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Determination of the specificity of monoclonal antibodies against *Schistosoma mansoni* CAA glycoprotein antigen using neoglycoconjugate variants

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Abstract

The immunogenic *O*-glycan of circulating anodic antigen (CAA) is a high-molecular-mass polysaccharide with the unique →6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→ repeating unit. To obtain information at the molecular level about the specificity of monoclonal antibodies against CAA, the immunoreactivity of two series of bovine serum albumin-coupled synthetic oligosaccharides related to the CAA *O*-glycan was monitored using ELISA and surface plasmon resonance spectroscopy. The importance of the axial hydroxyl group of β-D-Gal*p*NAc for antibody binding was investigated using the following series of analogues: β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→O); β-D-Glc*p*NAc-(1→6)-[β-D-Glc*p*A-(1→3)]-β-D-Glc*p*NAc-(1→O); and β-D-Glc*p*A-(1→3)-β-D-Glc*p*NAc-(1→6)-[β-D-Glc*p*A-(1→3)]-β-D-Glc*p*NAc-(1→O). In the second series of analogues, β-D-Glc*p*6*S*-(1→3)-β-D-Gal*p*NAc-(1→O), β-D-Gal*p*NAc-(1→6)-[β-D-Glc*p*6*S*-(1→3)]-β-D-Gal*p*NAc-(1→O), and β-D-Glc*p*6*S*-(1→3)-β-D-Gal*p*NAc-(1→6)-[β-D-Glc*p*6*S*-(1→3)]-β-D-Gal*p*NAc-(1→O), the native β-D-Glc*p*A moiety was replaced by β-D-Glc*p*6*S* to evaluate the influence of the nature of the charge on antibody recognition. Comparison of the immunoreactivity of these series with that measured for conjugates containing corresponding synthetic CAA fragments showed that the antibody binding levels can be correlated to the antibody specificity to CAA fragments. For the most reactive antibodies, the structural changes chosen (β-D-Gal*p*NAc replaced by β-D-Glc*p*NAc, and β-D-Glc*p*A replaced by β-D-Glc*p*6*S*) completely eradicated the binding.

Keywords: circulating anodic antigen; monoclonal antibody recognition; *Schistosoma mansoni*; surface plasmon resonance.

Introduction

Schistosomiasis, otherwise known as bilharzia, is one of the main parasitic infections affecting man in (sub)tropical areas. An estimated 200 million people are infected and another 500–600 million are at risk of exposure and/or infection (Brooker and Michael, 2000; Chitsulo et al., 2000). The disease is caused by digenic trematodes of the genus *Schistosoma*, which reside in the portal and mesenteric veins of the host (Sturrock, 1993). The three major schistosome species infecting man have a complex life cycle that includes a freshwater snail as intermediate host and a definitive vertebrate host (Sturrock, 1993).

Reliable and sensitive diagnostic procedures are crucial for decisions on individual or community treatment, estimation of prognosis and assessment of morbidity, control measures, and evaluation of chemotherapy of schistosomiasis (Berquist, 1992; Feldmeier and Poggensee, 1993; Rabello, 1997). Microscopic demonstration of the parasite's eggs in faeces or urine remains the most widespread tool to diagnose schistosomiasis, mainly due to its low operational costs (Rabello et al., 2002). However, the demonstration of eggs in stools can be very difficult when the infections are not intense, due to the large day-to-day variation in egg output. A large variety of immunotechniques have been described in the literature as alternative diagnostic methods (de Jonge et al., 1989; Krijger et al., 1994; Nourel Din et al., 1994; van Lieshout et al., 2000). Two immunological methods are most widely accepted: (i) detection of specific antibodies; and (ii) determination of circulating antigens, in particular the gut-associated circulating anodic antigen (CAA) and circulating cathodic antigen (CCA). Despite the high sensitivity and specificity of the determination of antibodies, this technique does not discriminate between active and previous infections or reinfection, and gives no information about the intensity of infection and the effects of chemotherapy (Dunne et al., 1992; Grogan et al., 1996). On the other hand, the detection of antigens can differentiate between a past infection and an active one, and a significant correlation between antigen levels and worm burden has been demonstrated (Barsoum et al., 1990; Agnew et al., 1995). Immunotechniques based on the detection of CAA and CCA, in urine and/or serum, have been developed to a routine technique applicable to schistosomiasis diagnosis (Nourel Din et al., 1994; van Lieshout et al., 2000). Quantitative determination of CAA in serum has so far shown a specificity of virtually 100% because of the unique nature of the immunoreactive glycan (Krijger et al., 1994).

The immunoreactive part of CAA from the parasite *Schistosoma mansoni* (adult worm stage) is a unique threonine-linked polysaccharide consisting of →6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→ repeating units (Bergwerff et al., 1994). Studies using bovine serum albumin (BSA)-coupled synthetic di- to pentasaccharide fragments of the CAA polysaccharide showed that some

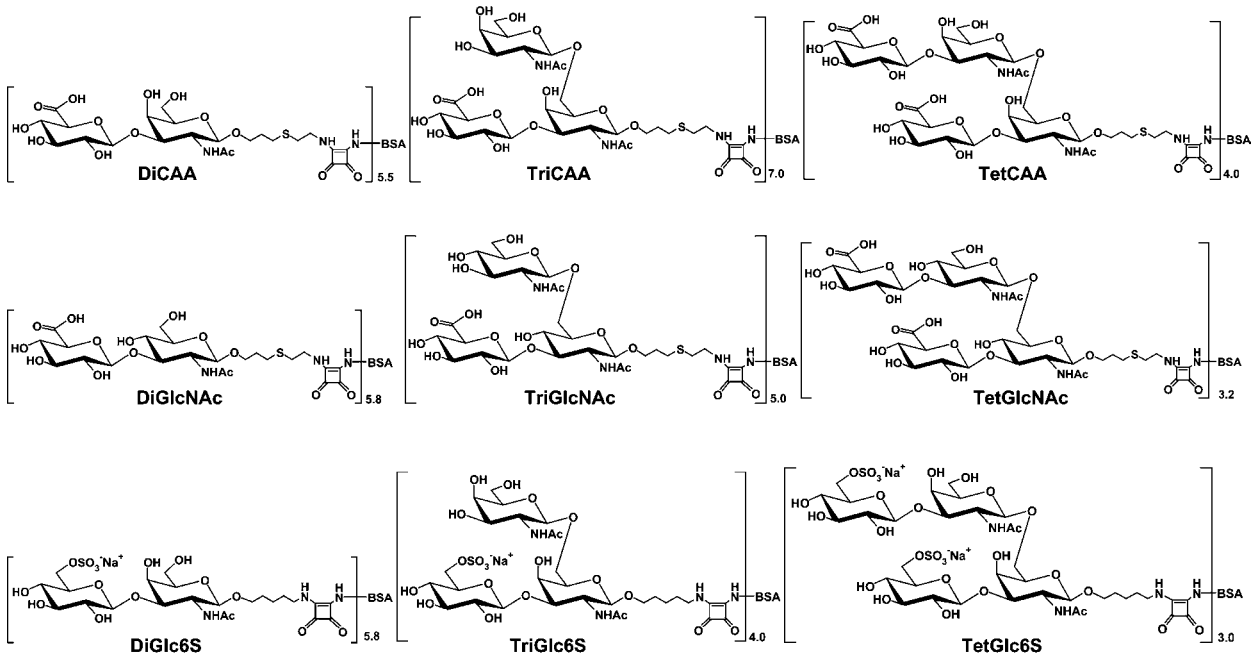

Figure 1 Neoglycoconjugates related to the *O*-glycan of CAA of *Schistosoma mansoni*.

anti-CAA IgM monoclonal antibodies recognise even the disaccharide repeating unit (Vermeer et al., 2000, 2003). These synthetic fragments, together with their matching set of monoclonal antibodies, could be useful tools for the further development of analytical and diagnostic methods. Therefore, it is imperative to obtain more information about antibody-carbohydrate recognition at the molecular level.

To analyse the specificity of a set of anti-carbohydrate monoclonal antibodies (mAbs) isolated from *Schistosoma*-infected mice, we compared BSA-conjugates of synthetic CAA fragments (DiCAA to TetCAA) with those of specific modifications of the CAA *O*-glycan fragments (Figure 1). To evaluate the importance of the HO4 group of the β-D-Gal*p*NAc residue of the CAA glycan for antibody binding, an analogue series denoted DiGlcNAc to TetGlcNAc has the native β-D-Gal*p*NAc residue replaced by β-D-Glc*p*NAc. In another series of analogues, named DiGlc6S to TetGlc6S, the native β-D-Glc*p*A moiety was replaced by β-D-Glc*p*6*S* to investigate the influence of the nature of the charge on antibody recognition. The interaction of the mAbs with these glycoconjugate analogues was monitored by ELISA and surface plasmon resonance spectroscopy. The results revealed that those mAbs that bind the strongest to their respective epitope allow no structural variations.

Results

Screening of mAbs by ELISA

To compare the immunoreactivity of the two series of BSA-conjugates containing synthetic oligosaccharide analogues to that of the BSA-conjugates containing synthetic CAA fragments, the binding of a selected panel of three uncharacterised mAbs and 14 anti-CAA mAbs (Table 1; entries 1–3 and 5–18, respectively) to DiCAA to TetCAA, DiGlcNAc to TetGlcNAc, and DiGlc6S to TetGlc6S was monitored by ELISA. Coated adult worm antigen treated with trichloroacetic acid (AWA-TCA), which is enriched for CAA, was used as a positive control.

The ELISA data for binding of the anti-CAA and the three uncharacterised mAbs to the disaccharide-containing conjugates DiCAA, DiGlcNAc and DiGlc6S indicate that binding is restricted to members of the IgM isotype; IgG mAbs did not bind to the conjugates. A number of anti-CAA IgM mAbs recognised the analogue DiGlcNAc with similar or higher binding levels than DiCAA (Table 1; entries 5–10). The mAbs showed no reactivity to analogue DiGlc6S, with the exception of mAb 25-2B6-A. In general, the mAbs did not bind to TriCAA, TriGlcNAc and TriGlc6S, with the exception of mAb 114-2G11-A, which recognised the TriCAA conjugate. As observed for the disaccharide-containing conjugates, binding of the mAbs to TetCAA, TetGlcNAc and TetGlc6S was restricted to the IgM isotype. Comparison of the data from screening with disaccharide- and tetrasaccharide-containing conjugates demonstrates that the group of anti-CAA IgMs preferentially recognising the DiGlcNAc analogue binds specifically to TetCAA (Table 1; entries 5–10). The anti-soluble egg antigen (SEA) mAb 100-2H5-A (entry 4) was not included in the ELISA screening due to detection problems.

Screening of mAbs by surface plasmon resonance spectroscopy

For a more detailed investigation of the interactions between the panel of 18 mAbs and the neoglycoconjugates, binding was monitored using surface plasmon resonance (SPR). Three chips were coated with the neoglycoconjugates and BSA as a negative control, with loadings of approximately 5000 resonance units (RU). SPR data obtained from the screening of undiluted mAb supernatants supported the ELISA results. Figure 2

Table 1 Monoclonal antibodies against *S. mansoni* antigens.

Entry	Monoclonal antibody	Isotype	Antigenic glycoconjugates	Oligosaccharide epitope
1	114-2G11-A	M	Unknown	n.d.
2	23-1F10-A	M	Unknown	n.d.
3	5-28-A	M	Unknown	n.d.
4	100-2H5-A	M	SEA	β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→[a]
5	25-7C11-A	M	CAA	→6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→[b]
6	25-3D10-A	M	CAA	→6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→[b]
7	25-9B10-A	M	CAA	→6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→[b]
8	25-2B6-A	M	CAA	→6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→[b]
9	27-2E2-A	M	CAA	→6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→[b]
10	5-25-B	M	CAA	→6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→[b]
11	120-1C4-A	M	CAA	→6)-[β-D-Glc*p*A-(1→3)]-β-D-Gal*p*NAc-(1→[b]
12	120-1C11-C	G	CAA	n.d.
13	120-1B10-A	G1	CAA	n.d.
14	147-1B4-A	G1	CAA	n.d.
15	141-5C9-A	G	CAA	n.d.
16	120-1B6-A	G	CAA	n.d.
17	147-4E5-A	G	CAA	n.d.
18	145-3C6-A	G	CAA	n.d.

[a]Oligosaccharide epitope determined by van Remoortere et al., 2000.
[b]Oligosaccharide epitope determined by Vermeer et al., 2003.

shows composite sensorgrams, illustrating binding characteristics representative for the binding of each group of mAbs to the neoglycoconjugate surfaces. As expected, no binding was detected when IgGs were injected (e.g., injection of IgG mAb 120-1B10-A to tetrasaccharide-containing surfaces; Figure 2A). In general, the tetrasaccharide-containing surfaces gave higher responses than surfaces containing di- or trisaccharides. Some anti-CAA IgMs bound to the TetCAA and TetGlcNAc surfaces to give moderate to low antibody-binding levels, whereas the TetGlc6S surface generally showed no response (e.g., injection of IgM mAb 25-7C11-A to tetrasaccharide-containing surfaces; Figure 2B). Injections of anti-CAA IgMs on the disaccharide-containing surfaces (DiCAA, DiGlcNAc, and DiGlc6S) showed low binding to the DiGlcNAc surface only (e.g., injection of IgM mAb 25-7C11-A to disaccharide-containing surfaces; Figure 2C). Comparison of the responses obtained for the anti-CAA IgMs to the tetrasaccharide and disaccharide surfaces showed that these mAbs bind with a relatively higher affinity to the larger oligosaccharides (Figure 2B,C). mAb 100-2H5-A showed binding to TriCAA and TriGlc6S only (Figure 2D). Injection of this antibody on the surfaces containing other conjugates resulted in no significant binding. The mAb 114-2G11-A presented remarkably high reactivity for TriCAA and TetCAA in comparison to DiCAA (Figure 2E).

Concentration-dependent characteristics of mAb binding

Based on the results obtained from ELISA and SPR screening, a smaller panel of mAbs (114-2G11-A, 100-2H5-A, 5-25-B, 25-9B10-A, 25-2B6-A and 27-2E2-A) was selected for investigation of the concentration-dependent characteristics of mAb binding. Overlay plots are presented in Figures 3–5 (antibody concentration 0.012–25 μg/ml).

Figure 3 shows the overlay plot of interactions of mAb 100-2H5-A with TriCAA, TriGlc6S, and TriGlcNAc. The TriCAA surface gave a response of approximately 3500 RU for antibody concentration of 25 μg/ml (Figure 3A). From Figure 3B it is evident that the binding affinity of this antibody to the TriGlc6S surface is much lower; a maximum response of approximately 600 RU was obtained for an antibody concentration of 25 μg/ml. The surface carrying TriGlcNAc showed no antibody binding (Figure 3C). It should be noted that mAb 100-2H5-A did not bind to any of the di- and tetrasaccharide-carrying surfaces.

As observed with the undiluted supernatants (Figure 2E), injections of mAb 114-2G11-A resulted in comparable sensorgrams for the TriCAA (Figure 4A) and TetCAA surfaces, whereas the DiCAA surface gave much lower binding levels (maximum of 300 RU at antibody concentration of 25 μg/ml) and faster dissociation rates. mAb 114-2G11-A appeared to be a highly specific antibody for the CAA fragments, as injections on the surfaces carrying GlcNAc or Glc6S conjugates resulted in negative responses only (e.g., concentration range of mAb 114-2G11-A to TriGlc6S surface; Figure 4B).

The overlay plots obtained after injection of selected anti-CAA IgMs on the trisaccharide-carrying surfaces did not show any detectable binding, even at the highest antibody concentration. From the selected anti-CAA IgMs, mAb 5-25-B showed a moderate response for the TetCAA surface (Figure 5A); however, no response was detected for other surfaces, such as DiCAA, DiGlcNAc and TetGlcNAc. The other three anti-CAA mAbs investigated (25-9B10-A, 25-2B6-A and 27-2E2-A, entries 7–9 in Table 1) showed much lower antibody binding levels for the DiCAA, DiGlcNAc, TetCAA and TetGlcNAc surfaces than those observed for mAb 5-25-B injection on the TetCAA surface. As observed in the ELISA and SPR screening, the tetrasaccharide-containing conjugates exerted a stronger reaction to these anti-CAA IgMs than the disaccharide-containing conjugates (data not shown). Comparison between the GlcNAc and CAA surfaces showed minimal differences in the antibody binding patterns (e.g., overlay plot of interaction of mAb 27-2E2-A with TetCAA and TetGlcNAc; Figure 5B,C). The anti-

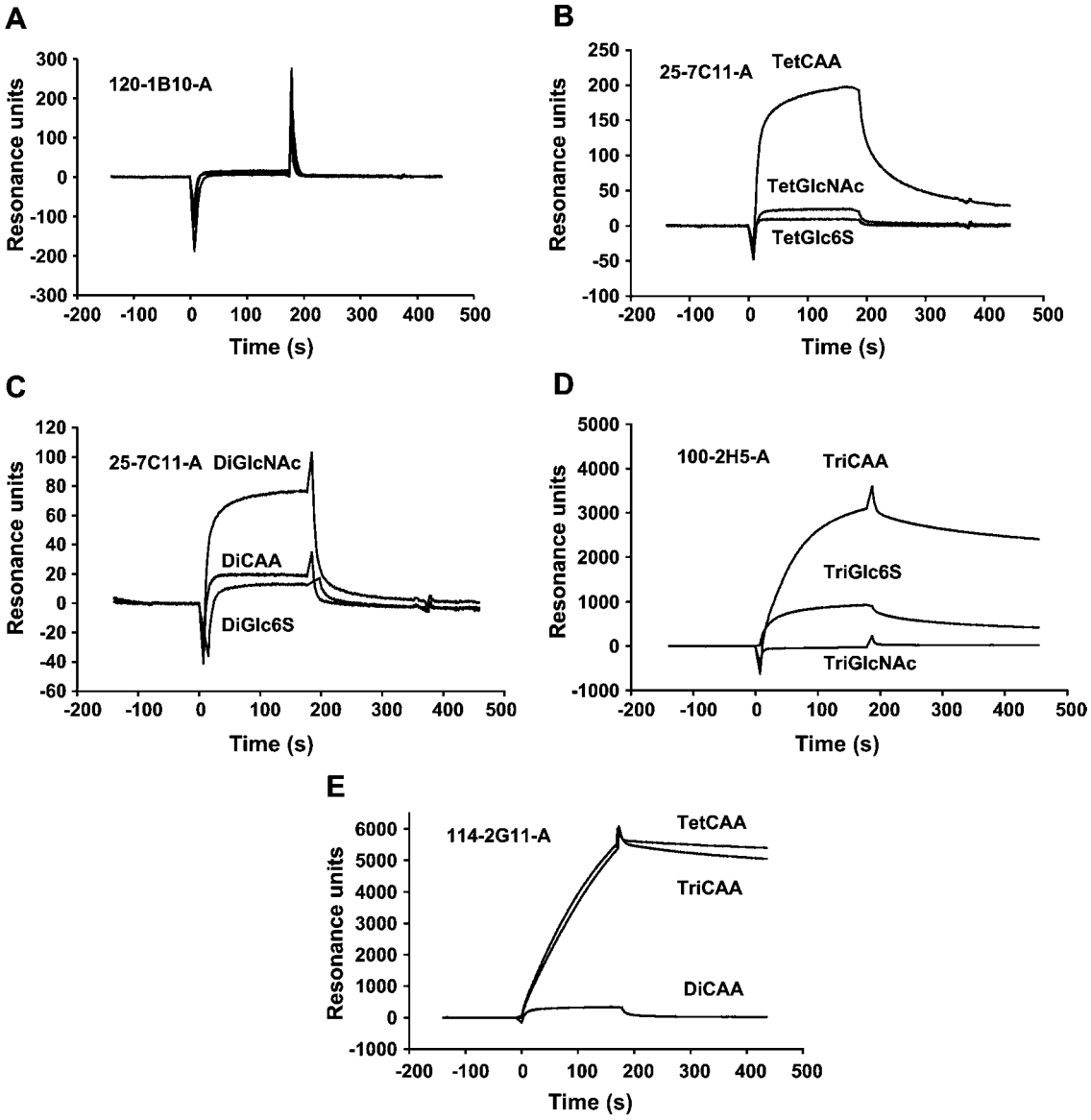

Figure 2 Composite sensorgrams illustrating binding characteristics representative for each group of mAbs from the selected panel to the immobilised neoglycoconjugates.
(A) Injection of 15 μl of undiluted 120-1B10-A on TetCAA, TetGlcNAc, and TetGlc6S surfaces. (B) Injection of 15 μl of undiluted 25-7C11-A on TetCAA, TetGlcNAc, and TetGlc6S surfaces. (C) Injection of 15 μl of undiluted 25-7C11-A on DiCAA, DiGlcNAc, and DiGlc6S surfaces. (D) Injection of 15 μl of undiluted 100-2H5-A on TriCAA, TriGlcNAc, and TriGlc6S surfaces. (E) Injection of 15 μl of undiluted 114-2G11-A on DiCAA, TriCAA, and TetCAA surfaces.

CAA IgMs gave only borderline binding to the Glc6S surface series.

Discussion

The main purpose of this study was to evaluate the importance of two functional groups of the immunoreactive CAA *O*-glycan of *S. mansoni*, namely, the carboxyl group in the side chains and the axial hydroxyl group in the main chain, for recognition by different *S. mansoni* monoclonal antibodies. To this end, small synthetic oligosaccharides, in which either native β-D-Gal*p*NAc was replaced by β-D-Glc*p*NAc (DiGlcNAc to TetGlcNAc) or native β-D-Glc*p*A was replaced by β-D-Glc*p*6*S* (DiGlc6S to TetGlc6S), were coupled to a carrier protein. The interaction of these neoglycoconjugates with a panel of monoclonal antibodies was investigated using ELISAs and SPR detection, and the data obtained were compared with those from conjugates containing CAA fragments (DiCAA to TetCAA).

The present ELISA and SPR data confirmed our earlier report (Vermeer et al., 2003), wherein several anti-CAA mAbs of the IgM class could recognise even the disaccharide β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→O) (DiCAA), the repeating unit of the CAA *O*-glycan. Moreover, these antibodies are more reactive to the dimer of this repeating unit (TetCAA) than to the monomer (DiCAA). No relevant immunoreactivity of the anti-CAA mAbs was detected against TriCAA and its analogues TriGlcNAc and

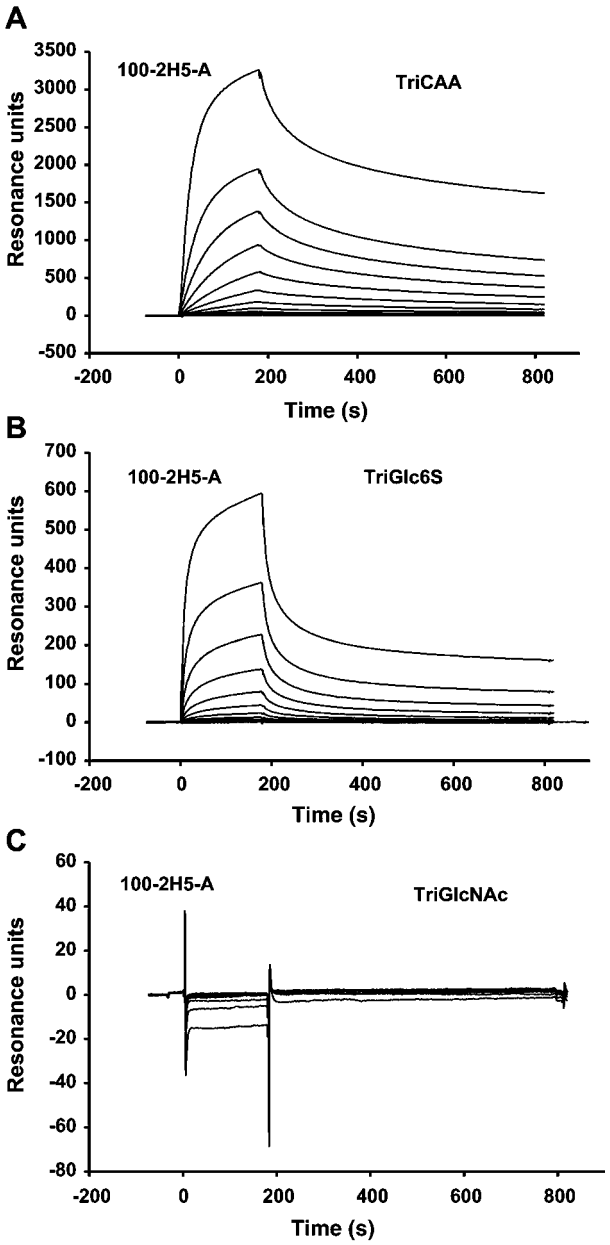

Figure 3 Composite sensorgrams of the concentration experiments for mAb 100-2H5-A-neoglycoconjugate interactions. (A) Overlay plot of 100-2H5-A interaction with TriCAA. (B) Overlay plot of 100-2H5-A interaction with TriGlc6S. (C) Overlay plot of 100-2H5-A interaction with TriGlcNAc. mAb concentration from top to bottom in μg/ml: (i) 25; (ii) 12.5; (iii) 6.25; (iv) 3.12; (v) 1.56; (vi) 0.78; (vii) 0.39; (viii) 0.19; (ix) 0.09; (x) 0.04; (xi) 0.024; and (xii) 0.012.

TriGlc6S. The presence of a terminal GalNAc residue in the oligosaccharides seems to interfere with binding of the mAb to the underlying disaccharide repeating unit (Vermeer et al., 2003). Some of the anti-CAA mAbs could recognise the DiGlcNAc and TetGlcNAc analogues. None of the anti-CAA mAbs of the IgG class binds to the CAA fragments, in agreement with our previous studies (Vermeer et al., 2003), or to the corresponding analogues. However, it should be noted that the same studies have shown that sera from schistosome-infected individuals contain both IgM and IgG antibodies to TriCAA and in particular to TetCAA.

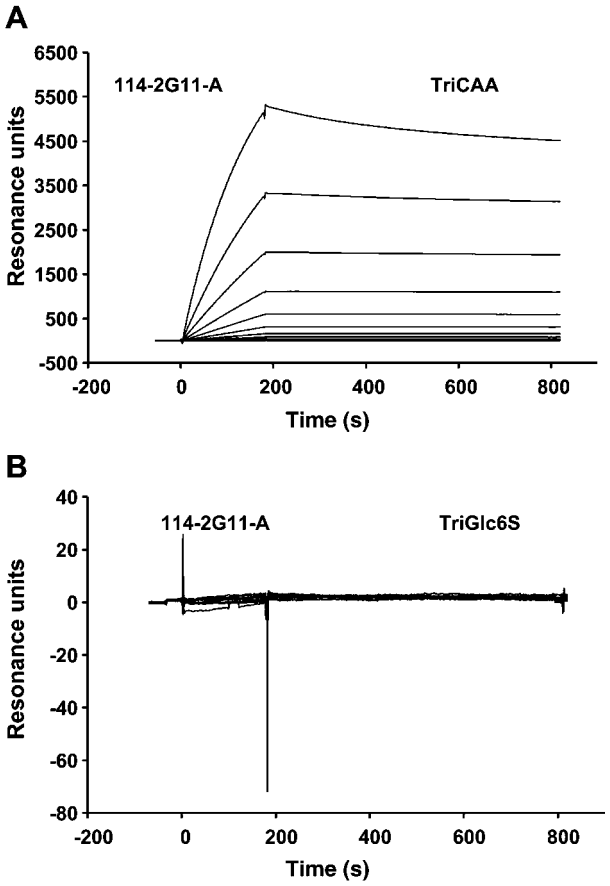

Figure 4 Composite sensorgrams of the concentration experiments for mAb 114-2G11-A-neoglycoconjugate interactions. (A) Overlay plot of 114-2G11-A interaction with TriCAA. (B) Overlay plot of 114-2G11-A interaction with TriGlc6S. mAb concentration from top to bottom in μg/ml: (i) 25; (ii) 12.5; (iii) 6.25; (iv) 3.12; (v) 1.56; (vi) 0.78; (vii) 0.39; (viii) 0.19; (ix) 0.09; (x) 0.04; (xi) 0.024; and (xii) 0.012.

Based on the ELISA and SPR screenings, a smaller panel of five mAbs that significantly bound one or more of the conjugates tested was chosen to investigate the concentration-dependent characteristics of mAb-glycoconjugate interactions. To this end, the five antibodies chosen were injected on sensor surfaces at 12 different concentrations.

An overlay plot of the interactions of mAb 100-2H5-A with the TriCAA surface showed high antibody-binding levels, whereas no recognition was observed on the surface carrying the analogue TriGlcNAc. The latter effect illustrates the importance of the β-D-Gal*p*NAc moiety for antibody binding to TriCAA. The fact that this antibody did not bind to DiCAA and TetCAA indicates that a terminal β-D-Gal*p*NAc moiety is essential for antibody recognition, in accordance with the previous observations for binding to β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→O) (van Remoortere et al., 2000). A significant reduction in binding level was observed for the injection of mAb 100-2H5-A to TriGlc6S. Apparently, the sulfate group on TriGlc6S hinders the binding of mAb 100-2H5-A to this glycoconjugate, resulting in a decrease in binding levels when compared to TriCAA (having a carboxyl function). It is interesting to note that in earlier studies (van Remoortere et al., 2000), the binding of mAb 100-2H5-A

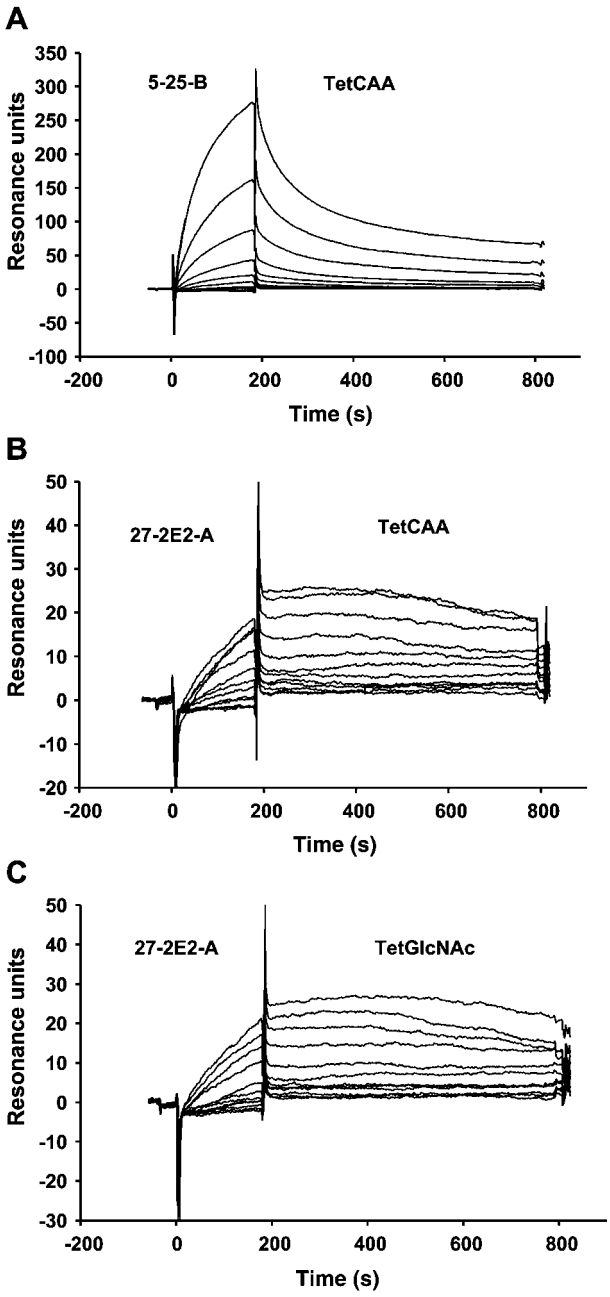

Figure 5 Composite sensorgrams illustrating binding characteristics representative for CAA-related mAbs to the immobilised neoglycoconjugates.
(A) Overlay plot of 5-25-B interaction with TetCAA. (B) Overlay plot of 27-2E2-A interaction with TetCAA. (C) Overlay plot of 27-2E2-A interaction with TetGlcNAc. mAb concentration from top to bottom in μg/ml: (i) 25; (ii) 12.5; (iii) 6.25; (iv) 3.12; (v) 1.56; (vi) 0.78; (vii) 0.39; (viii) 0.19; (ix) 0.09; (x) 0.04; (xi) 0.024; and (xii) 0.012.

to the enzymatically synthesised β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→O) was abolished after addition of an α-L-Fuc*p*-(1-3)-linked unit to the GlcNAc residue.

An interesting finding is the high-affinity binding of mAb 114-2G11-A to TriCAA and TetCAA. A considerable decrease in binding levels was detected with the DiCAA surface. The high specificity of this antibody for CAA structures is confirmed by the fact that it did not bind to any of the analogues, suggesting that both the axial HO4 group in the galactosamine residue and the carboxyl group in the glucuronic acid unit are irreplaceable for this high-affinity antibody-carbohydrate binding. Another interesting feature is the specific binding of anti-CAA mAb 5-25-B to TetCAA. This antibody did not bind to the smaller CAA structures. Probably, longer CAA oligosaccharides are needed for more efficient recognition by mAb 5-25-B.

In the concentration-dependent studies, the last three anti-CAA mAbs studied (25-9B10-A, 25-2B6-A and 27-2E2-A) appeared to be much less reactive to the glycoconjugates. In addition, no significant differences were observed in the responses measured for the analogues DiGlcNAc and TetGlcNAc and surfaces containing DiCAA and TetCAA. However, no recognition was observed for the analogues with sulfated oligosaccharides, indicating that the carboxyl group of β-D-Glc*p*A plays an important role in mAb binding.

The analysis of synthetic glycoconjugates related to CAA using ELISA and SPR techniques provided valuable information about the importance of the carboxyl and axial HO4 functional groups in the CAA *O*-glycan for mAbs recognition. In conclusion, we can correlate the specificity of the mAbs and their affinity to the oligosaccharide structures. The mAbs presenting highest binding levels could not tolerate changes to the carbohydrate backbone, whereas antibodies with lower binding levels could still recognise some of the analogues. mAbs 114-2G11-A and 5-25-B appear to be promising candidates for more detailed research into the role of the different functional groups of the carbohydrate epitope for antibody recognition.

Materials and methods

Preparation of neoglycoconjugates

The syntheses of BSA conjugates containing di- to tetrasaccharide fragments of CAA (Vermeer et al., 2000), DiCAA to TetCAA, and their corresponding analogues DiGlcNAc to TetGlcNAc and DiGlc6S to TetGlc6S (Carvalho de Souza et al., 2004) have been reported previously.

Production of monoclonal antibodies

Monoclonal antibodies were produced from hybridomas derived from the spleen cells of schistosome-infected mice or mice immunised with antigen preparations of *S. mansoni*, *S. haematobium*, or *S. japonicum* (Deelder et al., 1996). From a previously investigated panel of 24 anti-CAA mAbs (Vermeer et al., 2003), 14 mAbs were selected for the present study (Table 1). The immunoreactivity of one mAb against soluble egg antigen and three uncharacterised mAbs against *S. mansoni* adult antigens were also selected for investigation. Culture supernatants containing the antibody were used without further purification. The concentration of mAbs in the supernatants was determined by ELISA.

ELISA screening

Flat-bottom 96-well polystyrene microtitration plates (Maxisorp; Nunc, Roskilde, Denmark) were coated with 50 μl of neoglycoconjugates, AWA-TCA, or BSA as a negative control (5 μg/ml) in 35 mM phosphate-buffered saline, pH 7.8 (PBS) for

15 min at 37°C with shaking. Wells were washed with 20-fold diluted 0.3% Tween-20 in PBS (PT) solution. Non-specific binding sites were blocked with 100 μl of 0.3% BSA in PBS. Subsequently, the wells were incubated for 15 min with 50 μl of hybridoma supernatants (5 μg/ml antibody concentration) in PT. After washing, the plates were incubated with peroxidase-conjugated $F(ab')_2$ fragments of rabbit anti-mouse antibodies (Dakopatts, Glostrup, Denmark) in PT (1:1000). After a thorough final washing, the peroxidase substrate solution [0.42 mM 3,3′,5,5′-tetramethylbenzidine (TMB; Polysciences, Warrington, PA, USA) and 1.4 mM H_2O_2 in 0.1 M sodium acetate buffer, pH 5.5; Hancock and Tsang, 1986] was added, and the absorption at 630 nm (A_{630}) was measured. The TMB and H_2O_2 concentrations used were shown to be present in excess (Bos et al., 1981). At least five A_{630} values were determined within intervals of 1 min.

Surface plasmon resonance spectroscopy

Preparation of the sensor surfaces All sensor surfaces were prepared using a BIAcore 2000 instrument (Biacore AB, Uppsala, Sweden). CM5 sensor chips and P-20 surfactant (Tween 20%) were obtained from Biacore AB. The immobilisation procedures were carried out in HEPES-buffered saline (HBS; 10 mM HEPES, 0.15 M NaCl, 3.4 mM EDTA and 0.05% surfactant P-20, pH 7.4) at 25°C, at a flow rate of 5 μl/min. For chip 1, after equilibration of the sensor surface with HBS, the carboxymethylated dextran surface was activated with 35 μl of an equimolar mixture of 0.1 M *N*-ethyl-*N*′-[3-(dimethylamino)-propyl]carbodiimide hydrochloride (EDC)/*N*-hydroxysuccinimide (NHS). The neoglycoconjugates DiCAA, DiGlcNAc, and DiGlc6S were immobilised at a flow rate of 5 μl/min in 10 mM sodium acetate (pH 4.6) onto the activated sensor surface until an increase of 4500, 4000, and 3500 RU, respectively, was observed. The remaining activated carboxyl groups were blocked by injection of 1.0 M ethanolamine hydrochloride (pH 8.5) for 7 min. A BSA control surface was prepared following the same procedure to an increase of 4000 RU. For chip 2, activation, immobilisation and deactivation were performed as described for chip 1. The neoglycoconjugates TriCAA, TriGlcNAc, and TriGlc6S were immobilised onto the activated surface until an increase of 5600, 5500, and 5500 RU, respectively, was observed. The BSA control surface presented an immobilisation level of 4600 RU. For chip 3, activation, immobilisation, and deactivation were performed as described for chip 1. The neoglycoconjugates TetCAA, TetGlcNAc, and TetGlc6S were immobilised onto the activated surface until an increase of 3800, 3500, and 3200 RU, respectively, was observed. The BSA control surface presented an immobilisation level of 4000 RU.

Binding experiments The screening experiments were performed at 25°C at a flow rate of 5 μl/min. Undiluted hybridoma supernatants (15 μl each) were injected using HBS as the running buffer. Surfaces were regenerated with a 180-s pulse of 1 M NaCl. After comparison of the results obtained from the SPR screening and the ELISA assays, a selected panel of mAbs was injected at different concentrations at 25°C at a flow rate of 10 μl/min for 3 min using PT as running buffer. The hybridoma supernatants were diluted in PT to an antibody concentration varying from 25 to 0.023 μg/ml.

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