*Immunochemistry*

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# Thiooligosaccharide Conjugate Vaccines Evoke Antibodies Specific for Native Antigens**

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Conjugate vaccines composed of polysaccharides or oligosaccharides covalently linked to immunogenic proteins successfully overcome the limitations of pure polysaccharide antigens, which are classical T-cell-independent antigens.[1] To date, the use of oligosaccharides to provide the recognition element of such conjugate vaccines has largely been limited to experimental, proof of concept investigations, whereas polysaccharide conjugates are successfully employed in efficacious vaccination strategies against life-threatening bacterial infections.[1–3] Intensive efforts are directed toward the synthesis and evaluation of glycopeptide and glycolipid vaccines that present tumour-associated carbohydrate antigens,[4–6] the epitopes of which may contain glycosidic linkages that are potentially susceptible to endogenous glycosyl hydrolases, for example, ganglioside-specific plasma-membrane sialidase.[7–9] Such structural features include, but are not limited to, terminal sialyl residues, the loss of which would destroy crucial recognition elements. A method for the formation of glycosidic linkages that are resistant to enzymatic or acid hydrolysis in vivo has been proposed; C-glycosides are one class of compound that has received attention.[10–12] Herein we present data for four distinct antigen systems that show, for the first time, conjugate vaccines constructed from oligosaccharides that contain a thioglycosidic linkage function as well as antigens that induce antibodies specific for O-linked oligosaccharides.

The choice of sulfur (over atoms such as carbon) to replace oxygen at the glycosidic center was based on three considerations: the ease of synthesis,[13] the existence of well-documented examples with a similar conformational preference about the thioglycosidic and aglyconic bonds both when in solution and when complexed with a protein,[14–17] and significantly lower susceptibility to enzymatic and acid hydrolysis.[18,19] Although the sulfur–carbon bond is longer than the carbon–oxygen single bond, the C–S–C bond angle is significantly smaller than the C–O–C angle, which often results in relatively small differences between the position of the carbon atoms of the glycosidic linkage.[20] However, the longer bonds and weaker stereoelectronic effect that result when oxygen is replaced with sulfur allow substantially greater flexibility.

This flexibility creates a potential obstacle to the use of thioglycoside immunogens. S-Glycosidic bonds are less constrained than the corresponding O-glycosides and can access the higher energy anti conformation in the unbound and bound state more readily. In the search for effective protective carbohydrate epitopes, it remains an open question as to whether such conformational flexibility would preclude the effective use of antigenic determinants that contain metabolically stable S-linkages. Although the structural similarity of O- and S-linked oligosaccharides is critical to this concept, their differences may also identify S-glycosides as non-self antigens and thus further enhance their immunogenicity.

To investigate the immunochemistry of S-linked oligosaccharides, we initially considered two microbial antigens and subsequently two tumour-associated ganglioside antigens (Scheme 1), in which the existence of a potentially labile sialoside linkage might account for the poor immune response toward synthetic conjugate vaccines. Amine-terminated saccharides were coupled to bovine serum albumin (BSA) or tetanus toxoid (TT) by using either of the squarate or adipate homobifunctional linkers shown in Scheme 2.[21,22] Protein

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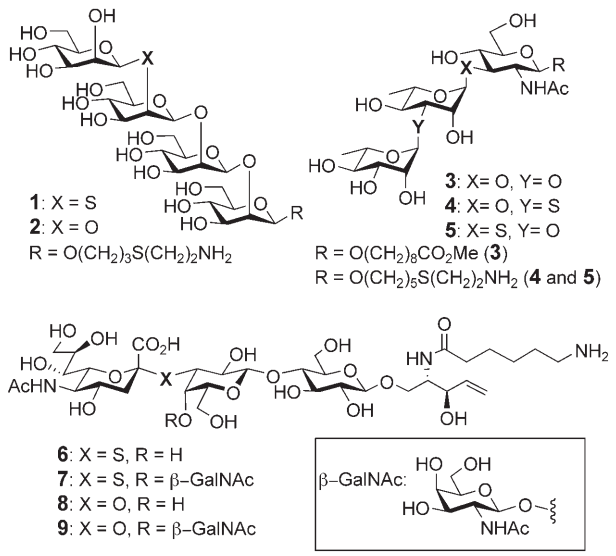
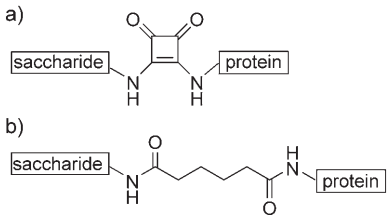



***Scheme 1.*** S- and O-linked synthetic carbohydrate antigens described herein.




***Scheme 2.*** Linkers used in the preparation of glycoconjugates: a) squarate linker and b) adipate homobifunctional linker.

conjugates of the O-glycosides, and for the first time S-glycosides, were evaluated for their ability to induce an antibody response. The antisera from each immunogen was then screened for binding to the immunizing and heteroatom-substituted oligosaccharides that were coupled to a heterologous protein carrier. In general, the S-glycosides induced high titer sera containing carbohydrate-specific immunoglobulin (Ig) and produced a class switch to the IgG isotype, which is associated with the development of immunological memory. Typically, the sera derived from either class of oligosaccharide displayed a similar affinity for the heterologous and homologous oligosaccharides.

Monoclonal antibodies specific for the β-1,2-mannan component of phospomannan and phospholipomannan homopolymers present in the cell wall of *Candida albicans*, protect mice against disseminated and vaginal candidiasis.[23–25] The minimal protective epitope appears to be a β-1,2-linked mannose trisaccharide.[26] Trisaccharide–TT conjugates are highly immunogenic in rabbits and bind to phospomannan when it is part of the cell wall.

Conjugation of oligosaccharides **1** and **2** to TT and BSA provided glycoconjugates for use as immunogens and ligands for ELISA screening (Table 1).[27] The results for the titrations of sera from rabbits, immunized with **10** or **12**, against a crude *C. albicans* cell-wall extract were comparable. The sera of

***Table 1:*** Glycoconjugates for immunization and screening.

| Conjugate | Oligosaccharide | Protein | Linker | $n$[a] |
|---|---|---|---|---|
| **10** | **1**(*S*) | TT | squarate | 33 |
| **11** | **1**(S) | BSA | squarate | 15 |
| **12** | **2**(O) | TT | squarate | 28 |
| **13** | **2**(O) | BSA | squarate | 13 |
| **14** | **3**(O) | BSA | see [29] | 42 |
| **15** | **4**(S) | BSA | squarate | 6 |
| **16** | **5**(S) | BSA | squarate | 7 |
| **17** | **6**(S) | TT | adipate | 8 |
| **18** | **6**(S) | BSA | squarate | 5 |
| **19** | **7**(S) | TT | adipate | 8 |
| **20** | **7**(S) | BSA | squarate | 3 |
| **21** | **8**(O) | TT | adipate | 6 |
| **22** | **8**(O) | BSA | squarate | 10 |
| **23** | **9**(O) | TT | adipate | 7 |
| **24** | **9**(O) | BSA | squarate | 6 |

[a] $n$ = incorporation.

rabbits immunized with **12** had a titre that was approximately tenfold higher than the sera from rabbits immunized with the corresponding S-linked antigen **10**, when screened against the *C. albicans* cell wall extract. Sera raised against **10** exhibited titer volumes that exceeded 1:100 000 demonstrating clearly that carbohydrate-specific antibodies to the thioglycoside cross-react with O-glycosides (Figure 1). Polyclonal sera

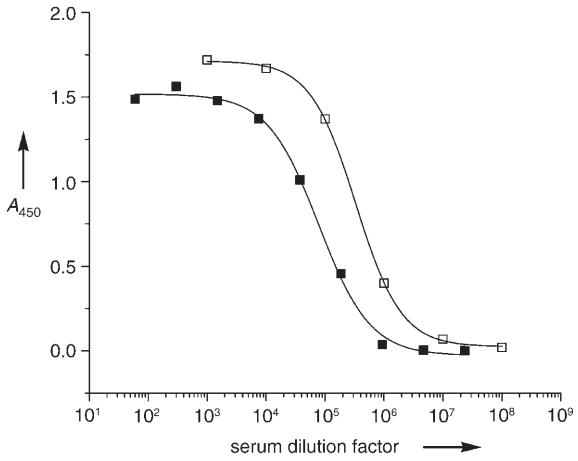



***Figure 1.*** Titration of rabbit sera against *C. albicans* cell-wall extracts. Sera are derived from S tetrasaccharide conjugate **10** (■) and O trisaccharide conjugate **12** (□). Data are averaged for two rabbits.

raised against the thiotetrasaccharide–TT conjugate **10** and the O-linked congener **12** were titrated against the corresponding BSA conjugates **11** and **13**. These data obtained with chemically defined antigens not only show production of high titer sera, but also confirm that the S-linked antigen **11** is recognized by antibodies that are produced in response to the corresponding O-linked immunogen **12**. Furthermore, the O-linked antigen **13** is bound tightly by antibodies induced by the S-linked immunogen **10** (Figure 2).

A lipopolysaccharide (LPS) O antigen provides the second proof of concept. Variant Y *Shigella flexneri* O poly-

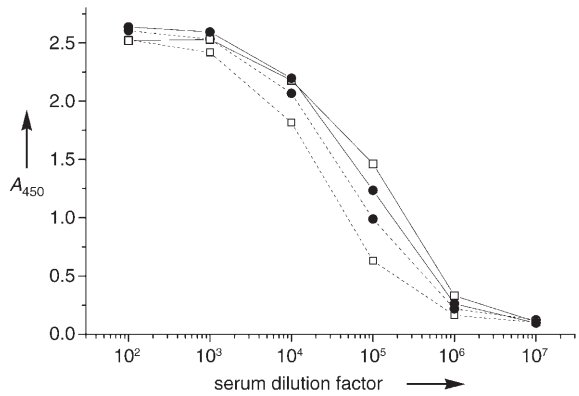



***Figure 2.*** Titration of rabbit sera, obtained after immunization with either **10** (-----) or **12** (——), against immobilized BSA conjugates **11** (□) and **13** (●). Data are averaged for two rabbits.

saccharide has a linear tetrasaccharide repeat [→2)α-L-Rha*p*(1→2)-α-L-Rha*p*(1→3)-α-L-Rha*p*(1→3)-β-D-GlcNAc*p*(1→]. Binding studies with a monoclonal antibody SYA/J6 has identified the trisaccharide α-L-Rha*p*(1→3)-α-L-Rha*p*(1→3)-β-D-GlcNAc*p* as the minimal recognition epitope. This trisaccharide was synthesized as the O-linked structure **3** and as two monothiooligosaccharide analogues, **4** and **5**.[28–30] BSA conjugates of the three trisaccharides were prepared, and murine sera derived from immunization with each trisaccharide–BSA conjugate **14**–**16** (Table 1) were titrated against purified LPS that had been extracted from the parent *S. flexneri* bacteria (Figure 3). Recognition of LPS

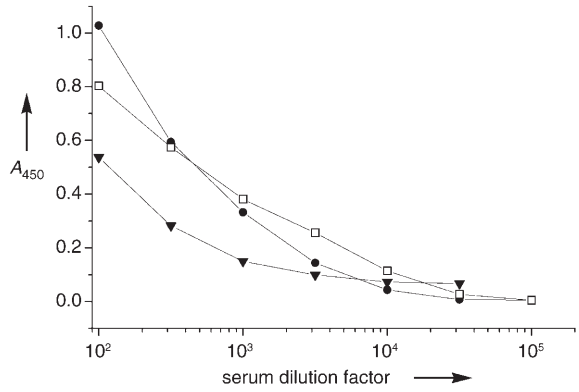



***Figure 3.*** Binding of antibodies from polyclonal sera, derived by immunization with conjugates **14** (▼), **15** (□), and **16** (●), to immobilized *S. flexneri* LPS. Data are averaged for groups of five mice.

by immune sera derived from S-linked oligosaccharides is (represented by specific IgG) to S-glycosides appears equivalent, if not superior, to the response to O-glycosides as judged by the antibody recognition of native LPS. Consistent with these observations, antibodies to the conjugates **15** and **16** were also shown to bind the trisaccharides **4** and **5** and the tetrasaccharide α-L-Rha*p*(1→3)-α-L-Rha*p*(1→3)-β-D-GlcNAc*p*(1→2)-α-L-Rha*p* when they were immobilized on microtiter plates by noncovalent adsorption (data not shown).

Aberrant expression of gangliosides on malignant cells has highlighted the potential of these glycolipids to serve as antigens in cancer immunotherapy.[4,6] Both the trisaccharide $GM_3$ (α-Neu5Ac-(2→3)-β-Gal-(1→4)-β-Glc-(1→1)-ceramide) and the tetrasaccharide $GM_2$ (α-Neu5Ac-(2→3)-[β-GalNAc-(1→4)]-β-Gal-(1→4)-β-Glc-(1→1)-ceramide) are considered promising vaccine targets, yet their status as self-molecules and their correspondingly poor immunogenicity have hampered progress toward the development of a successful conjugate vaccine. Although endogenous enzyme hydrolysis of $GM_2$ is not a concern, at least in serum, extracellular enzymatic degradation of $GM_3$ is known.[7] We have modified the common terminal glycosidic linkage of $GM_3$ and $GM_2$ by replacement of the interresidue oxygen atom with sulfur to give the ganglioside analogues for use as antigens with potentially enhanced immunogenicity. The thiogangliosides **6** and **7**[31] and their O-linked counterparts **8** and **9**[32] bear a truncated ceramide aglycon for coupling to proteins.

The in vivo immunogenicity of ganglioside glycoconjugates (Table 1) was evaluated by ELISA. Immunization of BALB/C mice with TT conjugates (**17** and **21**) of the S- and O-linked $GM_3$ analogues **6** and **8** yielded a carbohydrate-specific IgG response in three out of five mice. Sera derived from either immunogen were found to cross-react with the homo- and heterosubstituted counterparts **18** and **22** (Figure 4). The

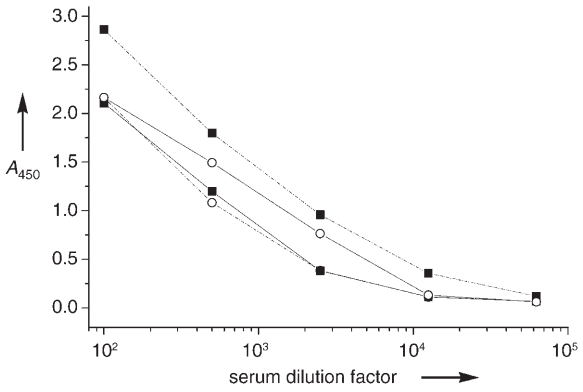



***Figure 4.*** Binding to immobilized **22** (○) and **18** (■) by murine IgG generated from immunization of mice with **21** (——) or **17** (-----). Data are averaged for three mice from each group.

S-linked conjugate **17** was the more immunogenic of the two $GM_3$ analogues when screened against the immunizing carbohydrate. Cross-reactivity of these antisera with the O-glycoside was further supported by its unambiguous recognition of a α-Neu5Ac-(2→3)-β-Gal-BSA–disaccharide conjugate (see Supporting Information). Furthermore, binding of the same sera to glycoconjugate **22** could be inhibited by the addition of synthetic O-linked oligosaccharides **25** and **26** (Figure 5).[31]

Immunization with the S-$GM_2$–TT conjugate **19** also generated a strong carbohydrate-specific IgG response, although recognition of the immobilized glycoconjugates was restricted to the S-linked $GM_2$ analogue **20**. In contrast, sera to the related O-$GM_2$–TT conjugate **23** bound both O-

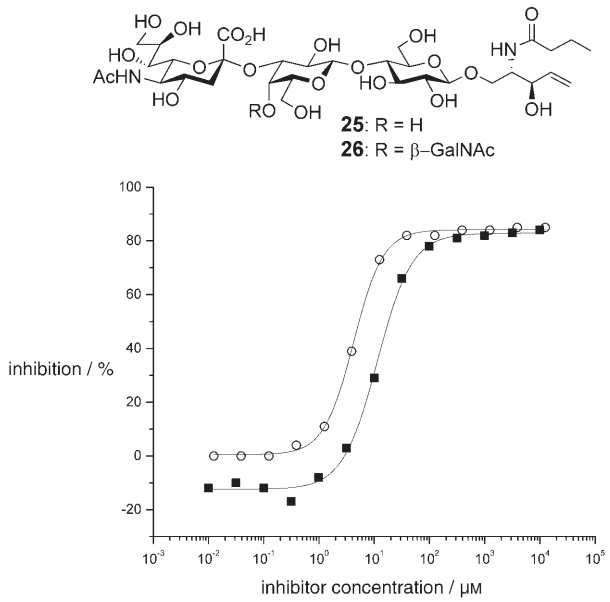



***Figure 5.*** Synthetic oligosaccharides **25** (○) and **26** (■) inhibit the binding of polyclonal sera (IgG) against conjugate **17** to immobilized **22**. $IC_{50}$ values were determined to be 4.2 μM (**25**) and 11.3 μM (**26**).

and S-glycosides and demonstrated equivalent or superior binding of the heterologous oligosaccharide (see Supporting Information). The apparent incongruity in the response to thioimmunogens **17** and **21**, evident in the inability of antibodies to S-$GM_2$ to recognize the O-glycoside, can be rationalized in terms of the flexibility of the thioglycosidic linkage in **7**. Although the *exo* anomeric effect would be expected to hold the O-linked GalNAc residue in its preferred family of low-energy conformations, the S-linked Neu5Ac should be less constrained and any steric demands imposed by vicinal substitution could be relieved by adoption of conformations that might not be populated as readily by O-linked $GM_2$.

S-linked immunogens, as components of conjugate vaccines, clearly generate an antigen-specific immune response that approaches (**10** and **19**) or exceeds (**15**, **16**, and **17**) the response to the native oligosaccharide. With the exception of antigens that have terminal, vicinal-branching residues (**19**), antibodies against S-glycosides demonstrate a clear cross-reactivity with the O-glycosides, which suggests a certain degree of conformational similarity. In the systems studied, superior or equivalent recognition of a heterologous oligosaccharide was demonstrated for either the O- or S-linked immunogens or both. The recognition of S-glycosides by sera derived from the native antigens may be explained by the flexibility of the thioglycosidic linkage, which may allow for an induced fit.

We have shown for the first time that carbohydrate epitopes of conjugate vaccines may be modified to contain S-linked residues and that immune responses to such vaccines are of comparable magnitude in terms of antibody titer. Furthermore, the specificity of these antibodies exhibits only a modest loss of fidelity with respect to recognition of the native O-linked epitope. We anticipate that antigens bearing critical residues attached through hydrolysis-resistant linkages will find application either in active immunotherapy or as ligands for immunoabsorption of pathological antiganglioside antibodies present in the sera of patients with certain autoimmune neuropathies.[33]

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