Novel Solutions for Vaccines and Diagnostics To Combat Brucellosis

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S *Supporting Information*
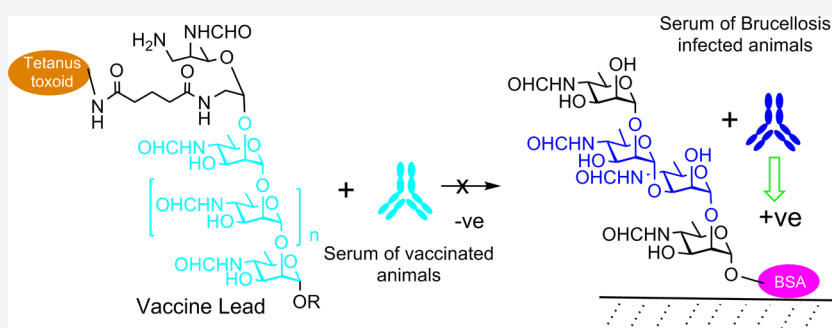
ABSTRACT: Brucellosis is diagnosed by detection of antibodies in the blood of animals and humans that are specific for two carbohydrate antigens, termed A and M, which are present concurrently in a single cell wall O-polysaccharide. Animal brucellosis vaccines contain these antigenic determinants, and consequently infected and vaccinated animals cannot be differentiated as both groups produce A and M specific antibodies. We hypothesized that chemical synthesis of a pure A vaccine would offer unique identification of infected animals by a synthetic M diagnostic antigen that would not react with antibodies generated by this vaccine. Two forms of the A antigen, a hexasaccharide and a heptasaccharide conjugated to tetanus toxoid via reducing and nonreducing terminal sugars, were synthesized and used as lead vaccine candidates. Mouse antibody profiles to these immunogens showed that to avoid reaction with diagnostic M antigen it was essential to maximize the induction of anti-A antibodies that bind internal oligosaccharide sequences and minimize production of antibodies directed toward the terminal nonreducing monosaccharide. This objective was achieved by conjugation of *Brucella* O-polysaccharide to tetanus toxoid via its periodate oxidized terminal nonreducing monosaccharide, thereby destroying terminal epitopes and focusing the antibody response on internal A epitopes. This establishes the method to resolve the decades-long challenge of how to create effective brucellosis vaccines without compromising diagnosis of infected animals.

INTRODUCTION

The World Health Organization ranks brucellosis among the top seven "neglected zoonoses".[1] The disease affects both domesticated (cattle, sheep, goats, and pigs) and wild (deer, bison, elk, moose, camels, caribou, and water buffalo) animals. Brucellosis causes abortions and infertility in these animals and undulant fever in humans, a grave disease that requires a long and costly antibiotic therapy.[2] Where animal production systems involve close contact with human populations, as occurs widely in developing regions, brucellosis is an endemic, insidious and embedded disease. It impacts both human and animal health, with a significant detrimental economic effect that perpetuates poverty. Three vaccines are available for use in ruminants, but there is no human vaccine and there is no recognized vaccine for swine.[3]

Control of endemic brucellosis is achievable via mass vaccination of animals although this is fraught with many difficulties due to significant inadequacies in the current vaccines.[4] They are all live vaccines and thus require refrigeration. They possess residual virulence in animals and can cause abortions. They may be excreted in milk and cause brucellosis in humans when consumed. Two of the three carry resistance to antibiotics that are important in the treatment of human brucellosis. However, chief among the shortfalls is that the most protective vaccines induce antibodies that react in serodiagnostic assays used to identify infected animals. Given that the available vaccines are most effective when combined with the removal of seropositive animals, these reactions are a major barrier to control and eradication. This frequently leads to campaign failure or reluctance to even initiate one.[5]

The most effective vaccines contain bacterial cell wall O-polysaccharide (OPS), but this defeats identification of infected animals because the reactive antibodies induced by these vaccines are primarily specific for the OPS and conventional serodiagnostic assays for these species rely upon the detection of anti-OPS antibodies. OPS is the outermost part of the lipopolysaccharide within the cell wall of these vaccines and the field strains of *Brucella abortus*, *Brucella melitensis*, and *Brucella suis*. Its presence gives the bacteria a smooth appearance on culture media, giving rise to the terms "smooth" strains and "smooth" (s) LPS. The OPS protrudes into the host environment, and antibodies against it dominate the humoral immune response.

Brucella is an intracellular pathogen, and effective protection requires the joint input of cell-mediated and humoral immunity. This is well documented in small animal models[6−8] and also seems certain to be the case in small ruminants where rough vaccines lacking OPS have been insufficiently protective.[9,10] A rough vaccine, *B. abortus* RB51, which provides protection in the target host under experimental conditions, is in field use for large ruminants and does not cause positive serology. However, its use is controversial[11] and it is considered by many to be less effective at controlling endemic disease than the smooth vaccine *B. abortus* S19.[12] It seems that maximum protection requires the presence of OPS. Thus, despite decades of research, efforts to develop vaccines that offer the combination of strong protection without diagnostic interference have been unsuccessful.

The inclusion of the native OPS in serodiagnostic assays to detect antibodies that recognize these antigens has been effective for the diagnosis of brucellosis for over 100 years.[2,13] However, several problems occur. Some bacteria possess OPS that is structurally related to the A antigen, and infection by these bacteria can cause false positive diagnosis.[14] It has been known since the 1930s that these two antigens could not be separated, but the precise molecular basis for this observation was only recently established.[15,16] *Brucella* OPS is a block copolymer of two distinct homopolysaccharide sequences composed of a single rare monosaccharide, 4-formamido-4,6-dideoxy-D-mannopyranose (Rha4NFo).[17] A longer inner sequence of α1,2-linked residues constitutes the A antigen, and this is capped by a shorter sequence that creates the M antigen, which consists of one or several tetrasaccharide sequences containing an α1,3-linked residue (Figure 1).[16]

We have shown previously that the capping M tetrasaccharide and an α1,3-linked disaccharide are sensitive and specific serodiagnostic antigens for the detection of antibodies in *Brucella* infected animals.[19,20] We hypothesized that antibodies specific for the longer internal A motif, although also present in sera, are not detected by this disaccharide antigen and may be protective. Therefore, having access to this A antigen in a pure form should provide the basis of a protective vaccine.

Here we create these vaccine leads and diagnostics in pure form by chemical synthesis, conjugate them to protein, and demonstrate that a hexasaccharide representing the A antigen can induce A-specific antibodies that react only weakly with a disaccharide representing the M antigen. Using these insights, we were able to identify an approach that uses OPS glycoconjugates containing long sequences of exclusively α1,2-linked Rha4NFo units and wherein the terminal residues, containing the M antigen, have been chemically modified. This OPS-based vaccine lead in combination with chemically synthesized diagnostics[19,20] permits differentiation of infected from vaccinated animals (DIVA) and breaks the decades-old scientific impasse for mass
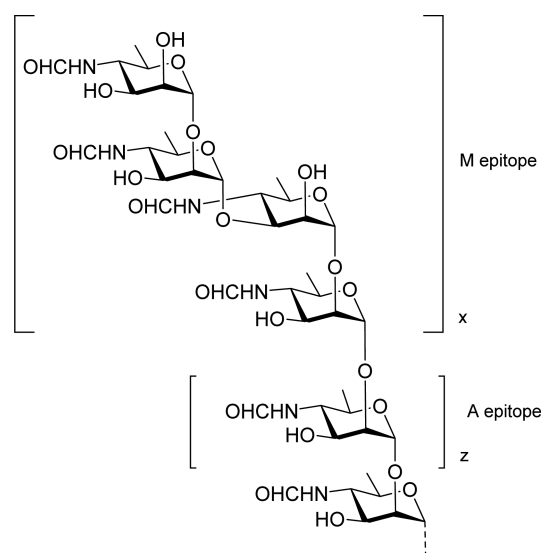

Figure 1. O-antigen structure of *B. abortus*, *B. melitensis*, and *B. suis* (except biovar 2) showing the two elements M and A of the block copolymer.[16] The capping tetrasaccharide containing a single 1,3-linkage defines the M epitope. The relative length of the M and A sections defined by the number of repeats, *x* and *z*, varies between strains. The OPS of *B. suis* biovar 2[18] is not terminated by a M tetrasaccharide.

brucellosis vaccination in animals. These observations suggest key features for vaccines suitable for use in animals and humans that have the potential to reduce the estimated multibillion dollar annual economic losses and human suffering caused by the disease.[21,22]

■ RESULTS AND DISCUSSION

Initially we selected a relatively large A type hexasaccharide conjugated to tetanus toxoid as a vaccine lead with the expectation that an immunogen of this type would induce antibodies reactive with the internal A antigen sequences and not with the terminal 1,3 linkage of the M antigen. We have described the syntheses of this A vaccine candidate **1** and **2** as well as its conjugate to BSA **3** as well as M type di- and tetrasaccharide BSA conjugates **6** and **7**.[19] In order to monitor the size of antibody combining sites in immune sera the 1,2-linked trisaccharide **4** and its BSA conjugate **5** were synthesized. These syntheses which closely follow previous work are referenced and described in the Supporting Information.

Immunization of mice with the A hexasaccharide **1** as its glycoconjugate **2** (Figure 2) gave a strong antibody response against the hexasaccharide epitope **1** when screened with the BSA conjugate **3**.[19] The sera also gave strong titers with the trisaccharide epitope **4** and exhibited strong cross reactivity with *B. abortus* LPS and weaker reactions with the LPS of *B. melitensis* and *Yersinia enterocolitica* O:9 (Figures 3 and S7). The O antigen of *Y. enterocolitica* O:9 LPS is an exclusively α1,2-linked polysaccharide of Rha4NFo[23] and virtually identical to that of A dominant *Brucella* O antigen (for example *B. abortus* S99)[17] but lacks the terminal M type tetrasacharide motif.[16] M dominant O antigen, exemplified by *B. melitensis* 16M, contains several M repeats that cap the inner A antigen.[16] The immune sera also exhibited cross reactions with the M disaccharide and tetrasaccharide antigens **6** and **7** (Figure 3) that were surprisingly high.

1 R₁ = O(CH₂)₅CO₂Me
2 R₁ = O(CH₂)₅CONH(CH₂)₂NH-dsg-TT
3 R₁ = O(CH₂)₅CONH(CH₂)₂NH-sq-BSA

4 R₁ = O(CH₂)₅CO₂Me
5 R₁ = O(CH₂)₅CONH(CH₂)₂NH-sq-BSA

6   7

R₁ = O(CH₂)₅CONH(CH₂)₂NH-sq-BSA

8 R₂ = (CH₂)₅NH₂
9 R₂ = (CH₂)₅NH-dsg-Tetanus toxoid
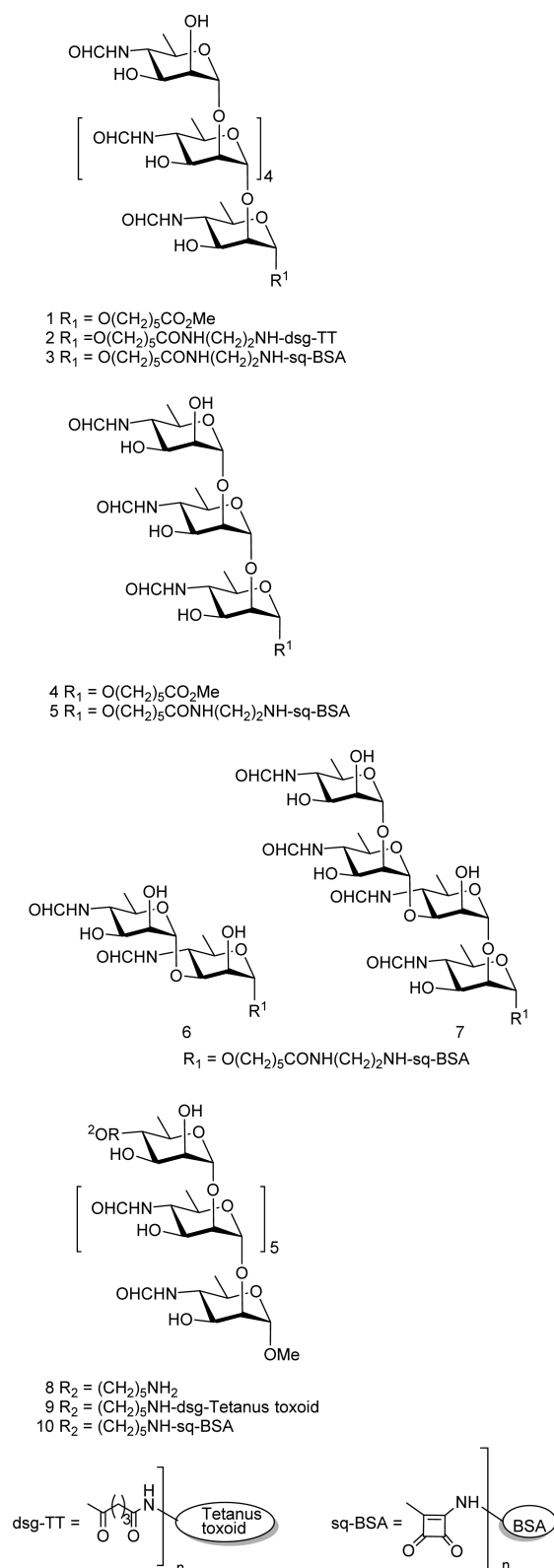10 R₂ = (CH₂)₅NH-sq-BSA

Figure 2. Key oligosaccharides and their protein glycoconjugates used in this study. Additional glycoconjugates are shown in the Supporting Information and carry the prefix "S": A type hexa- and trisaccharide **1** and **4**, and their protein conjugates **2**, **3**, and **5**; M type di- and tetrasaccharide BSA glycoconjugates **6** and **7**; heptasaccharide **8** carries a terminal rhamnose residue equipped with an amino tether to effect attachment of an A type hexasaccharide repeat to protein via the terminal nonreducing end; as in BSA and tetanus toxoid conjugates **9** and **10**.

Because tetrasaccharide **7** is terminated by a 1,2-linked disaccharide, some of this reactivity could be expected. Screening of a monosaccharide conjugate (**S28**) was consistent with the presence of a population of antibodies that were able to bind the terminal monosaccharide. A wider panel of glycoconjugates of more complex structures, in which the terminal Rha4NFo residue was mono- or di-O-methylated (glycoconjugates **S19**–**S21**) or capped by D-mannose (**S22**–**S24**), blocked binding of this population of antibodies as evidenced by their correspondingly lower titers. This was consistent with a terminal epitope in which the formamido residue likely plays an immunodominant role.

To study this possibility in more detail, sera from six mice vaccinated with **2** and exhibiting high and intermediate titers against *B. abortus* LPS were studied by inhibition ELISA. Six pentasaccharide inhibitors were used in which Rha4NFo residues were replaced by D-Rha. These were originally designed to investigate antigen frame shifting for antibodies that recognize internal epitopes[24,25] and are as follows: (where P = Rha4NFo and R = Rha): P_4R; P_2RP_2; P_2RPR; PRPRP, PR_2PR; RP_3R (Table S1). The most effective inhibition was achieved with P_4R, P_2RP_2, and P_2RPR, all of which were terminated by a Rha4NFo disaccharide. Two mouse sera showed weak inhibition (IC$_{50}$ = 0.1 mM) against pentasaccharides (PRPRP, PR_2PR) with a single Rha4NFo residue; however, RP_3R, with a single terminal rhamnose, is inactive at 1 mM. Also noteworthy is that the antibody levels of the ten mice were tightly clustered with BSA–hexasaccharide conjugate **3**, but the responses when measured against the LPS and conjugates **5**–**7** varied over a log range of 2.5. Glycoconjugate **2** would clearly fail to differentiate infected from vaccinated animals (DIVA) as it generates anti-O-antigen serum antibodies with low but significant reactivity with the M disaccharide antigen **6** and tetrasaccharide **7**.

The observation that, despite the absence of any 1,3-linkages in glycoconjugate **2**, the substantial titers observed for tetrasaccharide **7**, M disaccharide **6**, and the monosaccharide **S28** prompted consideration that the terminal D-Rha4NFo residue may be particularly influential. As discussed above, this proved to be the case. Similar antibodies that target the nonreducing end have been described before, although this has previously been associated with heteropolymers (*Francisella tularensis*)[26] or unique terminal residues that differ from those within the polymer (*Vibrio cholera* O1 serotype Ogawa).[27]

A seldom, if ever, used approach with synthetic glycoconjugate vaccines and one that could avoid the induction of antibodies to the terminal capping residue of an A type epitope involves location of a tether at the nonreducing end as shown in heptasaccharide **8** (Figure 2). Its synthesis employing the key intermediates **11**–**13** is outlined in Scheme 1, and syntheses of synthons (**S1**–**S17**) using known chemistry[19] are outlined in the Supporting Information. Thioglycoside **11** serves as the glycosyl donor for a series of iterative glycosylations that begin with glycosylation of the known methyl glycoside **S12**[28] to afford disaccharide **14**. Transesterification provides the disaccharide alcohol **15**, which serves as the acceptor for a second glycosylation reaction. Sequential glycosylation by **11** followed by de-O-acetylation of the resultant oligosaccharide was repeated four times to provide, in succession, fully protected oligosaccharides **16**, **18**, **20**, and **22** (Scheme 1) all in yields of 89% or higher. The selectively protected hexasaccharide alcohol **23** was glycosylated by the rhamnopyranosyl donor **13** equipped with a protected 5-aminopentanyl tether. The resulting heptasaccharide **25** was transformed to **8** by a series of previously described
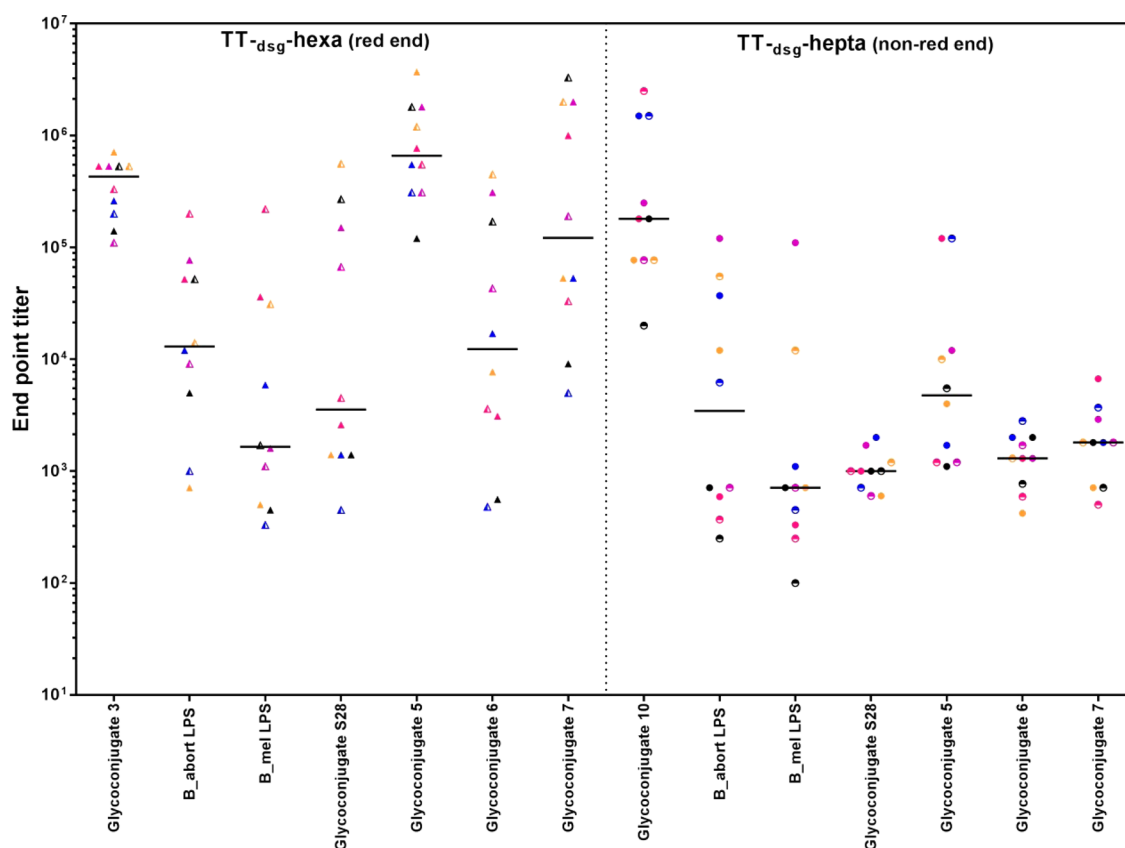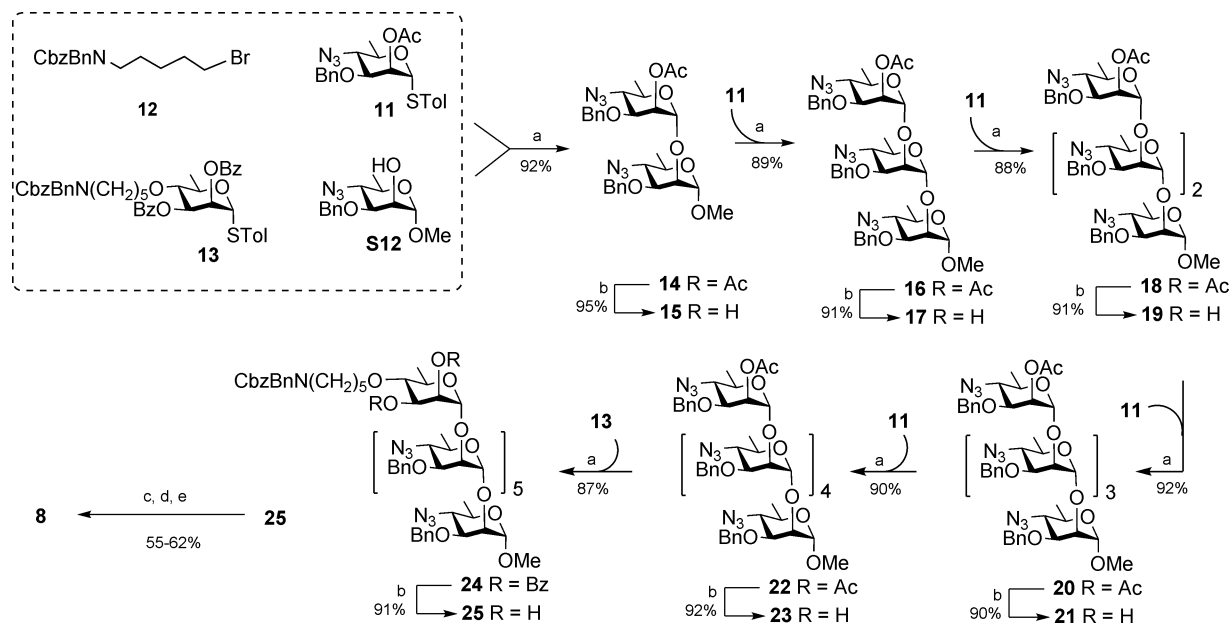
Figure 3. Comparison of the antibody binding profiles generated in mice immunized with **2** and **9** screened against synthetic antigens and *Brucella* LPS.

Scheme 1. Synthesis of 'A' Type Heptasaccharide 8 with a Terminal Nonreducing Tether[a]

[a]Conditions: (a) TMSOTf, NIS, 4 Å MS, CH_2Cl_2 −20 °C to rt, 3 h; (b) NaOMe, MeOH, rt, 6 h; (c) H_2S, Py/H_2O, 40 °C, 16 h; (d) $(HCO)_2O$,MeOH, −20 °C, 3 h; (e) H_2, Pd(OH)$_2$, MeOH/H_2O, rt, 16 h.

reactions involving azide reduction, *N*-formylation, and deprotection reactions (Scheme 1).[19] The amine **8** was activated with disuccinimidyl glutarate or dibutyl squarate and covalently linked to tetanus toxoid and BSA to provide glycoconjugates **9** and **10**.

Mice were immunized with **9** using a protocol identical to that employed with **2**. After three injections, very high titers were

observed for the sera of all ten mice measured against the immunizing hapten, BSA conjugate **10** (Figure 3). A similarly wide distribution of titers against the three LPS antigens (*B. abortus*, *B. melitensis* and *Y. enterocolitica* O:9; Figure S7) was observed although the median titer for the ten mice was 2−3-fold lower than the mice vaccinated with **2**. Most striking was the

markedly confined distribution of titers against the M disaccharide and tetrasaccharide antigenic determinants **6** and **7**. Whereas titers from sera of mice vaccinated with glycoconjugate **2** spanned a three-log range against **6** and **7**, sera of mice vaccinated with **9** were restricted to a single log span and furthermore were 1−2 logs lower. This low titer against the diagnostic M antigen compound **6** comes closer to fulfilling the objective for a DIVA vaccine.

High titers against the immunizing haptens **1** and **8** for mice vaccinated with **2** or **9** compared to the corresponding titers against the 1,2-linked trisaccharide **4** suggested a significant proportion of nonreducing end-specific antibodies generated by **2** and a modest to good level against internal 1,2-linked Rh4NFo residues in the group immunized with **9**. We reasoned that a glycoconjugate similar to **9** but with a larger A epitope should increase the titers against internal 1,2-linked Rh4NFo residues. If the terminal epitope was also eliminated from this structure, the presence of antibodies that bind M antigens (**6** and **7**) would be reduced further.

This objective could be achieved by conjugating the exclusively α1,2-linked O antigen produced by *B. suis* biovar 2.[18] Kubler-Kielb and Vinogradov reported the structure of the *Brucella* OPS and the oligosaccharide through which it is attached to the inner core KDO (Scheme 2).[29] There are two primary sites susceptible to mild periodate oxidation: the terminal Rha4NFo residue and the reducing end KDO residue, which remains attached to the OPS after the mild acid hydrolysis to cleave lipid A from the lipopolysaccharide molecule. Reductive amination of aldehydes created at these sites followed by reaction with disuccinimidyl glutarate allows conjugation of the activated polysaccharide to tetanus toxoid (Scheme 2). The scheme also shows how the terminal Rh4NFo is degraded by the process of oxidation followed by amination. Therefore, regardless of whether the OPS is conjugated to the protein via the terminal or reducing end, the modified OPS does not possess a tip epitope. The OPS from *B. suis* biovar 2 (strain Thomsen) and *B. abortus* strain S99, with 98% α1,2- and 2% α1,3-linkages, were conjugated to tetanus toxoid in this manner to yield respectively OPS−TT2 and OPS−TTS99. Notably, these α1,3-linkages occur near the modified nonreducing end of the OPS.[16] However, they are sufficiently accessible on the OPS−TTS99 to bind the anti-M specific monoclonal antibody BM40[30] as shown by Western blot (Figure S1). This antibody can bind the disaccharide **7**, tetrasaccharide **6**, and hexasaccharide **3**, but not the exclusively α1,2-linked hexasaccharide **S37** BSA conjugate (unpublished data).

Mice were immunized three times with each OPS tetanus toxoid conjugate (OPS−TT2 and OPS−TTS99) (Scheme 2). All eight mice immunized with the modified *B. abortus* OPS conjugate produced titers against both *B. abortus* S99 and *B. melitensis* 16M sLPS, despite the latter being M dominant, having OPS with 20% α1,3-links (Figure 4). Sera from all eight mice also reacted against *B. abortus* S99 and *B. suis* (Thomsen) whole cells although three did not react against the *B. melitensis* 16M whole cells. Not only was there was no response against the disaccharide **6** but there was also no response against the tetrasaccharide **7**.

Binding to the hexasaccharide antigen **3** compared to that of the 1,3-linked hexasaccharide **S37** shows that antibodies to the A epitope dominate. The lack of antibodies against the M epitope (as detected by binding to conjugates **6** and **7**), even though 2% of the linkages are of type α1,3, may be due to the occurrence of these linkages near the site of terminal conjugation resulting in their reduced accessibility to the immune system. Antibodies that

Scheme 2. Conjugation of *B. suis* Biovar 2 OPS to Tetanus Toxoid[a]
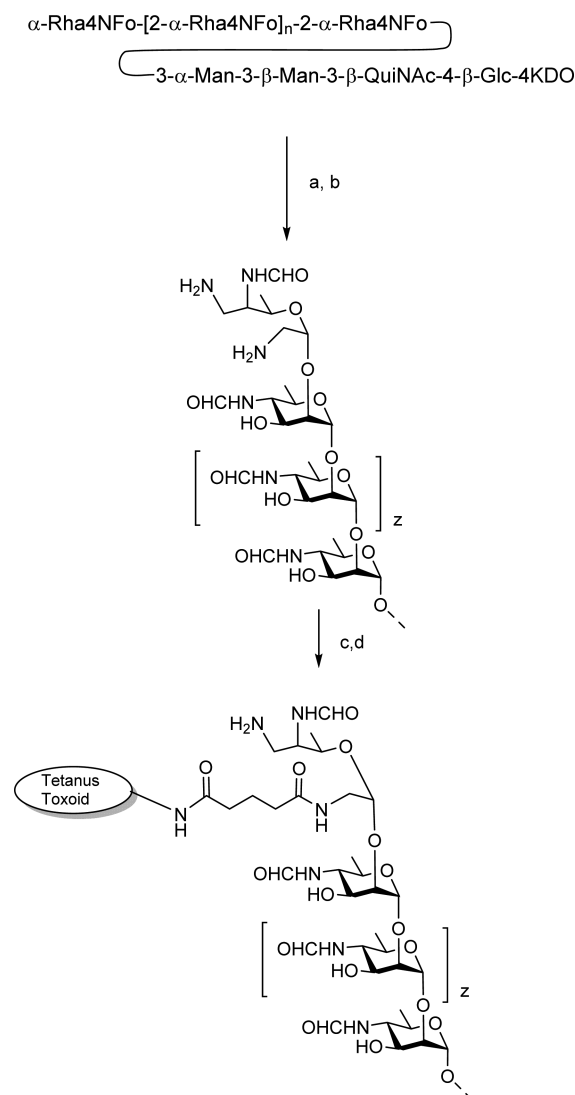
[a]Conditions: (a) 10 mM NaIO₄, 50 mM NaOAc, pH 5.5, 4 °C 1 h; (b) 0.5 M NH4Cl, 0.1 M NaCNBH₃, 37 °C; (c) aminated OPS 5 mg/mL in PBS containing 10% DMSO and 5 mg/mL disuccinimidal glutarate (dsg) 45 min, rt; (d) activated OPS, tetanus toxoid 2.5 mg/mL PBS.

are induced against this region may also have epitopes that overlap with the modified terminal residue and fail to bind in its absence, or the antibodies require epitopes that are longer than four Rha4NFo units.

The absence of antibodies that bind to singular M epitopes would account for the average 5-fold reduction in binding of the antibodies to the M dominant (*B. melitensis* 16M sLPS) O antigen compared to the A dominant (*B. abortus* S99 sLPS) O antigen. Despite this reduction, the average titer was still approximately 1/7,000. Most of the sera also reacted against whole cell antigens although with lower titers. Importantly the results show that the OPS tetanus toxoid conjugate can induce antibodies that bind to M dominant cells via the A epitope of their OPS. It has also been shown previously that antibodies with anti-A specificity can provide protection against challenge with M dominant strains of *Brucella*.[31]
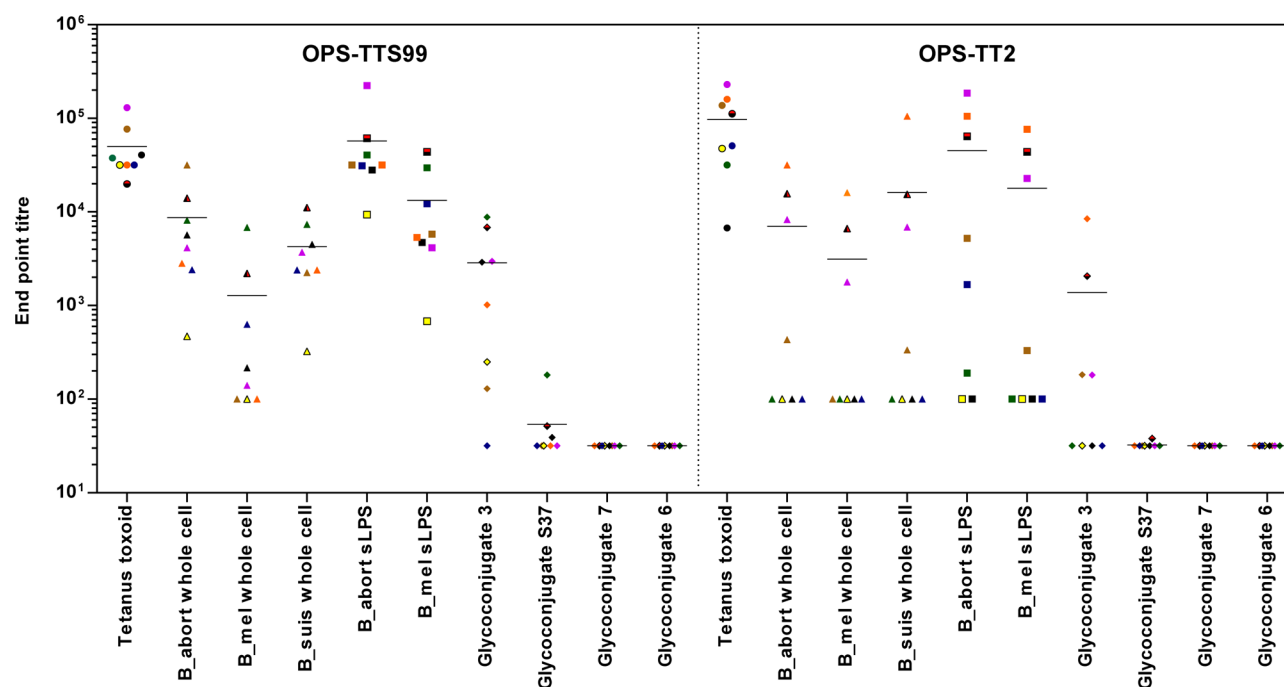
Figure 4. Final bleed titers from eight CD1 mice immunized with modified *Brucella* OPS TT conjugates (left panel, *B. abortus* S99 OPS−TTS99; right panel, *B. suis* bv2 OPS−TT2). The antigens used for antibody detection are shown on the *x*-axis.
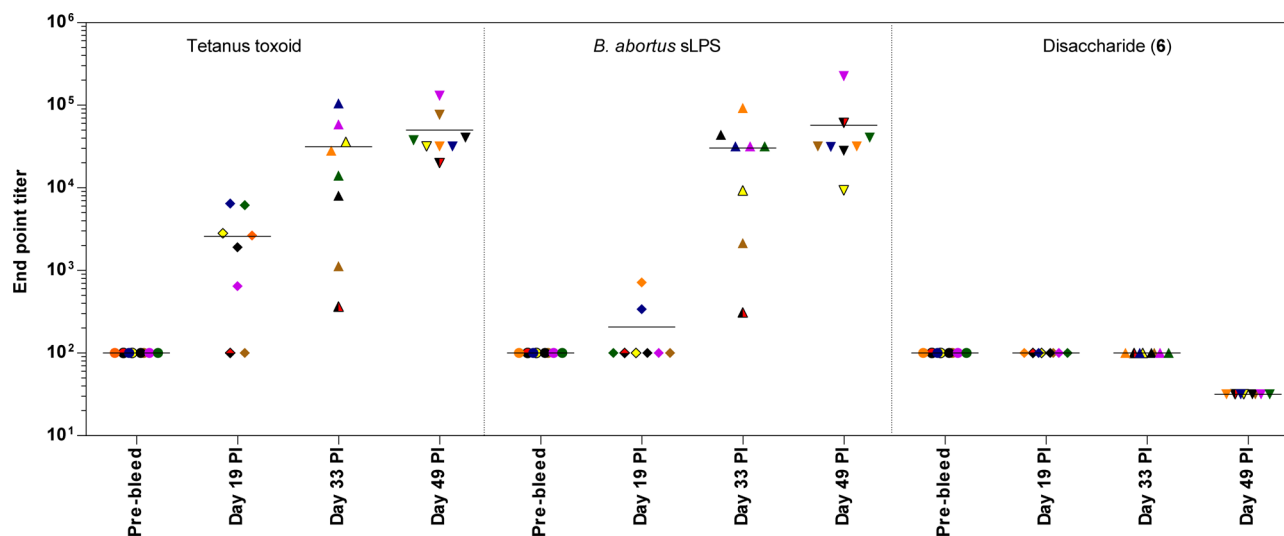

Figure 5. Antibody binding end point titer (*y*-axis) of sera from eight CD1 mice immunized with TT−*B. suis* biovar 2 modified OPS conjugate evaluated against different antigens at four different time points on the *x*-axis (shown in days postimmunization [PI]). Central horizontal bar shows the mean titer. The range of titers tested was $\log_{10}$ 2 to 4.5, except for the final bleed evaluation of the disaccharide **6**.

Immunization with OPS−TT2 gave lower titers than the OPS−TTS99 conjugate although five of the eight mice developed antibodies against *B. abortus* S99 sLPS and four against *B. melitensis* 16M sLPS. Notably, those mice with low or nondetectable antibody response to the sLPS antigens were those that also showed a lower, yet still substantial, response against the tetanus toxoid. The lower average titers generated by OPS−TT2 are most likely due to less efficient conjugation of OPS. Evaluation of the glycoconjugates by SDS−PAGE (Figure S3) and MALDI-ToF (Figures S4−S6) are consistent with this interpretation. More efficient conjugation may also address the higher variability of titers observed with the OPS−TT2 conjugate.

Antibodies against whole cell antigens were also generated. As with the OPS−TTS99, no antibodies were detectable that bound disaccharide and tetrasaccharide M antigens **6** and **7**. Although the antibody titers generated by OPS−TT2 are lower than that induced by OPS−TTS99, the results demonstrate that antibodies that bind linear epitopes and are induced by exclusively 1,2-linked Rha4NFo can bind to these internal epitopes present in M dominant OPS. The binding to the M dominant OPS presumably occurs toward the reducing end of this block copolymer where longer stretches of α1,2-linked Rha4NFo units exist.

The development of the antibody response required a boost before all mice demonstrated an anti-sLPS or TT response (Figure 5). A second boost increased the antibody titers further

until the mean anti-*Brucella* A dominant OPS antibody titer (demonstrated by reaction against *B. abortus* S99 sLPS) was in excess of 1/30,000. At no point was there a response to the disaccharide **7** antigen, even at a 1/32 dilution ($10^{1.5}$).

CONCLUSION

Polysaccharide conjugates such as the one as described here create a long-sought *Brucella* vaccine component that is able to produce the anti-O-antigen antibodies that are considered to be a vital element of the most protective vaccines. Unlike the various whole cell live vaccines, these glycoconjugates produce a dominant immune response against only the α1,2-linked Rha4NFo units that make up the A epitope present in the *Brucella* OPS antigen. Thus, diagnostics based upon the M and terminal epitopes, including disaccharide and tetrasaccharide conjugates (**6** and **7**), provide a gold standard universal diagnostic that can discriminate infected from vaccinated animals. We have shown elsewhere the excellent sensitivity and discriminator power of this diagnostic test.[20] The existence of anti-*Brucella* OPS antibodies that do not bind these M and tip epitope-specific conjugates (**6** and **7**) has been shown previously.[32] We have now demonstrated the means to reliably and exclusively induce high titers of polyclonal antibodies with these particular characteristics; i.e., no cross-reactivity to the M and terminal epitopes. As shown in both cases, OPS tetanus toxoid conjugate induced antibodies can bind whole cell antigens of A and M serotype.[33] The combination of diagnostic test and a vaccine that does not generate antibodies that bind to short M type conjugates establishes the important principles of a viable DIVA.

One could envisage various scenarios for a successful, cost-effective vaccine for ruminants with diverse immune systems. Combining anti-*Brucella* OPS antibody induction with an effective cell-mediated immune response will give the most effective protection against brucellosis.[34] This could be achieved via OPS conjugation to or inclusion of a relevant *Brucella* protein, of which many have been reported.[35] Avoiding growth of *Brucella* to harvest OPS which mandates level 3 containment could be achieved with conjugate vaccines derived from *Y. enterocolitica* O:9, which shares the same Rha4NFo polymer structure as *B. suis* biovar 2[18,23] but requires less stringent containment and likely represents the most cost-effective route to a human vaccine.

Brucellosis is a zoonotic infection that is passed to humans by contact with infected animals but is not spread by human-to-human contact. Eradication of human disease can only be achieved by mass vaccination of animals combined with test and slaughter.[36] Our work is the first to establish a ground-breaking concept by which an OPS-based brucellosis vaccine can be applied in a DIVA format. Moreover, this work has described the main elements of a safe, viable and thermostable glycoconjugate vaccine that could protect humans against the disease. Finally, the study identifies principles that can guide biotechnology approaches for creation of a genetically engineered live DIVA vaccine that exploits known OPS biosynthetic mechanisms.[37−39]

ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscentsci.7b00019.

> Details of chemical syntheses, glycoconjugation, spectroscopic proof of structure including mass spectrometry, immunization, and ELISA protocols (PDF)

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Author Contributions

J.M. and D.R.B. jointly conceived and secured funding for the study, designed the experiments, and prepared the manuscript; S.S.M. and N.V.G. synthesized oligosaccharides and their glycoconjugates; L.D., S.S., and P.J.H. performed immunology and ELISA experiments, and L.D. and L.H. prepared polysaccharide conjugates and performed mass spectrometry experiments on polysaccharide and polysaccharide protein conjugates. All authors have given approval to the final version of the manuscript.

Notes

The authors declare the following competing financial interest(s): None of the authors hold a direct financial interest in the work but disclose that a joint patent application between the University of Alberta and the APHA has been filed for the glycoconjugation process and glycoconjugate products described in this manuscript. Consequently, it is possible that they may benefit from patent licensing.

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