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Immunochemical characterization of polyclonal and monoclonal *Streptococcus* group A antibodies by chemically defined glycoconjugates and synthetic oligosaccharides

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ABSTRACT

Synthetic oligosaccharides of increasing complexity that represent different epitopes of the *Streptococcus* Group A cell-wall polysaccharide were used as haptens and glycoconjugates of bovine serum albumin (BSA) and horse hemoglobin (HHb) to characterize polyclonal and monoclonal antibodies. Rabbits were immunized with the BSA glycoconjugates of a linear trisaccharide, branched trisaccharide, and branched pentasaccharide. The binding specificities of the polyclonal antisera were determined by a series of inhibition ELISA studies in which disaccharide through pentasaccharide haptens were used as inhibitors of antibody–glycoconjugate binding. Monoclonal antibodies derived from mice immunized with a killed bacterial vaccine were selected for their binding to native polysaccharide antigen coupled to BSA and the BSA glycoconjugates of the di- and linear tri-saccharides. Polyclonal antibodies were moderately specific for the oligosaccharide epitope of the immunizing glycoconjugate and only those antibodies raised to the branched pentasaccharide antigen showed cross-reaction with the bacterial antigen. The behaviour of selected monoclonal antibodies parallels the binding profile of polyclonal antibodies in that the two highest-titre antibodies were directed toward an epitope displayed by the branched pentasaccharide.

INTRODUCTION

The Gram-positive β-hemolytic *Streptococcus* Group A are widespread bacteria which are pathogenic in humans. Infection with this organism can result in the common condition of streptococcal pharyngitis or strep throat [1]. The initial streptococcal infection has been implicated in the development of the more serious condition of rheumatic fever and other diseases such as heart valve disease, acute

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glomerulonephritis, and rheumatoid arthritis [1–3]. Cross-reactivity between streptococcal Group A carbohydrate antigens and tissue antigens has been implicated in the pathogenesis of these disease states [4–7]. However, the exact basis for such correlations is far from clear [1–3]. Well defined antibodies directed against carbohydrate epitopes could be used to clarify and provide a molecular basis for the correlations.

We describe herein the use of glycoconjugates to induce polyclonal antibodies that bind the native polysaccharide antigen. The binding specificities were further defined by synthetic oligosaccharide inhibitors. The same materials were also used to select and characterize mouse monoclonal antibodies generated by a bacterial vaccine.

EXPERIMENTAL

Bacteria.—Streptococcus pyrogens Group A type 4, strain J17A4 bacteria were heat-killed. The pepsin digest was performed according to Krause [8] and yielded a vaccine whose concentration was determined to be 300 μg/mL rhamnose by use of a phenol–H_2SO_4 test [9].

Glycoconjugates and oligosaccharide haptens.—Oligosaccharides were synthesized as their 1-propyl and 8-(methoxycarbonyl)octyl glycosides [10–13]. The latter were converted via hydrazide derivatives to acyl azide intermediates which were coupled to bovine serum albumin (BSA), as previously described [13–15]. One of the hydrazides, **8**, was also used as an inhibitor. Horse hemoglobin (HHb) glycoconjugates were prepared in analogous fashion to the BSA conjugates. Incorporation levels ranged between 8–18 molecules of hapten per molecule of BSA or HHb, as determined by carbohydrate analysis [9]. The polysaccharide–BSA conjugate was prepared according to Herbst et al. [16].

Preparation of BSA affinity gel.—To a stirred slurry of Sepharose 6B gel (100 mL) (Pharmacia, Uppsala, Sweden) 2 M in Na_2CO_3 (100 mL) at 5°, was added a CH_3CN–CNBr solution (10.0 mL, 2.0 g/mL). The slurry was stirred for 3 min at 5°, and then poured into a sintered-glass funnel and rinsed with 0.1 M $NaHCO_3$, followed by a rinse with distilled water (1000 mL). The activated gel was then transferred to a beaker, by use of 0.2 M $NaHCO_3$–Na_2CO_3, pH 9.5 (100 mL). To this suspension was added a solution of BSA, crystallized 99%, (400 mg in 5.0 mL of 0.1 M $NaHCO_3$–Na_2CO_3, pH 9.5) (ICN Biochemicals, Cleveland, OH). The solution was kept at room temperature, with occasional stirring, for 2.5 h. The BSA-gel was poured into a sintered-glass funnel and filtered. The protein content of the filtrate was determined by measuring the A_{280}, and it was estimated that 98% of the BSA was bound. The gel was then taken up in 0.2 M $NaHCO_3$–Na_2CO_3, pH 9.5, (200 mL) containing 0.2 M glycine to block any unbound activated sites. The solution was kept at room temperature for 3 h with occasional stirring. The gel was then filtered on a sintered-glass funnel, and washed with 0.1 M $NaHCO_3$, pH 8.2 (200 mL), followed by 0.1 M NaOAc, pH 4.0 (200 mL). The cycle of washings

was repeated four times, and then the gel was taken up in PBS, containing NaN_3 (0.05%), and stored at 4° until needed.

Immunization of rabbits with BSA glycoconjugates.—Each of the three glycoconjugates **1a**, **2a**, and **3a** (12 mg) were dissolved in 0.01 M phosphate buffered saline (PBS) (12.0 mL, pH 7.0) and emulsified with Freund's Complete Adjuvant (15.0 mL) (Difco Laboratories, Detroit, MI). For each glycoconjugate vaccine, one group of four New Zealand White rabbits were given 0.1 mL/rabbit via subcutaneous injections twice a week for a period of three weeks. Rabbits were exsanguinated 8–10 days after the last injections and the serum was centrifuged prior to being frozen at −20°.

Purification of rabbit sera.—Samples of the antisera from each of the 4 rabbits in each group were pooled to give three separate samples of serum, one from each glycoconjugate immunization. Serum samples (5.0 mL) were applied to a protein A–Sepharose CL-4B column system (Pharmacia Canada Inc., Dorval, PQ) which was maintained at 4°. The IgG fraction was bound by use of 1.5 M glycine, containing 3 M NaCl adjusted to pH 8.9 by use of 5 M NaOH. The bound antibodies were eluted by use of citric acid (100 mM), adjusted to pH 4.0 by use of 5 M NaOH. The antibody-containing fractions were pooled and dialyzed against 0.1 M Tris · HCl, pH 8.5, containing 0.5 M NaCl and concentrated to the original 5-mL volume, by use of an Amicon ultrafiltration apparatus. The sample was then loaded onto a BSA–Sepharose 6B affinity column (prepared as already described) in the same buffer, and the effluent fractions were combined and dialyzed against 50 mM Tris · HCl, pH 8.0, containing 150 mM NaCl, and concentrated, by use of an Amicon ultrafiltration apparatus. The protein concentrations for the purified antibody preparations were determined by measuring A_{280} ($\epsilon = 1.35$ mL cm^{-1} mg^{-1} for IgG immunoglobulin).

Immunizations of mice.—A/J Mice and BALB/c mice were given a series of 6 and 7 injections, respectively, over periods of 53 and 67 days. All injections were intraperitoneal except for the final one given intravenously 3 days prior to fusion. The injections consisted of 0.1 mL of the pepsin-treated cell vaccine in PBS; the equivalent to 30 μg rhamnose.

Fusions, ELISA screening, cloning and isotyping.—Two separate fusion experiments were carried out, in which spleen cells from two immunized mice were fused with the nonimmunoglobulin-producing Sp2/0 plasmacytoma cell-line [17] (Institute for Medical Research, Camden, NJ), as previously described [18,19]. The cell colonies were screened initially with a *Streptococcus* Group A polysaccharide BSA glycoconjugate (GAP–BSA) [16], and further screenings were carried out with disaccharide and trisaccharide glycoconjugates as well. The screenings were carried out by indirect ELISA [18,19]. Bound antibody was detected by use of an alkaline phosphatase-labelled goat anti-mouse immunoglobulin M (IgM) and IgG reagent (Zymed, Cedarlane Laboratories Ltd., Hornby, ON). Hybridomas judged to be positive in the ELISA were cloned by limiting dilution, using mouse spleen cells as feeders. All hybrids were cloned twice to ensure clonality and stability before cell

samples were frozen and ascites fluid was raised. The fusion experiment with A/J mice produced six stable hybridomas, and the BALB/c fusion experiment produced one stable hybridoma. Heavy and light chain class were established by ELISA conducted with spent culture supernatant and monoclonal antibody isotyping kit (Amersham Canada Ltd., Oakville, ON).

Ascites fluid.—BALB/c mice were primed by intraperitoneal injection with 0.5 mL of 2,6,10,14-tetramethylpentadecane (pristane). After 14 days, the mice were injected (intraperitoneal) with 10^6 hybridoma cells, and tapped for ascites fluid after a further 7–10 days. The ascites fluid was centrifuged, filtered and stored at −20° until further use.

Purification of monoclonal antibodies by $(NH_4)_2SO_4$ precipitation.—The ascites fluid was centrifuged for 30 min at 4° (10000 *g*), and then filtered through a 0.22-μm filter. An equal volume of saturated $(NH_4)_2SO_4$ was added dropwise with stirring to the filtrate. The solution was left stirring for 16 h at 4°, and then centrifuged for 30 min at 4° (10000 *g*). The pellet was resuspended in PBS (0.5 times the original volume of ascites fluid) and then dialyzed against PBS containing 0.02% NaN_3. The protein concentrations of the antibody solutions were determined by measuring A_{280} ($\epsilon = 1.2$ mL cm^{-1} mg^{-1} for IgM immunoglobulin).

Indirect enzyme-linked immunosorbent assay.—In a typical indirect ELISA, 96 well microtitre plates (Linbro; Flow Laboratories, Missisauga, ON) were coated with a glycoconjugate solution, 10 μg/mL in PBS, 100 μL/well. The glycoconjugate solution was allowed to bind for 3 h at room temperature, and then washed three times with PBS, containing 0.05% Tween 20 and 0.02% NaN_3 (PTA). Alternatively, the plates could be stored at 4° until needed. To the washed plates, an antibody solution diluted in PBS was then added, 100 μL/well. The plates were left for 3 h at room temperature and then washed three times with PTA. An alkaline phosphatase-labelled reagent, either goat anti-rabbit IgG (Miles Scientific, Rexdale, ON) or goat anti-mouse IgM and IgG (Zymed) diluted in PBS containing 1% BSA was added, 100 μL/well. The labelled antibody was allowed to react for 1 h at room temperature and then the plates were washed three times with PTA. The enzyme substrate *p*-nitrophenyl phosphate (*p*-NPP) at 1.0 mg/mL in 0.05 M $NaHCO_3$–Na_2CO_3, pH 9.8, containing 0.001 M $MgCl_2$ was added, 100 μL/well. After 1 h at room temperature, the absorbance was read at 405 nm.

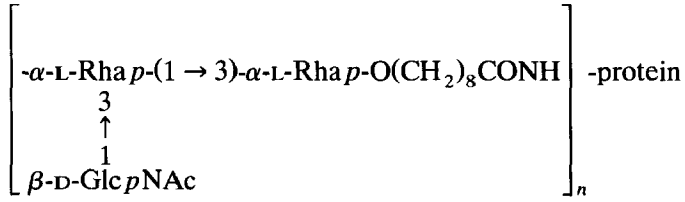
Indirect inhibition ELISA by use of rabbit polyclonal antibodies.—Prior to the inhibition assay, the appropriate concentrations to use for each glycoconjugate–antibody combination were determined in an indirect ELISA in which the concentrations of the coating glycoconjugate and the antibody to be tested, were varied simultaneously in a checkerboard fashion. Following this determination, the EIA plates were coated with an appropriate glycoconjugate concentration, 1–10 μg/mL in PBS, 100 μL/well. The glycoconjugate solution was allowed to bind for 3 h at room temperature and the plates were then washed three times with PTA. To the washed plates, solutions of inhibitors were added at 2 × the final concentration, 50 μL/well, serially diluted in PBS containing 0.1% BSA (dilution factor used was

√10). The antibody solution was then added at 2 × the determined final concentration, 1.5–10 μg/mL, 50 μL/well in PBS containing 0.1% BSA. All inhibitor concentrations were run in triplicate on the same plate. Several wells were used as reference wells, where in place of an inhibitor solution, 50 μL of PBS containing 1% BSA was added. These wells were used to calculate the 0% inhibition value. The plates were left at room temperature for 16–18 h and then washed three times with PTA. Alkaline phosphatase-labelled goat anti-rabbit IgG antibody was added, 100 μL/well, diluted 1:1000 in PBS containing 1% BSA and the plates were developed according to the above protocol.

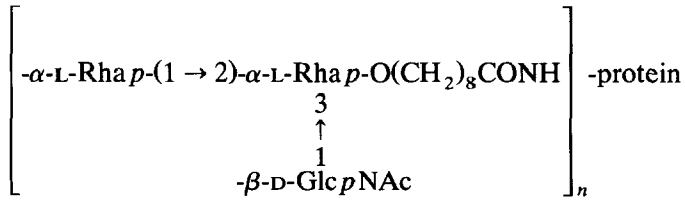
Inhibition studies of monoclonal antibodies.—The inhibition studies were carried out by use of an indirect inhibition ELISA, as already described. Concentration levels for each glycoconjugate–antibody combination were determined in an indirect ELISA where the concentrations of the coating glycoconjugate and the antibody to be tested were varied simultaneously in a checkerboard fashion. Bound antibody was detected by use of an alkaline phosphatase-labelled goat anti-mouse IgM reagent (Zymed) diluted 1:1000 in PBS containing 1.0% BSA.

RESULTS

The antigens **1a**, **2a**, and **3a** were used to immunize three groups of rabbits. The corresponding horse hemoglobin (HHb) conjugates were then used to assay carbohydrate-specific antibody.



Antigen **1a**: Protein = BSA
Antigen **1b**: Protein = HHb



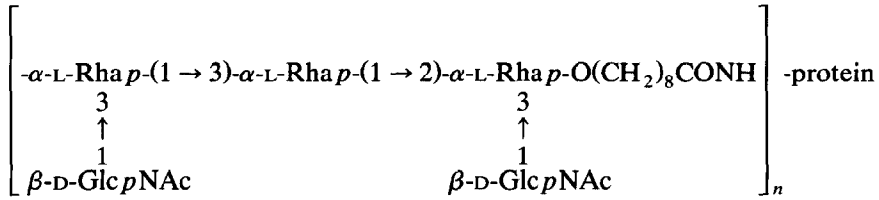
Antigen **2a**: Protein = BSA
Antigen **2b**: Protein = HHb

TABLE I

Inhibition data [a] for rabbit polyclonal antisera with synthetic di- through penta-saccharides

Compound	Antisera		
	A (μM)	B (μM)	C (μM)
4	> 24400	n.a. [b]	1700
5	40.5	n.a. [c]	> 18000
6	n.a. [c]	37.8	> 18000
7	3500 [d]	2000 [d]	3500 [d]
8	2400 [d]	870	6.0

[a] Concentration of inhibitor compound (μM) required for 50% inhibition, using solid phase antigen **1b**, **2b**, and **3b** for antisera A, B, and C, respectively. [b] No inhibitory activity at 2400 μM. [c] No inhibitory activity at 1800 μM. [d] Extrapolated values.



Antigen **3a**: Protein = BSA

Antigen **3b**: Protein = HHb

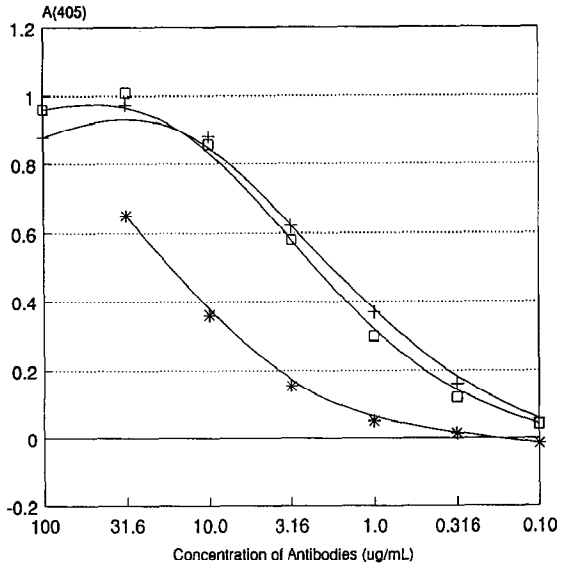


Fig. 1. Titration of three different rabbit polyclonal antisera, using glycoconjugate **1b** as the solid-phase antigen in the indirect ELISA: (+) antiserum A raised against glycoconjugate **1a**; (∗) antiserum B raised aginst glycoconjugate **2a**; (□) antiserum C raised against glycoconjugate **3a**.

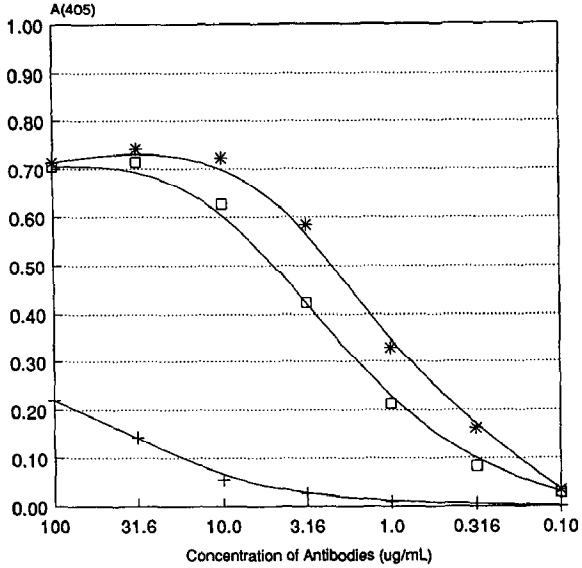


Fig. 2. Titration of three different rabbit polyclonal antisera, using glycoconjugate **2b** as the solid-phase antigen in the indirect ELISA: (+) antiserum A raised against glycoconjugate **1a**; (*) antiserum B raised against glycoconjugate **2a**; (□) antiserum C raised against glycoconjugate **3a**.

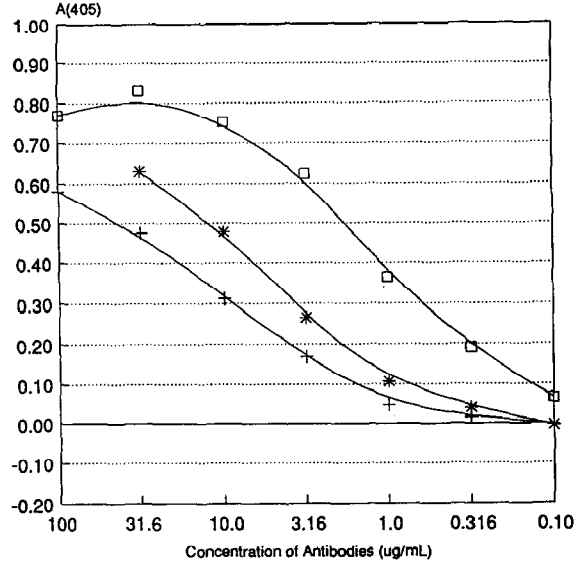


Fig. 3. Titration of three different rabbit polyclonal antisera, using glycoconjugate **3b** as the solid-phase antigen in the indirect ELISA: (+) antiserum A raised against glycoconjugate **1a**; (*) antiserum B raised against glycoconjugate **2a**; (□) antiserum C rasied against glycoconjugate **3a**.

TABLE II

Inhibition data [a] for monoclonal antibody SA-3

Monoclonal Ab	Inhibitors (μM)				
	4	**5**	**6**	**7**	**9** [b]
SA-3	97.8 (1.0) [c]	126 (1.1)	113 (1.1)	67.6 (0.8)	17.8 (0.0)

[a] Concentration of inhibitor compound (μM) required for 50% inhibition, using solid-phase antigen **1a**. [b] This inhibitor was used as the reference for the indicated series. [c] The values in parentheses are for $\Delta(\Delta G)$ in kcal mol^{-1}, determined from the expression $\Delta(\Delta G) = RT \ln([I_1]/[I_2])$, where $[I_2]$ is the concentration of the reference inhibitor, $[I_1]$ is the concentration of the other inhibitors both measured at 50% inhibition, $R = 1.98$ cal K^{-1} mol^{-1}, and $T = 295$ K; more positive values for $\Delta(\Delta G)$ indicate poorer binding.

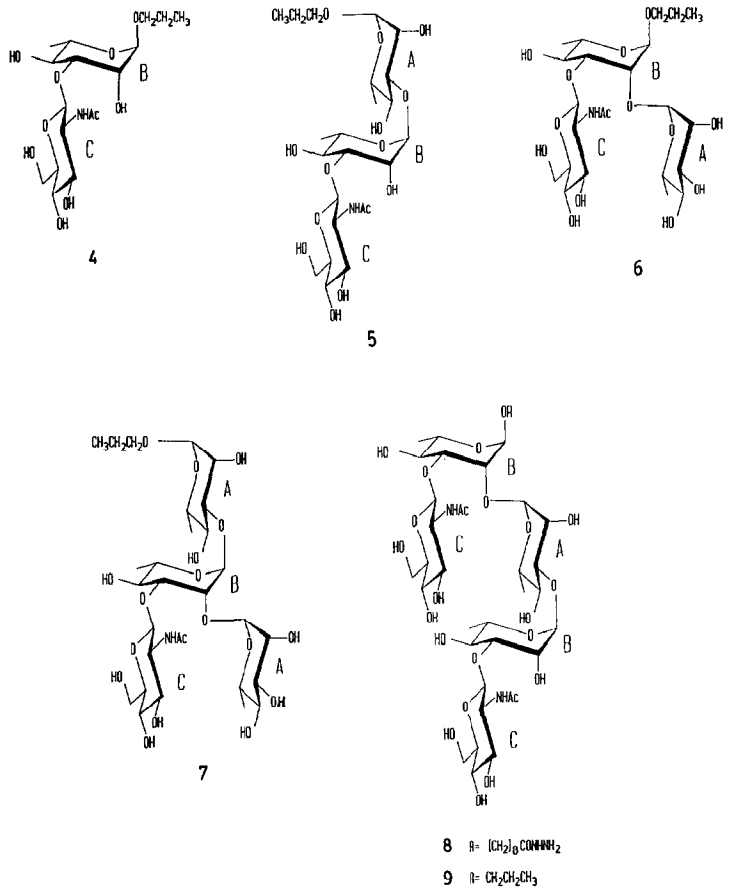


Fig. 4. Panel of inhibitors used in binding studies with rabbit polyclonal antisera and mouse monoclonal antibodies. The letters A, B, and C identify the three pyranose residue types present in the bacterial polysaccharide repeating-unit (A-B-[C]-)$_n$.

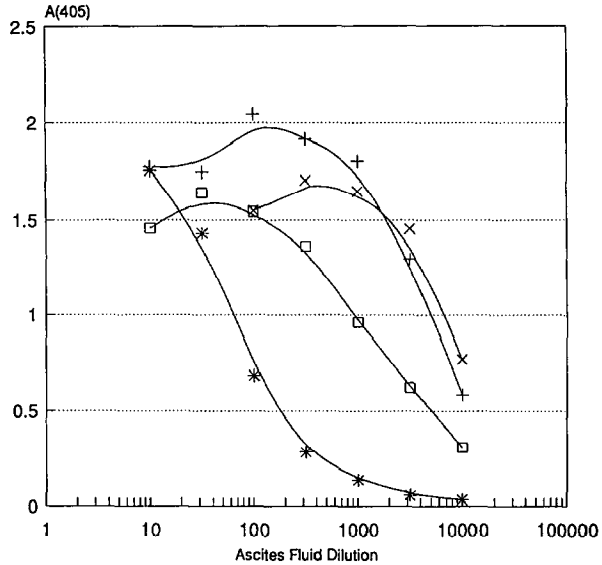


Fig. 5. Titration of the 4 ascites fluids containing monoclonal antibodies: SA-1 (+); SA-2 (□); SA-3 (×); and SA-4 (*); using an indirect ELISA with a Group A polysaccharide–BSA glycoconjugate as the solid phase antigen.

Pre-immune sera screened by ELISA, using the three BSA-glycoconjugates, showed no background activity to the glycoconjugates. Immune sera from rabbits within each group were then pooled and the antibody pools were labelled "A", "B", and "C" corresponding to BSA-glycoconjugates **1a**, **2a**, and **3a**, respectively. The IgG antibody fraction of these pools was enriched by sequential protein A chromatography and BSA affinity chromatography to remove BSA-specific antibodies. Each antibody was titrated against the three glycoconjugates **1b**, **2b**, and **3b** (Figs. 1–3). Antibody raised to linear trisaccharide (**1a**) and branched pentasaccharide (**3a**) behave identically with glycoconjugate **1b**. Antibody to the branched epitopes **2a** and **3a** binds with similar titres to the glycoconjugate **2b**. The pentasaccharide screening conjugate **3b** reacts well with antibody from the pool C, as expected.

Inhibition ELISA experiments were performed for each of the three antibody pools with the synthetic oligosaccharides 4–8 (Fig. 4 and Table I). In all cases the most active inhibitor was complementary to the oligosaccharide hapten of the immunizing antigen.

Two separate hybridoma experiments were performed with BALB/c and A/J mice. In both cases the animals were immunized with killed whole-cell vaccine and the resultant hybrid clones were screened in ELISA using Group A polysaccharide–BSA (GAP-BSA) conjugate and the disaccharide and trisaccharide–BSA conjugates. A total of seven clones were selected (6 from A/J mice and 1 from BALB/c) for binding to the GAP-BSA and of these four also bound to glycoconju-

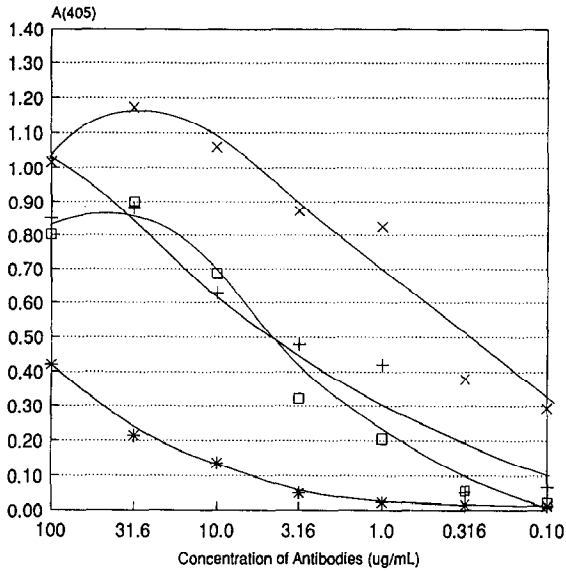


Fig. 6. Titration of the monoclonal antibodies: SA-1 (+); SA-2 (□), SA-3 (×); and SA-4 (*); obtained from $(NH_4)_2SO_4$ precipitation of the corresponding ascites fluid, using glycoconjugate **1a** as the solid-phase antigen in the indirect ELISA.

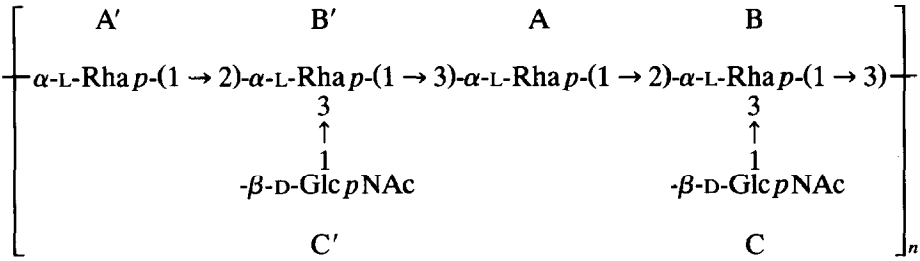
gate **1a** (Figs. 5 and 6). Isotyping showed all four to belong to the IgM class; SA-1, SA-2, and SA-3 have κ light chains, while SA-4 has a λ chain. ELISA titres showed that the ascites fluids containing antibodies SA-1, SA-2, and SA-3 have comparable end-point titres with GAP-BSA (Fig. 5). After ammonium sulfate precipitation, SA-3 gave a titre with **1a** that was an order of magnitude higher than those of SA-1 and SA-2.

The oligosaccharide inhibitor power was measured for SA-3 using the five synthetic oligosaccharides **4–7** and **9** (Fig. 4). The concentration of inhibitor required to reach 50% inhibition (Table II) established the following order of inhibitory potency: pentasaccharide > tetrasaccharide > disaccharide ≈ branched trisaccharide ≈ linear trisaccharide.

DISCUSSION

The *Streptococcus* Group A cell-wall polysaccharide consists of a poly-L-rhamnopyranosyl backbone composed of alternating α-L-(1 → 2), and α-L-(1 → 3) linkages. The 3-positions of the poly-rhamnopyranosyl backbone have branching *N*-acetyl-β-D-glucosamine residues [20]. It was envisaged that a variety of polyclonal as well as monoclonal antibodies could be generated to various epitopes present within this structure. Towards this end, oligosaccharide portions of this structure were prepared by chemical synthesis [10–13]. A disaccharide sequence, two trisaccharide sequences, and a pentasaccharide sequence were prepared with the 8-

(methoxycarbonyl)octyl linking arm [14] as the aglycon [10,11,13]. This linking arm permitted [14,15] the attachment of the oligosaccharides to the protein carriers BSA and HHb.



Polyclonal antibodies raised against a particular glycoconjugate exhibited high specificity for the oligosaccharide epitope of that immunizing antigen (Figs. 1–3). Since the pentasaccharide **8** contains within its structure both epitopes represented by the linear trisaccharide **5** and branched trisaccharide **6**, the antibodies generated by glycoconjugate **1a** and **2a** cross-react with the branched pentasaccharide antigen **3b** (Fig. 3).

Only the antibodies generated to the pentasaccharide glycoconjugate **3a** cross-reacted with the native Group A polysaccharide. Titration curves (Fig. 3) and inhibition data (Table 1) further indicate that these antibodies recognize a more extended epitope than any of the lower homologous linear trisaccharide **5**, branched trisaccharide **6** or branched tetrasaccharide **7**.

Similar conclusions can be reached for the monoclonal antibody SA-3, despite the fact that it was generated from a bacterial vaccine. This antibody binds to both the polysaccharide conjugate GAP-BSA (Fig. 5) and the linear trisaccharide conjugate **1a** (Fig. 6). Oligosaccharides of increasing complexity exhibited increasing potency. These data (Table II) suggest that both the size and branch point are essential features of the Group A epitope.

Previous workers [21] have produced monoclonal antibodies to a streptococcal group A vaccine. They concluded that there were two basic types of antibody produced. One type was a high-affinity antibody directed at an extended portion of the polysaccharide chain, with the binding site probably resembling a shallow groove. The other type was a low-affinity antibody with a small binding site probably directed at the tips of the polysaccharide chain, and able to accommodate only single sugar residues. Extensive inhibition studies were not carried out with these antibodies; however, both types were shown to bind *N*-acetylglucosamine. The SA-3 antibody would appear to have similar binding characteristics to the high-affinity antibodies directed at internal sequences which were identified by Braun et al. [21].

Glycoconjugates prepared from oligosaccharides of increasing complexity have been shown to provide good selectivity and discrimination in producing and characterizing carbohydrate-specific antibody. In particular, the branch point of the *Streptococcus* Group A antigen appears to be a crucial element of the epitope

recognized by both polyclonal and monoclonal antibodies that are able to bind the native antigen. Synthetic methodology has now been developed to readily furnish high order oligosaccharides that span two or more branch points. Glycoconjugates prepared from the latter structures can facilitate the selection and characterization of antibodies with binding profiles for defined epitopes. These glycoconjugates should also provide a more accessible source of defined antigens that can be used in a variety of diagnostic formats.

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