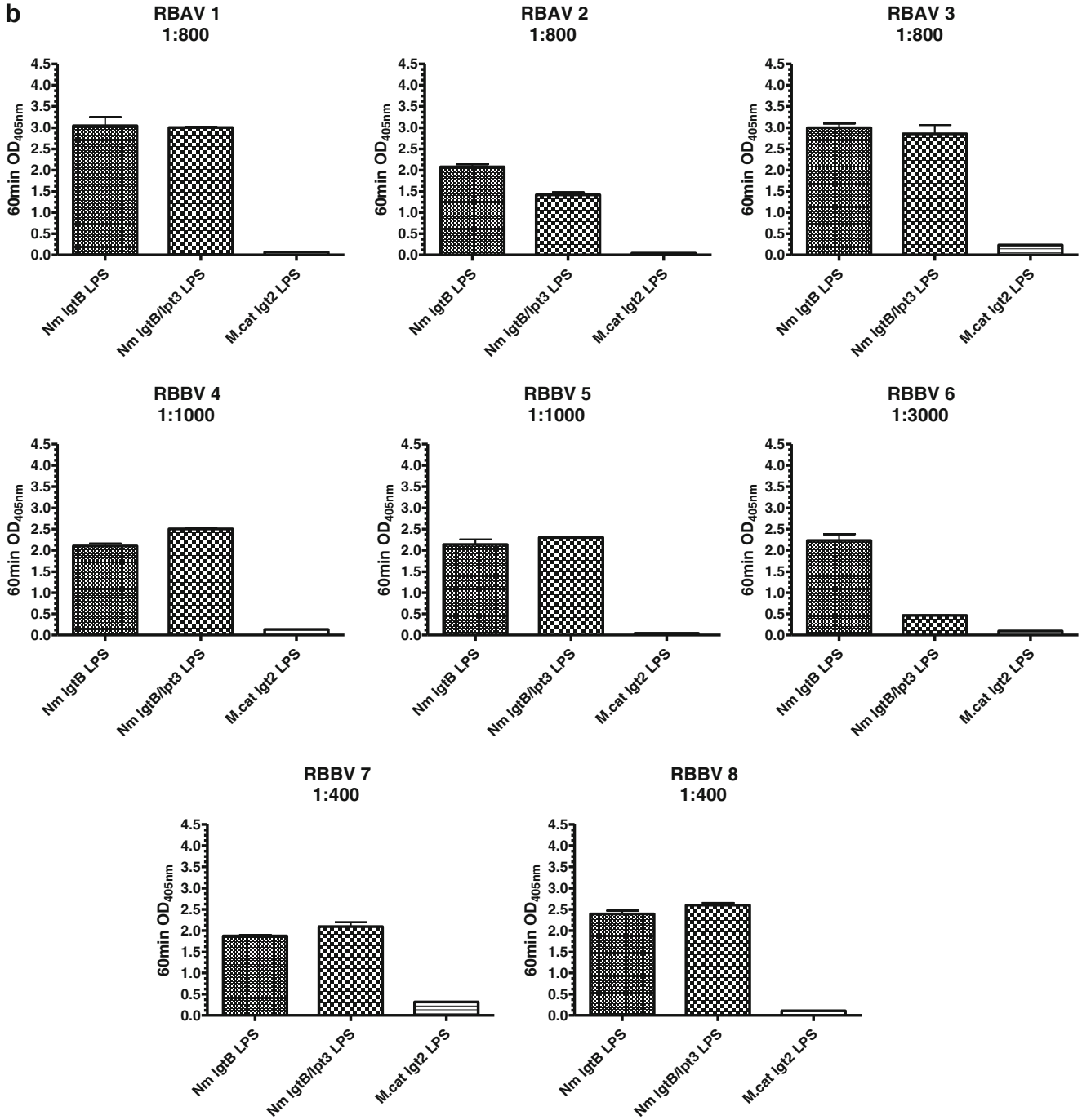


Fig. 4 Cross-reactivity and specificity of rabbit sera (as detailed) derived from immunisation with *lgtB*-BMPH-SATP-ADH-CRM and *lgtB*-ADH-CRM conjugates; **a** Recognition of purified LPS and proteins with and without linkers as indicated; **b** Recognition of purified LPS with and without (*lpt3* mutants) phosphoethanolamine as indicated. Dilution of each individual serum is shown in *parentheses*

observed in conjugates prepared *via* direct reductive amination. As the loading levels seemed acceptable, perhaps it was the case that in the absence of linkers there was insufficient spacing between the carrier protein and carbohydrate antigen to generate an immune response. The conformation of inner core epitopes of the directly linked carbohydrate antigens had not been compromised as these conjugates were recognised by the B5 mAb (data not shown). Although the derived immune response was specific for meningococcal LPS, recognition of a significant immune response to the maleimide and hydrazido functionalities of the linkers was disappointing, suggesting that they were relatively immunogenic as presented to the host in the context of the conjugates. In many cases the response to the linkers was the immunodominant response and clearly, as we had observed with our previous style conjugates, this would be to the detriment of an immunological response to our target



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inner core antigen. This was borne out in bactericidal assays, which indicated good killing of the homologous immunising antigen but limited evidence for killing of wild-type meningococcal strains. Although higher titers to the immunising antigen were achieved with the *lgtA* and *lgtB* conjugates, it was clear that the immune response was more specific to the distal carbohydrate residues, whereas although titers were lower to the *galE* antigen containing glycoconjugates particularly, it was clear that a more broadly cross-reactive response was obtained. Furthermore it was clear that the immune response to these antigens was very much dependent upon key inner core residues, namely phosphoethanolamine (PEtn) at the 3-position and *N*-acetyl-glucosamine (GlcNAc) at the 2-position of the distal heptose residue. We had previously identified the importance of these same residues for the ability of mAb B5 to recognise meningococcal inner core LPS. Similar to the sera

Table 2 Whole cell ELISA data of D70 post-immunisation rabbit sera from conjugates against *N. meningitidis* mutant and wild-type strains and *M. catarrhalis* (*Mc*) *lgt2* mutant strain

Conjugate Sera/strain	*galE*	*lgtA*	*lgtB*	MC58	*Mc lgt2*
icsB conjugates					
icsB-BMPH-SATP-ADH-CRM					
RIBV1 (1:400)[a]	1.3[b]	1.5	0.8	1.1	0.9
RIBV2 (1:100)	1.2	1.4	1.4	1.3	1.4
RIBV3 (1:800)	2.1	2.1	2.2	1.8	0.7
icsB-ADH-CRM					
RIAV4 (1:100)	1.8	1.7	2.4	2.1	1.3
RIAV5 (1:200)	2.1	2.0	2.2	1.6	1.2
RIAV6 (1:100)	1.4	1.5	1.6	1.5	1.5
galE conjugates					
galE-BMPH-SATP-ADH-CRM high load					
RG50V4 (1:100)	2.5	2.0	1.8	1.6	1.4
RG50V5 (1:100)	2.0	1.2	1.2	1.0	0.9
RG50V6 (1:100)	2.8	1.5	2.0	1.1	1.2
galE-BMPH-SATP-ADH-CRM low load					
RG15V1 (1:100)	2.6	1.6	1.5	1.0	1.3
RG15V2 (1:100)	2.3	1.3	1.3	1.5	1.0
RG15V3 (1:100)	2.5	1.4	1.3	1.1	1.2
galE- ADH-CRM					
RGCV1 (1:100)	3.2	2.1	2.1	1.3	1.6
RGCV2 (1:100)	3.0	0.9	1.2	0.8	1.1
RGCV3 (1:100)	2.4	0.9	0.9	0.8	1.0
lgtA conjugates					
lgtA-BMPH-SATP-ADH-CRM high load					
RNAV1 (1:6,400)	0.1	2.3	2.1	2.0	0.1
RNAV2 (1:6,400)	0.0	1.7	0.9	0.9	0.1
RNAV3 (1:6,400)	0.0	2.8	2.3	1.7	0.2
RNAV4 (1:6,400)	0.3	2.5	2.2	2.0	0.1
RNAV5 (1:6,400)	0.4	2.5	2.0	2.0	0.2
RNAV6 (1:6,400)	1.9	3.3	3.7	3.8	0.2
lgtA-BMPH-SATP-ADH-CRM low load					
RABV1 (1:100)	1.3	2.8	2.3	1.8	1.7
RABV2 (1:200)	0.7	2.6	2.2	1.8	1.5
RABV3 (1:800)	1.7	3.3	2.7	2.5	1.0
lgtB conjugates					
lgtB-BMPH-SATP-ADH-CRM high load					
RBBV4 (1:100)	1.2	1.3	2.3	2.2	1.4
RBBV5 (1:100)	1.5	1.3	2.3	2.0	1.3
RBBV6 (1:100)	1.6	1.5	2.3	2.0	1.3
lgtB-BMPH-SATP-ADH-CRM low load					
RBBV7 (1:100)	1.6	1.6	2.4	2.6	1.2
RBBV8 (1:100)	1.7	1.5	2.3	2.0	1.3
lgtB- ADH-CRM					
RBAV1 (1:100)	1.3	2.1	2.7	2.4	1.1
RBAV2 (1:100)	1.3	1.3	2.6	2.7	1.0
RBAV3 (1:100)	1.5	1.5	2.5	2.3	1.4
wt conjugates					
wt-BMPH-SATP-ADH-CRM					
RWCV4 (1:100)	1.2	1.2	1.2	1.4	0.8
RWCV5 (1:100)	1.2	1.1	1.2	2.4	1.0
RWCV6 (1:100)	1.4	1.6	1.5	1.6	1.2
wt-ADH-CRM					
RWCV1 (1:100)	1.1	1.0	1.9	2.1	1.2
RWCV2 (1:100)	0.9	1.0	1.0	1.9	0.8
RWCV3 (1:100)	1.1	0.8	1.0	2.2	1.2

Cells were killed with formalin, washed with water and resuspended at the same OD prior to plating

[a] Dilutions are shown after each serum in parentheses

[b] 60 min $OD_{405\ nm}$ readings are detailed

derived here, mAb B5 was raised to a *galE* mutant meningococcal strain. Recent glycoconjugate studies with LPS antigens from *Moraxella catarrhalis* and *Mannheimia haemolytica* illustrated that despite elaborating similar neo-epitopes and linkers to those utilised in our meningococcal work, we were able to induce antibodies that could facilitate a functional response capable of killing wild-type Moraxella and Mannheimia strains [14, 15]. Contrastingly, the ability to kill meningococcal wild-type strains has not been achieved with similar approaches, and clearly different species are likely to be variable in their susceptibility to antibody-mediated killing. The capsular polysaccharide of *Neisseria meningitidis* probably contributes somewhat to the resistance we have encountered, although the homologous immunising strains which have been killed efficiently in this study also elaborate capsules, so the elaboration of a capsule alone is not a guarantee of avoidance of LPS-specific antibody-mediated killing. Although we still believe that it should be possible to kill wild-type meningococcal strains with a LPS-based vaccine if the appropriate antigens can be presented in such a way to the immune system, this current study raises reasonable doubt that this is an achievable objective. Our results from our conjugation strategies that avoided immunodominant linkers whilst maintaining reasonable carbohydrate loading did not derive a functional immune response to homologous let alone wild-type meningococcal strains, whereas conjugates utilizing linkers to enable higher carbohydrate loading were only capable of killing the homologous and not wild-type strains. It is clear that ingenious strategies will be required to obtain the necessary immune response.

Acknowledgments We thank our NRC colleagues, Perry Fleming (core Bacterial Culture Facility) for large scale biomass production and Jacek Stupak and Xianyu Li for mass spectrometry. This work was supported by Novartis Vaccines. We thank Drs. E. Richard Moxon, Paolo Costantino and Francesco Berti for helpful discussions.

Table 3 Bactericidal titers (bactericidal titers expressed as the reciprocal of the serum dilution yielding > = 50 % killing when compared to the corresponding pre-immune sera) of pre- and post-immunisation rabbit sera from core style conjugate against *N. meningitidis* wild type and mutant strains. Baby rabbit complement was used as the source of complement at a dilution of 1/8

Sera/vs. strain	Pre/post	*icsB*	*galE*	*lgtA*	H44/76	MC58	NZ 98/254
icsB conjugates							
icsB-BMPH-SATP-ADH-CRM high load							
RIBV1	Pre	4	4	4	nd[a]	4	4
	Post D42	**>2048**	4	4	nd	16	nd
	Post D70	**>2048**	4	4	nd	8	4
RIBV2	Pre	4	4	4	nd	8	<4
	Post D42	**>2048**	4	4	nd	8	nd
	Post D70	**>2048**	4	4	nd	16	4
RIBV3	Pre	4	32	64	nd	8	4
	Post D42	**>2048**	64	**1024**	nd	16	nd
	Post D70	**>2048**	32	**512**	nd	32	4
icsB-ADH-CRM							
RIAV4	Pre	nd	nd	nd	nd	nd	nd
	Post D42	nd	nd	nd	nd	nd	nd
	Post D70	nd	nd	nd	nd	nd	nd
RIAV5	Pre	8	4	4	nd	4	nd
	Post D42	32	4	4	nd	32	nd
	Post D70	**1024**	8	4	nd	16	nd
RIAV6	Pre	nd	nd	nd	nd	nd	nd
	Post D42	nd	nd	nd	nd	nd	nd
	Post D70	nd	nd	nd	nd	nd	nd
galE conjugates							
galE- BMPH-SATP-ADH-CRM low load[b]							
RG15V1	Pre	32	4	<4	16	<4	<4
	Post D70	**256**	**>2048**	**32**	32	4	<4
RG15V3	Pre	64	8	32	64	<4	4
	Post D70	**512**	**>2048**	32	64	4	<4
galE- BMPH-SATP-ADH-CRM high load[b]							
RG50V4	Pre	32	8	8	8	4	<4
	Post D70	**>2048**	**1024**	16	16	4	4
RG50V6	Pre	nd	<4	nd	16	<4	nd
	Post D70	nd	**64**	nd	32	4	nd
galE-ADH-CRM[b]							
RGCV1	Pre		<4	nd	16	<4	nd
	Post D70		**64**	nd	16	4	nd
lgtA conjugates							
lgtA-BMPH-SATP-ADH-CRM low load							
RABV1	Pre	nd	nd	4	nd	8	nd
	Post D42	nd	nd	**1024**	nd	8	nd
	Post D70	nd	nd	**512**	nd	8	nd
RABV2	Pre	nd	nd	4	nd	8	nd
	Post D42	nd	nd	**>2048**	nd	16	nd
	Post D70	nd	nd	**>2048**	nd	16	nd
RABV3	Pre	nd	nd	4	nd	16	nd
	Post D42	nd	nd	**1024**	nd	16	nd

Table 3 (continued)

Sera/vs. strain	Pre/post	*icsB*	*galE*	*lgtA*	H44/76	MC58	NZ 98/254
	Post D70	nd	nd	**>2048**	nd	16	nd
lgtA- BMPH-SATP-ADH-CRM high load							
RNAV1	Pre	nd	nd	4	8	16	nd
	Post D70	nd	nd	**2048**	8	16	nd
RNAV2	Pre	nd	nd	8	8	32	nd
	Post D70	nd	nd	**1024**	16	32	nd
RNAV3	Pre	nd	nd	4	8	16	nd
	Post D70	nd	nd	**128**	16	8	nd
RNAV4	Pre	nd	nd	8	8	16	nd
	Post D70	nd	nd	**2048**	32	16	nd
RNAV5	Pre	nd	nd	16	8	16	nd
	Post D70	nd	nd	**2048**	16	8	nd
RNAV6	Pre	nd	nd	16	8	16	nd
	Post D70	nd	nd	**2048**	**64**	16	nd
lgtB conjugates							
lgtB-ADH-CRM							
RBAV1	Pre	16	4	nd	16	8	nd
	Post D42	32	8	nd	64	8	nd
	Post D70	32	8	nd	32	8	nd
RBAV2	Pre	8	<4	nd	16	4	nd
	Post D42	32	8	nd	128	16	nd
	Post D70	64	8	nd	32	8	nd
RBAV3	Pre	32	8	nd	16	4	nd
	Post D42	128	4	nd	64	4	nd
	Post D70	256	4	nd	32	<4	nd
lgtB-BMPH-SATP-ADH-CRM high load							
RBBV4	Pre	16	<4	nd	16	8	nd
	Post D42	32	4	nd	64	4	nd
	Post D70	8	<4	nd	32	4	nd
RBBV5	Pre	>128	32	nd	8	4	nd
	Post D42	128	128	nd	**128**	<4	nd
	Post D70	128	128	nd	**128**	<4	nd
RBBV6	Pre	4	<4	nd	16	4	nd
	Post D42	8	<4	nd	**128**	8	nd
	Post D70	8	<4	nd	**256**	8	nd
lgtB-BMPH-SATP-ADH-CRM low load							
RBBV7	Pre	8	<4	nd	4	4	nd
	Post D42	32	16	nd	16	4	nd
	Post D70	8	8	nd	32	4	nd
RBBV8	Pre	8	<4	nd	8	4	nd
	Post D42	8	4	nd	32	4	nd
	Post D70	8	<4	nd	16	4	nd
wt conjugates							
wt-ADH-CRM conjugate[b]							
RWCV1	Pre	nd	nd	nd	64	4	<4
	Post D70	nd	nd	nd	128	8	<4

Table 3 (continued)

Sera/vs. strain	Pre/post	*icsB*	*galE*	*lgtA*	H44/76	MC58	NZ 98/254
wt-BMPH-SATP-ADH-CRM conjugate[b]							
RWCV5	Pre	nd	nd	nd	64	<4	4
	Post D42	nd	nd	nd	**256**	8	nd
	Post D70	nd	nd	nd	**1024**	**128**	**32**

Bold titers indicate evidence for bactericidal activity

[a] *nd* not determined

[b] Only the rabbits detailed for each conjugate developed sufficient titers to the homologous antigen to be tested in SBA

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