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Citation
Dam, G. J. van. (1995, February 9). *Circulating gut-associated antigens of Schistosoma mansoni : biological, immunological, and molecular aspects*. Retrieved from https://hdl.handle.net/1887/41317

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Author: Dam, G.J. van
Title: Circulating gut-associated antigens of Schistosoma mansoni : biological, immunological, and molecular aspects
Issue Date: 1995-02-09

Chapter 7

The immunologically reactive O-linked polysaccharide chains derived from circulating cathodic antigen isolated from the human blood fluke *Schistosoma mansoni* have Lewis x as repeating unit

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European Journal of Biochemistry **1994**; **225**:467-482

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Chapter 7

The immunologically reactive O-linked polysaccharide chains derived from circulating cathodic antigen isolated from the human blood fluke *Schistosoma mansoni* have Lewis x as repeating unit

Abstract

The gut-associated excretory antigen circulating cathodic antigen (CCA) was isolated by immunoaffinity chromatography from adult *Schistosoma mansoni* worms, which were collected from infected golden hamsters. This antigen is probably involved in protection of the schistosome gut and is increasingly used in highly sensitive and specific immunodiagnostic assays. Amino acid analysis before and after alkaline borohydride treatment of CCA and monosaccharide analysis indicated that CCA is O-glycosylated mostly via GalNAc-Thr. After reductive alkaline treatment, the O-linked carbohydrate chains were fractionated by gel-permeation chromatography, followed by normal-phase HPLC on LiChrosorb-NH_2. Carbohydrate-positive fractions were investigated by one-dimensional and two-dimensional ^{1}H-NMR spectroscopy, fast atom bombardment mass spectrometry and collision-induced-dissociation tandem mass spectrometry. The analyses showed that the low molecular mass O-linked oligosaccharide alditols (the minor fraction) consist of disaccharides to hexasaccharides having the Galβ(1-3)GalNAc-OL core in common. The major carbohydrate fraction comprises a population of polysaccharides, containing Lewis x repeating units (-3)Galβ(1-4)[Fucα(1-3)]GlcNAcβ(1-). CCA-specific monoclonal antibodies and IgM antibodies in patient sera recognized the fucosylated O-linked carbohydrate antigenic structures. Since CCA evokes a strong IgM antibody response and carbohydrate structures containing repeating Lewis x units are found on circulating neutrophils, it is proposed that the antigenic poly-Lewis x polysaccharide of CCA is involved in the induction of auto-immunity against granulocytes, resulting in the mild to moderate neutropenia observed during schistosome infection.

Introduction

Schistosomiasis is a parasitic disease caused by blood flukes of the genus *Schistosoma*, afflicting about 200 million individuals in the tropics. Antigen analysis plays an essential role in elucidating the immunological and immunopathological interactions between *Schistosoma mansoni* and its host. Despite the apparent importance of the carbohydrate moieties of the various antigens involved in these interactions [20,22,41,48], characterization of the primary structures has been hampered due to the availability of only limited amounts of parasite antigen. Studies have been performed using either indirect methods applying McAbs which recognize epitopes present on different schistosome life-stages or other antigens [31,32], or metabolically radio-labeled carbohydrate structures of adult worms [49–52,66,67].

In schistosomiasis, there is a strong humoral immune response of the host directed against tegument antigens [36,55,59,70,77] but also against antigens originating from the schistosome gut [9,19,47,54,60]. Gut-associated antigens are regularly released by the schistosome into the circulation of the host, when the parasite regurgitates the undigested contents of the gut. In this context, the detection of the gut-associated antigens circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) is increasingly used in seroepidemiology for specific immunodiagnosis of active schistosomiasis [13–16,76]. Moreover, CCA seems to be particularly useful as target antigen in specific antibody-detecting ELISA or ELISA-type assays [20,56,57,73]. The immunoreactive part of both antigens is thought to be located in the glycoconjugate glycans, as indicated by the observed stability of the antigen when treated with protein-denaturing agents, and the reduction of antigenicity after periodate treatment ([7,8,12,19,47,48] and unpublished results). Antigen levels in sera of schistosomiasis patients may increase to physiologically significant concentrations (*e.g.* for CAA from 0.3 μg/ml to 1 μg/ml, [33,74]).

Although several suggestions have been made for the function of CCA and/or CAA none of these has been experimentally verified. As the origin of these predominantly carbohydrate antigens is the gut of the parasite, it is obvious to propose a role in protection of the luminal surface of the schistosome gut [8,46]. Deelder *et al.* [20], after having demonstrated that the predominant IgM response against gut-associated antigens in humans was directed against CCA, reported the possibility of modulation of the host's immune response by evoking blocking antibodies. Complement activation was shown by Van Egmond *et al.* [75], using partly purified CCA preparations. In general, identification of the primary structure of these antigens would allow more detailed functional analyses, which would ultimately lead to a better understanding of the host-parasite interaction.

Previously, the purification, immunochemical and biochemical characterization of an antigen which is assumed to be for the most part identical to CCA have been described [8]. Although it was shown that this antigen contained O-linked carbohydrate chains, primary structures were not presented and a direct connection of these carbohydrate moieties with antigenicity was not demonstrated. In this study we present the primary structure of the major antigenic O-linked carbohydrate chains in immunopurified *S. mansoni* CCA.

Materials and Methods

Isolation of antigens

Adult *Schistosoma mansoni* worms (Puerto Rico strain) were collected from golden hamsters by perfusion of the hepatic portal system with a balanced salt solution, seven weeks after infection with 1500 cercariae. A trichloroacetic acid (TCA)-soluble (7.5% mass/vol.) fraction of homogenized adult worm antigen (AWA-TCA) was prepared as described [17], and used as a reference antigen preparation, shown to contain 3% CCA as determined using the immunopurified preparation discussed in this paper.

Washed and lyophilized worms (8 g) were homogenized in NaCl/P_i (3.5 mM KH_2PO_4, 32 mM Na_2HPO_4, pH 7.8, 0.15 M NaCl). The suspension was centrifuged at 25 000 × g for 20 min at 4°C, and $(NH_4)_2SO_4$ was added to the supernatant to a final concentration of 40% (mass/vol.). After centrifugation of the obtained suspension for 20 min at 10 000 × g and 4°C, the pellet was washed twice with 40% (mass/vol.) $(NH_4)_2SO_4$. The collected supernatant was pooled and partly desalted in an Amicon (Amicon Corporation) concentration cell, using a PM10 filter. The preparation was dialyzed against water for 2 days at 4°C, and the non-dialyzable material was lyophilized, yielding 2 g of solid material, including a sediment which had been formed during the dialysis. A turbid solution of the lyophilizate in water was centrifuged for 20 min at 10 000 × g and 4°C, and the pellet was washed twice with water. The CCA-containing supernatants were pooled and buffered by addition of Tris and NaCl to final concentrations of 0.1 M and 0.15 M, respectively, pH 7.6.

CCA was further purified on protein-A-based immunoaffinity columns [61], using as capturing antibody murine McAb 54-5C10-A (IgG3, CCA-specific, as determined by immunoelectrophoresis against AWA-TCA and immunofluorescence on adult *S. mansoni* worms [14,19]). Bound CCA was eluted with 75 mM Hepes/NaOH buffer, pH 7.2, containing 25% (mass/vol.) ethylene glycol and 3.0 M $MgCl_2$ [71]. The CCA solution was dialyzed under pressure against water, and desalted by chromatography on a column (2.6 cm × 35 cm) of Bio-Gel P-2 (Bio-Rad), eluted with water, after which the CCA-containing void volume fraction was lyophilized.

During the isolation, the purity of the antigen was checked by ELISA and expressed as percentage of the reference antigen preparation AWA–TCA.

Enzyme–linked immunosorbent assays

The antigen–capture ELISA was performed essentially as described [14] with some minor alterations. Among these are the use of a rapid shaking incubator system [45] allowing incubations to be shortened to 15 min and a simplification of the buffer system using NaCl/P_i/0.3% Tween–20 (PT). Briefly, the antigen was captured in various concentrations onto McAb 54–5C10–A–coated ELISA–plates (Maxisorp, Nunc) and detected using biotin–labeled McAb 8.3C10 (IgM, CCA–specific, characterized in a similar way as for McAb 54–5C10–A). After incubation with a streptavidin–alkaline phosphatase conjugate (Dakopatts), color was developed using *p*–nitrophenylphosphate as a substrate and absorbances were measured at 405 nm. The relative CCA concentration was read against a standard curve of AWA–TCA.

Generally, in the ELISA described below, incubations were performed for 15 min in a shaking incubator at 37°C, unless otherwise stated. For direct antigen detection, different antigen preparations (AWA–TCA, purified and/or alkaline–borohydride–treated CCA) in NaCl/P_i were coated in various concentrations (dilution series) onto the ELISA–plate. After thorough washing with a 20–fold diluted NaCl/P_i (which was performed between all subsequent steps without further mentioning), the antigen was detected using an appropriate dilution of biotin–labeled McAb 8.3C10 in PT, after which conjugate and substrate incubations were performed as described above. In the ELISA for the determination of the periodate sensitivity of epitopes recognized by different anti–CCA McAbs (specificied as described above for 54–5C10–A and 8.3C10), after periodate treatment and blocking of the plates, McAb solutions (5 μg/ml in PT) were incubated followed by rabbit anti–mouse immunoglobulins conjugated with horseradish peroxidase (Dakopatts). Immunobilized enzyme was quantified using 3,3′,5,5′–tetramethylbenzidine ($(Me_2NH_2C_6H_2–)_2$) as substrate, with detection at 630 nm [26]. In the ELISA for the determination whether various McAbs and/or lectins bound to CCA, purified CCA was coated in a ten–fold dilution series in NaCl/P_i starting at 2 μg/ml. After blocking with 0.1% BSA in NaCl/P_i, mouse McAb or biotinylated lectin solutions in PT were incubated at the following concentrations: McAb anti–CD15 (IgM, DAKO C3D–1, Code no. M 733, dialysed culture supernatant, Dakopatts) at 2 μg/ml, McAb anti–carcinoembryonic antigen (IgG1, DAKO A5B7, Code no. M773, dialysed culture supernatant, Dakopatts) at 0.5 μg/ml, McAb 8.3C10 (see above, IgM, hydroxyapatite–purified from mouse ascitic fluid) at 1 μg/ml, McAb 54–5C10–A (see above, IgG3, protein A–purified from mouse ascitic fluid) at 1 μg/ml, *Ulex europaeus* I–biotin (Catalog no. BA–2201, known combining oligosaccharide Fuc(α1–2)Gal(β1–4)–GlcNAc, E–Y Laboratories) at 5 μg/ml, and *Lotus tetragonolobus* agglutinin–biotin (Catalog no. BA–1601, known combining oligosaccharide Fuc(α1–2)Gal(β1–4)–[Fuc(α1–3)]–GlcNAc, E–Y Laboratories) at 5 μg/ml. Bound McAbs and lectin–biotin conjugates were detected as above by respectively rabbit anti–mouse immunoglobulins conjugated with horseradish peroxidase (Dakopatts) and streptavidin–horseradish

peroxidase conjugate (Dakopatts). Color was developed using $(Me_2NH_2C_6H_2-)_2$ substrate and absorbances were measured at 630 nm.

To determine whether CCA could be recognized by anti-i or anti-I antibodies, purified CCA was coated (2.5 μg/ml) directly on the ELISA-plates followed by post-coating with 0.3% BSA in NaCl/P_i. The plates were then incubated shaking for 60 min at 4°C (as these antibodies react in the cold) with dilution series of two anti-i and two anti-I antisera (kindly provided by the Central Laboratory of Blood Transfusion, Amsterdam, The Netherlands; these sera contain IgM anti-i or anti-I antibodies as determined by erythrocyte agglutination assays). Positive and negative control sera were incubated at 37°C in a 1/200 dilution. Bound antibodies were detected using peroxidase conjugated $F(ab')_2$ fragments of rabbit anti-human IgM antibodies (Dakopatts). Color was developed using $(Me_2NH_2C_6H_2-)_2$ as substrate and absorbances measured at 630 nm. To account for aspecific binding of serum antibodies, the absorbances of wells without CCA were subtracted.

To study inhibition of an anti-CCA McAb by specific trisaccharides, biotin-labeled McAb 8.3C10 was prior incubated with solutions in water (80 μl) containing respectively no trisaccharide, 5 μg of Lewis x (Le^x) trisaccharide (Galβ(1-4)[Fucα(1-3)]-GlcNAcβ-O-Ethyl) [2] or 5 μg of a modified Le^x in which Gal is replaced by GalNAc (GalNAcβ(1-4)[Fucα(1-3)]GlcNAcβ-O-Methyl) [2]. Bound McAb was detected using streptavidin-peroxidase conjugate and $(Me_2NH_2C_6H_2-)_2$ substrate. As the amount of purified CCA was limited, lower amounts of CCA were coated as a positive control for inhibition.

Liberation and isolation of the O-linked carbohydrate chains

A solution of 6 mg CCA in 6 ml 0.1 M NaOH, containing 1 M $NaBH_4$, was incubated for 16 h at 40°C under nitrogen. Then the solution was adjusted to pH 6.0 with formic acid, and fractionated on a column (2.2 cm × 135 cm) of Bio-Gel P-6, eluted with 25 mM NH_4HCO_3 at a flow rate of 22 ml/h. The carbohydrate-containing fraction, eluting after the void volume, was lyophilized and loaded onto a column (0.9 cm × 155 cm) of Bio-Gel P-2, eluted with water at a flow rate of 11 ml/h. In each case, runs were monitored by detection at 205 nm.

Subfractionation of oligosaccharide alditols by HPLC was carried out on a 10 μm LiChrosorb-NH_2 column (0.46 cm × 25 cm, Chrompack, The Netherlands) in a Kratos Spectroflow 400 system, monitored at 205 nm. Elutions were carried out isocratically with a mixture of 31.3% water/68.7% acetonitrile (by vol.) for 10 min, followed by a linear gradient to a mixture of 39.4% water/60.6% acetonitrile (by vol.) in 35 min, at a flow rate of 120 ml/h and at ambient temperature.

Monosaccharide analysis

Monosaccharide analysis of 100 μg purified CCA was carried out [29] by GLC of trimethylsilylated methyl glycosides, which were prepared by methanolysis (1.0 M methanolic HCl, 24 h, 85°C), re-*N*-acetylation, and trimethylsilylation.

Mild periodate oxidation

Periodate sensitivity of the epitopes recognized by several anti-CCA McAbs was evaluated according to Woodward *et al.* [81]. In brief, antigen samples, immobilized on an ELISA plate (Maxisorp, Nunc), were treated with a concentration series of sodium metaperiodate (0 – 20 mM $NaIO_4$ in 50 mM sodium acetate, pH 4.5) in the dark. After blocking the aldehyde groups with 1% glycine to prevent non-specific cross-linking of antibody to antigen, the plate was further processed as described for the ELISA for antigen detection (see above). A decrease in binding by CCA-recognizing McAbs was expressed as a percentage of the background-corrected absorbance of the wells without periodate.

Defucosylation using mild acid hydrolysis

AWA-TCA (containing CCA) was hydrolyzed in 0.1 M TCA for 1 h at 100°C [64], neutralized with 0.4 M NaOH, and tested in antigen-capture ELISA (see above).

Amino acid analysis

Samples of 100 μg material were hydrolyzed with 6.0 M HCl for 22 h at 110°C under nitrogen. Amino acid analyses were performed on an LKB 4151 Alpha Plus Amino Acid Analyzer, using a five-buffer lithium citrate system [11].

500-MHz and 600-MHz ^{1}H-NMR spectroscopy

Carbohydrate samples were repeatedly exchanged in 99.8% ^2H_2O (MSD Isotopes) at p^{2}H 7 with intermediate lyophilization. Finally, they were dissolved in 99.96% ^2H_2O [78]. The 500-MHz and 600-MHz one-dimensional and two-dimensional ^{1}H-NMR spectra were recorded on Bruker AMX-500 and AMXT-600 spectrometers (Bijvoet Center, Department of NMR-spectroscopy, Utrecht University), at a probe temperature of 300 K, unless indicated otherwise. Chemical shifts are expressed relative to internal acetone ($\delta = 2.225$ ppm). In the case of two-dimensional NMR experiments, data sets of 512 × 2048 points were recorded at 500-MHz, or otherwise indicated. The $^1HO^2H$ signal was presaturated for 1 s during the relaxation delay. Phase-sensitive handling of the data in the f_1 dimension became possible by the time-proportional phase increment method [40]. The time domain data of the scalar shift correlated spectroscopy (COSY), homonuclear Hartmann-Hahn (HOHAHA), and nuclear Overhauser enhancement

spectroscopy (NOESY) experiments were zero-filled to 1024 × 2048 data matrices prior to multiplication with a squared-bell function, phase shifted by $\pi/3$.

Two-dimensional HOHAHA spectra were recorded using MLEV-17 mixing sequences of 120 ms [1,39] at 300 K (fraction **P**) and at 315 K (fraction **O1**). Spin-lock field-strength corresponding to 90° ^{1}H pulse-widths of 27.8 μs and 27.5 μs were applied to fractions **P** and **O1**, respectively. In the case of **P**, the data matrix represented a spectral width of 4505 Hz in each dimension, and in the case of **O1** this was 4033 Hz in each dimension.

The two-dimensional NOESY [28] spectrum of **P** was recorded with a mixing time of 75 ms. This relatively short mixing time was chosen to prevent spin-diffusion as a result of molecular rotational correlation times (τ_c), which were expected to be relatively long. The data set represented a spectral width of 4032 Hz in each dimension.

The double-quantum-filtered ^{1}H-^{1}H two-dimensional COSY spectrum of **P** of 450 × 2048 data points was obtained as described [58], using a spectral width of 4032 Hz in each dimension.

Gas chromatography–mass spectrometry (GC–MS)

Trimethylsilylated monosaccharide derivatives, obtained from the methanolyzed fraction **P**, were analyzed by GC-MS, using a JEOL JMS-AX505W mass spectrometer (Bijvoet Center, Department of Mass Spectrometry, Utrecht University) fitted with a Hewlett Packard 5890 gas chromatograph using an on-column injector and helium as the carrier gas. The derivatives were separated on an SE-54 column (30 m × 0.25 mm, Alltech) with the following temperature program: holding at 90°C for 3 min, then increasing at 40°C/min to 130°C, and holding for 2 min, then increasing at 4°C/min to 200°C and holding for 15 min. Mass spectra were obtained using electron ionization and were recorded using linear scanning from *m/z* 50-800 at an accelerating voltage of 3 kV.

Mass spectrometry

Positive-ion fast atom bombardment mass spectrometry (FAB-MS) of underivatized or permethylated carbohydrate samples was performed using MS1 of a JEOL JMS-SX/SX102A tandem mass spectrometer (Bijvoet Center, Department of Mass Spectrometry, Utrecht University), using 6 kV or 10 kV accelerating voltage. The FAB gun was operated at an emission current of 10 mA, with Xe as bombarding gas. The spectra were scanned at a speed of 30 s for the full mass range specified by the accelerating voltage used, and were recorded and averaged on a Hewlett Packard HP9000 data system operating JEOL complement software. Collision-induced-dissociation tandem mass spectra (CID-MS/MS) were obtained on the same instrument using 10 kV accelerating voltage with He as the collision gas at a pressure sufficient to reduce the parent ion to one third of its original intensity.

Table 1. Summary of ions observed, with their assignments, on FAB–MS and CID–MS/MS analysis of permethylated carbohydrate-containing fractions isolated after reductive β-elimination of CCA.

Sample	Ion			Assignment
	$M+H^+$	CID–MS/MS	fragment	
	m/z			
O11	961			$M+H^+$ for $Hex_2HexNAc_2$–OL
O13	961 (major)			$M+H^+$ for $Hex_2HexNAc_2$–OL
	1410 (minor)			$M+H^+$ for $Hex_3HexNAc_3$–OL
	1206 (trace)			$M+H^+$ for $Hex_2HexNAc_3$–OL
			669	Hex–HexNAc–Hex^+
			464	Hex–$HexNAc^+$
			432	β-elimination of MeOH from *m/z* 464
O14	1165 (major)	→ 702		$M+H^+$ for $Hex_3HexNAc_2$–OL HO–Hex–HexNAc–OL generated by β-cleavage | Hex
			668	Hex–HexNAc–Hex^+
			498	HO–HexNAc–OL generated by β-cleavage | Hex
			464	Hex–$HexNAc^+$
			432	β-elimination of MeOH from *m/z* 464
	961 (major)	→ 668		$M+H^+$ for $Hex_2HexNAc_2$–OL Hex–HexNAc–Hex^+
		464		Hex–$HexNAc^+$
		432		β-elimination of MeOH from *m/z* 464
		294		HO–HexNAc–OL generated by β-cleavage
	1410 (less intense)	→ 464		$M+H^+$ for $Hex_3HexNAc_3$–OL Hex–$HexNAc^+$
		431		β-elimination of MeOH from *m/z* 464
	1206			$M+H^+$ for $Hex_2HexNAc_3$–OL
	1135	→ 638		$M+H^+$ for $Deoxyhex_1Hex_2HexNAc_2$–OL Hex–$HexNAc^+$ | Deoxyhex
		432		β-elimination of Deoxyhex from C-3 of HexNAc
		294		HO–HexNAc–OL generated by β-cleavage
O15	1410			$M+H^+$ for $Hex_3HexNAc_3$–OL
			668	Hex–HexNAc–Hex^+
			464	Hex–$HexNAc^+$
			432	β-elimination of MeOH from *m/z* 464

P	432	β-elimination of MeOH from *m/z* 464 and/or Deoxyhex from *m/z* 638
	464	$Hex-HexNAc^+$
	638	$Hex(Deoxyhex)-HexNAc^+$
	881	β-elimination of MeOH from *m/z* 913 and/or Deoxyhex from *m/z* 1087
	913 (minor)	$Hex-HexNAc-Hex-HexNAc^+$
	1055	β-elimination of MeOH from *m/z* 1087 and/or Deoxyhex from *m/z* 1261
	1087	$Deoxyhex_1Hex_2HexNAc_2^+$
	1261	$Deoxyhex_2Hex_2HexNAc_2^+$
	1504	β-elimination of MeOH from *m/z* 1536 and/or Deoxyhex from *m/z* 1710
	1536 (very minor)	$Deoxyhex_1Hex_3HexNAc_3^+$
	1678	β-elimination of MeOH from *m/z* 1710 and/or Deoxyhex from *m/z* 1884
	1710	$Deoxyhex_2Hex_3HexNAc_3^+$
	1884	$Deoxyhex_3Hex_3HexNAc_3^+$
	2333	$Deoxyhex_3Hex_4HexNAc_4^+$
	2507	$Deoxyhex_4Hex_4HexNAc_4^+$
	2956	$Deoxyhex_4Hex_6HexNAc_6^+$

Results

Characterization of intact CCA

Starting from 8 g dried *S. mansoni* worm-pairs, the immunoaffinity-based isolation procedure yielded 6 mg purified CCA, which is about 50% of the total amount detectable by ELISA in the starting preparation. During the purification procedure it was found that CCA, but not CAA, dissolved in water. This phenomenon was used to achieve an initial separation of these two related antigens which are otherwise difficult to separate due to their similar characteristics and the presence of cross-reacting determinants on both antigens. This cross-reactivity would have led to a contamination of CAA in the immunopurified CCA-preparation. Monosaccharide composition analysis of the intact CCA sample revealed the presence of Fuc, Gal, Man, GlcNAc, GalNAc, rhamnose (Rha), Xyl and Glc in the molar ratios 5.4:6.0:0.5:10.8:1.0:1.2:0.1 :1.0. The carbohydrate content of intact CCA was estimated to be 78% by mass.

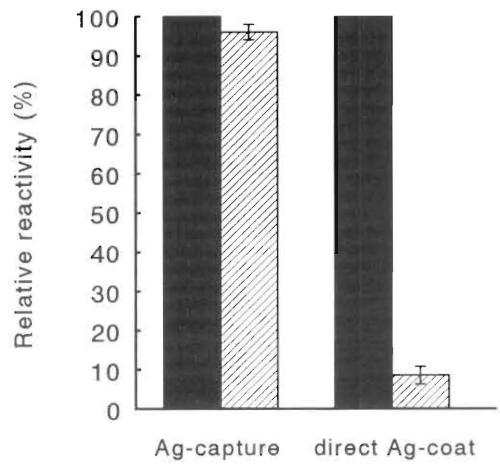


Figure 1. Detection of CCA before (closed bars) and after (shaded bars) reductive β-elimination (1 mg/ml CCA in 0.1 M NaOH, and 1 M $NaBH_4$). Untreated and treated samples were tested in concentration series starting from 30 ng/ml in both the antigen-capture ELISA and the direct antigen-coated ELISA as described in Materials and Methods. The responses of the treated samples were expressed as a percentage of the responses of the untreated samples and were averaged from at least three concentrations.

The detection of a small amount of Man may indicate the presence of N-linked carbohydrate chains on CCA. However, no release of N-glycans could be detected with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (*Flavobacterium meningosepticum*) under the usual range of conditions applied (see *e.g.* [10,69]). Importantly, the antigenic determinant was located on the O-linked glycans, since the antigen, as recognized by McAb 8.3C10, could be released from the protein by alkaline β-elimination. In Fig. 1 it is shown that after reductive alkaline treatment, the immunoreactivity of CCA remained unchanged as assessed by the antigen-capture ELISA, but the coating efficiency was markedly decreased. This indicates that the carbohydrate chains were released from the protein backbone. Only some breakdown of the polypeptide backbone without complete carbohydrate release as a cause of reduction in coating efficiency is unlikely, as suggested by the observation that fraction P contained only GalNAc-OL and no GalNAc (monosaccharide analysis), and less than 2% (by mass) amino acids (amino acid analysis). Amino acid analyses of CCA before and after reductive alkaline treatment showed that the amount of detectable Thr was decreased while that of 2-aminobutyric acid increased, indicating that the chemically released carbohydrate chains were originally attached to the protein backbone in O-linkage, mostly via Thr. Therefore, in order to study further the structure(s) of the most abundant antigenic

carbohydrate chains, CCA was submitted to preparative reductive alkaline treatment to release O-linked carbohydrate chains.

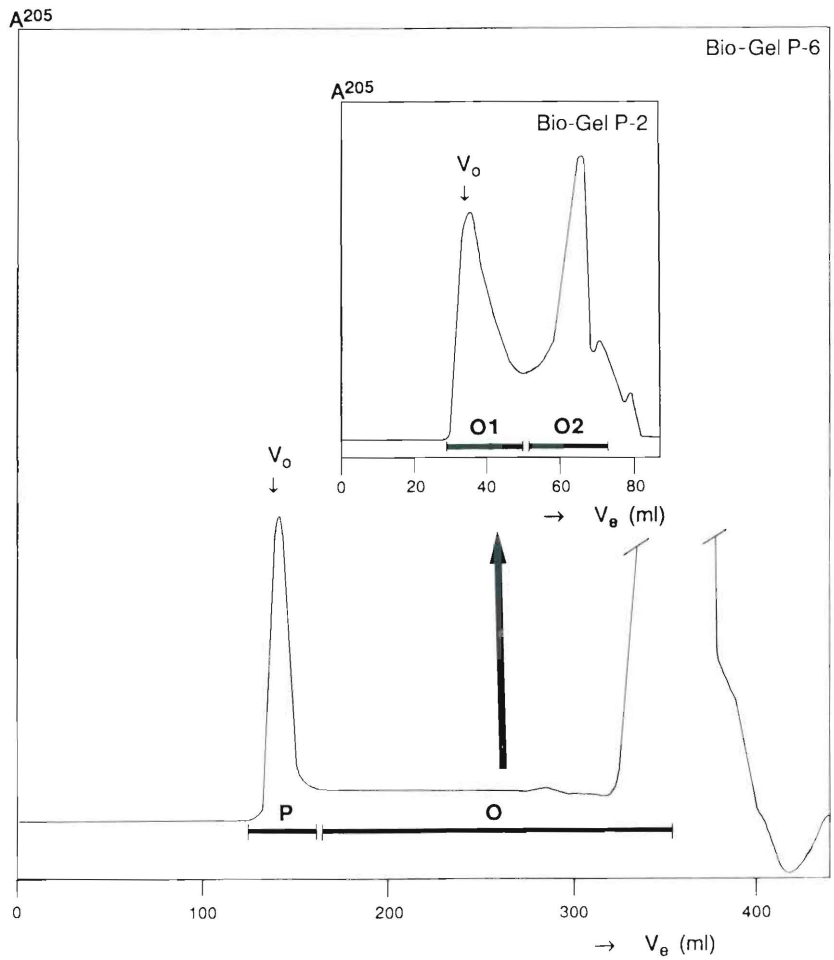


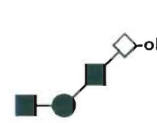
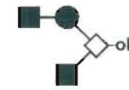
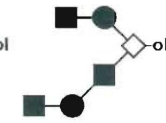
Figure 2. Elution pattern of reductive alkaline treated *S. mansoni* CCA on a column (2.2 cm × 135 cm) of Bio-Gel P-6, eluted with 25 mM NH_4HCO_3; the insert shows the elution pattern of fraction O on a column (0.9 cm × 155 cm) of Bio-Gel P-2 eluted with water. Runs were monitored by detection at 205 nm.

Isolation of released O-glycans

Gel-permeation chromatography of the β-elimination products on Bio-Gel P-6 gave rise to one major carbohydrate-positive fraction (Fig. 2), denoted **P** (polysaccharide material). Fraction **O**, being the remainder, eluted after the

excluded peak, was further separated on Bio-Gel P-2 into two carbohydrate-positive fractions, as demonstrated by ^{1}H-NMR analysis, which are denoted **O1** and **O2** (Fig. 2). HPLC fractionation of **O1** and **O2** on LiChrosorb-NH_2 resulted in the isolation of the carbohydrate-positive (orcinol/H_2SO_4) subfractions **O11**–**O15**, and **O21**–**O23**, respectively. These HPLC subfractions and **P** were subjected to one-dimensional and two-dimensional ^{1}H-NMR spectroscopy as well as FAB-MS and, where appropriate, CID-MS/MS analyses.

Table 2. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides for oligosaccharide alditols derived from *S. mansoni* CCA. Chemical shifts are given relative to internal acetone (δ 2.225) in ^2H_2O at 300 K and at p^{2}H 7 [78]. Compounds are represented by short-hand symbolic notation: (●), D-GlcNAc; (◇-ol), D-GalNAc-OL, (■), D-Gal, n.d., not determined. The first superscript at the name of a monosaccharide residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. A second superscript is used to discriminate between identically linked residues, by indicating the type of linkage of the neighbouring residue in the sequence.

Residue	Reporter group	Chemical shift			
					
		O23	**O13A**	**O13B**	**O15**
		ppm			
GalNAc-OL	H-2	4.393	4.395	4.395	4.400
	H-3	4.064	4.050	4.061	4.050
	H-4	3.507	3.497	3.466	3.451
	H-5	4.193	4.184	4.281	4.268
	NAc	2.050	2.047	2.066	2.067
Gal^3	H-1	4.478	4.464	4.464	4.452
	H-4	3.902	4.126	3.900	4.127
GlcNAc^3	H-1	—	4.688	—	4.684
	H-6	—	3.953	—	3.953
	NAc	—	2.041	—	2.038
Gal^{4,3}	H-1	—	4.480	—	4.483
	H-4	—	3.928	—	4.925
GlcNAc^6	H-1	—	—	4.560	4.555
	H-6	—	—	3.998	3.996
	NAc	—	—	2.064	2.058
Gal^{4,6}	H-1	—	—	4.470	4.467
	H-4	—	—	n.d.	3.925

Relevant FAB–MS and CID–MS/MS data are compiled in Table 1 and ^{1}H–NMR data in Tables 2 and 3. Compounds will be discussed in order of increasing complexity. The amount of material in fractions **O21** and **O22** was too low for reliable structure determination.

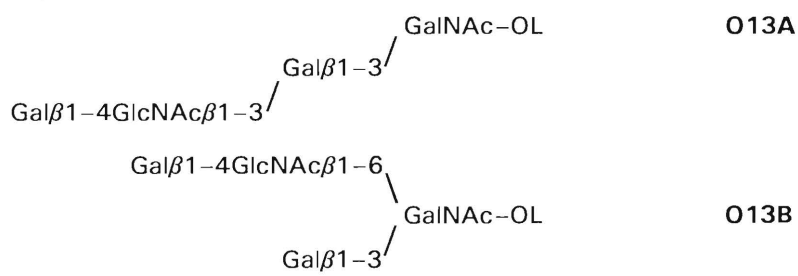
Structural analysis of oligosaccharide alditols

The ^{1}H–NMR data of fraction **O23** are in good agreement with those reported for the disaccharide alditol, Galβ(1–3)GalNAc–OL, compound **2** in [30].

Although the quantities of fraction **O11** were too low for ^{1}H–NMR analysis, an aliquot was subjected to permethylation and analyzed by FAB–MS. A single $M+H^+$ pseudomolecular ion of low intensity was observed at *m/z* 961 corresponding to a composition of $Hex_2HexNAc_2$–OL.

FAB–MS analysis of permethylated fraction **O13** revealed the presence of one major $M+H^+$ ion at *m/z* 961, corresponding to a composition of $Hex_2HexNAc_2$–OL, as well as a minor ion at *m/z* 1410 (for $Hex_3HexNAc_3$–OL) and a very minor ion at *m/z* 1206 (for $Hex_2HexNAc_3$–OL). The spectrum also contained fragment ions which may, from their intensities, be assumed to arise from the major species: *m/z* 668 corresponds to an A^+–type ion [21] with composition $Hex_2HexNAc_1^+$; a more intense fragment ion was observed at *m/z* 464 for $HexHexNAc^+$, and was accompanied by an ion derived from it by β–elimination of the methyl substituent on C–3 [21] at *m/z* 432. These fragment ions demonstrate that the major component in this fraction is a linear tetrasaccharide Hex–4/6HexNAc–Hex–HexNAc–OL.

^{1}H–NMR analysis of fraction **O13** showed the presence of a minor (25%) and a major (75%) component, being a branched (**O13B**) and a linear (**O13A**) tetrasaccharide alditol, respectively. The spectral data of **O13A** and **O13B** are in accordance with those of reference structures **12** and **25**, respectively [30], namely:



O13A

O13B

Probably as a result of a non–complete HPLC separation, the structures for **O13A** and **O13B** were also identified in fraction **O12**, but now in a ratio of 1:4.

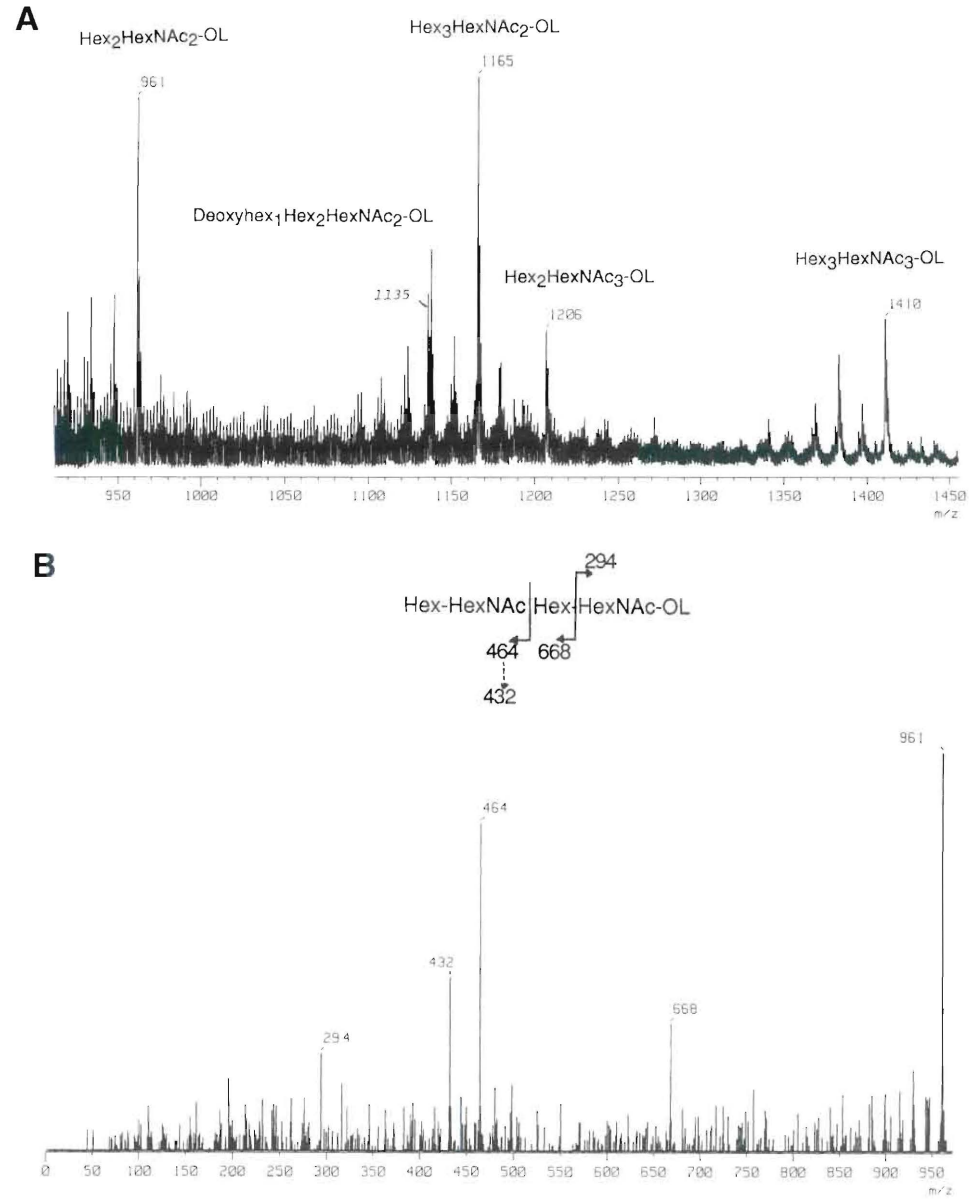


Figure 3. Partial FAB mass spectrum of permethylated fraction O14 (A), CID mass spectrum and fragmentation scheme for *m/z* 961 from permethylated fraction O14 (B) and CID mass spectrum and fragmentation scheme for *m/z* 1165 from permethylated fraction O14 (C). It should be noted that in each of the mass spectra the scale for the relative intensities of the ions remains constant across the depicted *m/z* range.

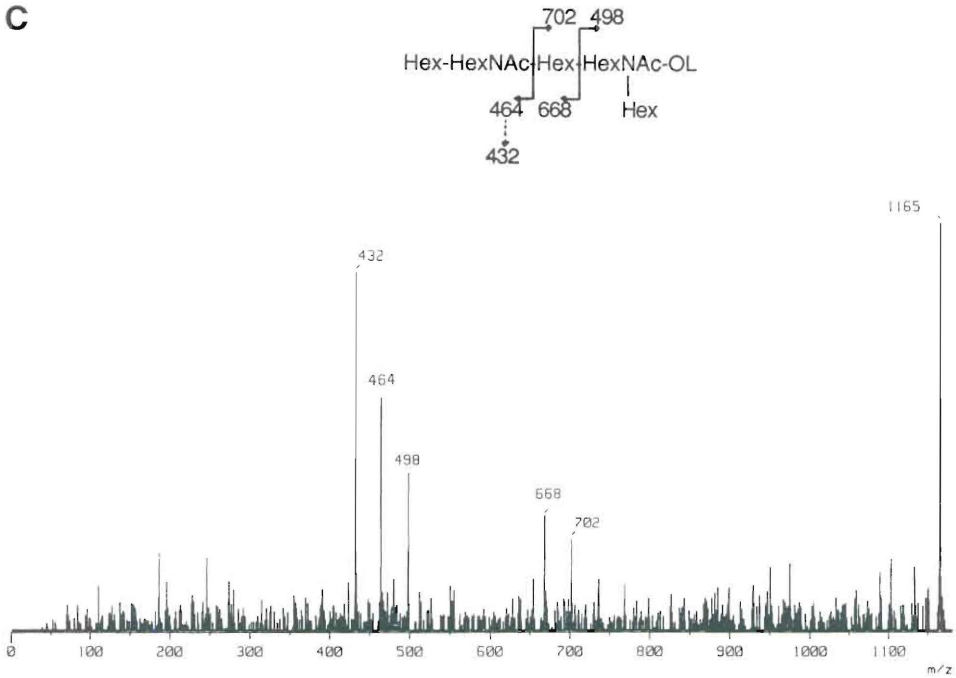
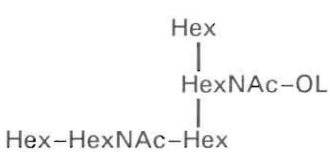
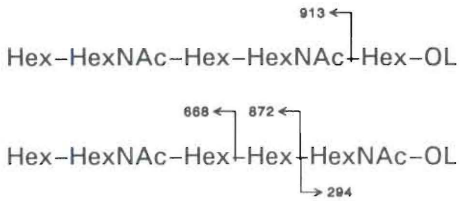


Figure 3. (continued)

FAB–MS analysis of permethylated fraction **O14** (Fig. 3A) revealed the presence of two predominant molecular species, as well as three minor components; the ion at *m/z* 1165 corresponds to $M+H^+$ for $Hex_3HexNAc_2$–OL, while an ion of almost equal intensity at *m/z* 961 corresponds to $M+H^+$ for $Hex_2HexNAc_2$–OL. Ions at *m/z* 1410 ($M+H^+$ for $Hex_3HexNAc_3$–OL), *m/z* 1135 ($M+H^+$ for $Deoxyhex_1Hex_2HexNAc_2$–OL), and *m/z* 1206 ($M+H^+$ for $Hex_2HexNAc_3$–OL) represent less abundant species. CID–MS/MS of the ion at *m/z* 961 gave ions indicating a linear structure (Fig. 3B). CID–MS/MS of the ion at *m/z* 1165 gave rise to a series of ions which suggests an unusual branching structure for the $Hex_3HexNAc_2$–OL component (Fig. 3C):

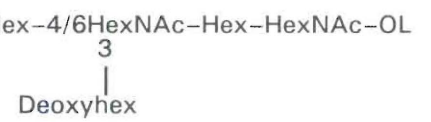


Two other structures would be formally possible for such a composition:



The first of these two structures may be expected to yield an intense ion at *m/z* 913, as shown, corresponding to the formation of an A^+–type ion on an *N*–acetylhexosamine residue, analogous to the ion at *m/z* 464. This is clearly absent from the CID spectrum of *m/z* 1165. The second structure may also be discounted since it may be expected to yield an ion at *m/z* 872 (analogous to that at *m/z* 668) as well as a *β*–cleavage ion [21] at *m/z* 294 (as seen in the CID spectrum of $M+H^+$ *m/z* 961, and analogous to that at *m/z* 498 in this spectrum).

CID–MS/MS of the $M+H^+$ molecular species at *m/z* 1410 yielded a spectrum with poor signal:noise due to the very small amounts of sample available. The only fragment ions observed (at *m/z* 464 and *m/z* 432, assigned as above) were insufficient to assign any unambiguous structure. CID–MS/MS of the $M+H^+$ molecular species at *m/z* 1135 yielded three fragment ions (*m/z* 638 for Hex–(Deoxyhex)–$HexNAc^+$; *m/z* 432 for the *β*–elimination of Deoxyhex from C-3 of HexNAc; and *m/z* 294 arising by *β*–cleavage of HexNAc–OL) which suggests the following structure:



^{1}H–NMR analysis of fraction **O14** indicated a complex mixture of oligosaccharide alditols. The low amount of material available did not permit fractionation of **O14** into separate components for further ^{1}H–NMR study.

FAB–MS analysis of underivatized fraction **O15** yielded pseudomolecular ions at *m/z* 1116 ($M+H^+$) and *m/z* 1138 ($M+Na^+$) corresponding to a composition of $Hex_3HexNAc_3$–OL. FAB–MS of permethylated fraction **O15** yielded a single $M+H^+$ molecular species at *m/z* 1410, corresponding to a composition of $Hex_3HexNAc_3$–OL. Fragment ions were observed at *m/z* 464 (for Hex–$HexNAc^+$), *m/z* 432 (*β*–elimination of methanol from 464) and *m/z* 668 (for Hex–HexNAc–Hex^+). The absence of an ion at *m/z* 913 (for Hex–HexNAc–Hex–$HexNAc^+$) suggests a branched structure:

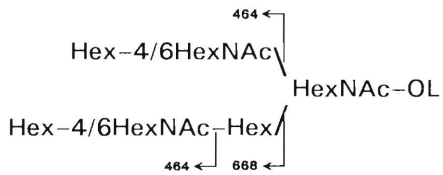
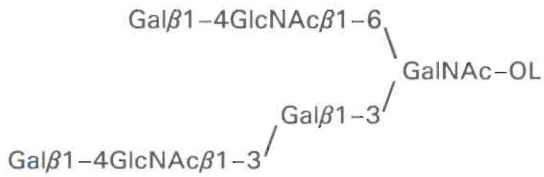


Table 3. ^{1}H-Chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the polysaccharide alditol fraction P, derived from *S. mansoni* CCA. Chemical shifts are given relative to internal acetone (δ 2.225) in ^2H_2O at 300 K and at p^{2}H 7 [78]. The underlined values are assignments derived from the two-dimensional HOHAHA and double quantum filtered COSY ^{1}H-NMR spectra. The terms internal and terminal refer to the position of the repeating fucosylated *N*-acetyllactosamine unit in the polysaccharide. ^{1}H-NMR signals for a distal unit and for a protein-linkage region were not observed. The term unsubstituted refers to a terminal non-reducing non-fucosylated *N*-acetyllactosamine unit in the carbohydrate chain; n.d., not determined.

Residue	Proton	Chemical shift in repeating unit		
		internal	terminal	unsubstituted
		ppm		
Gal	H-1	4.445	4.462	4.480
	H-2	3.500	3.500	n.d.
	H-3	3.697	3.655	3.664
	H-4	4.093	3.892	3.921
	H-5	3.585	n.d.	n.d.
	H-6/6'	3.7[a]	n.d.	n.d.
GlcNAc	H-1	4.705	n.d.	n.d.
	H-2	3.956	n.d.	n.d.
	H-3	3.859	n.d.	n.d.
	H-4	3.942	n.d.	n.d.
	H-5	3.571	n.d.	n.d.
	H-6	3.94[a]	n.d.	n.d.
	H-6'	3.740	n.d.	n.d.
	NAc	2.014	n.d.	n.d.
Fuc	H-1	5.118	5.132	—
	H-2	3.682	3.682	—
	H-3	3.882	3.90[a]	—
	H-4	3.773	3.787	—
	H-5	4.805	4.832[b]	—
	CH_3	1.145	1.174	—

[a] Values are given with less accuracy because of overlapping cross-peaks.
[b] Fuc H-5 in reference compound **164** (δ 4.851) was measured at 7°C [30], which explains the difference in chemical shift.

The ^{1}H-NMR spectrum of fraction **O15** is identical to that of the branched hexasaccharide alditol reference compound **26** [30]:



Structural analysis of polysaccharide alditol

Monosaccharide analysis of fraction **P** showed the presence of Fuc, Man, Gal, and GlcNAc in the molar ratios 1.1:0.07:1.0:0.8. However, GC–MS analysis allowed the additional identification of a single trimethylsilylated alditol residue corresponding to GalNAc–OL, suggesting that the polysaccharide alditol resulted from reductive alkaline β-elimination. The presence of GalNAc could not be demonstrated. Amino acid analysis showed trace amounts of amino acids (less than 2% (by mass) in total).

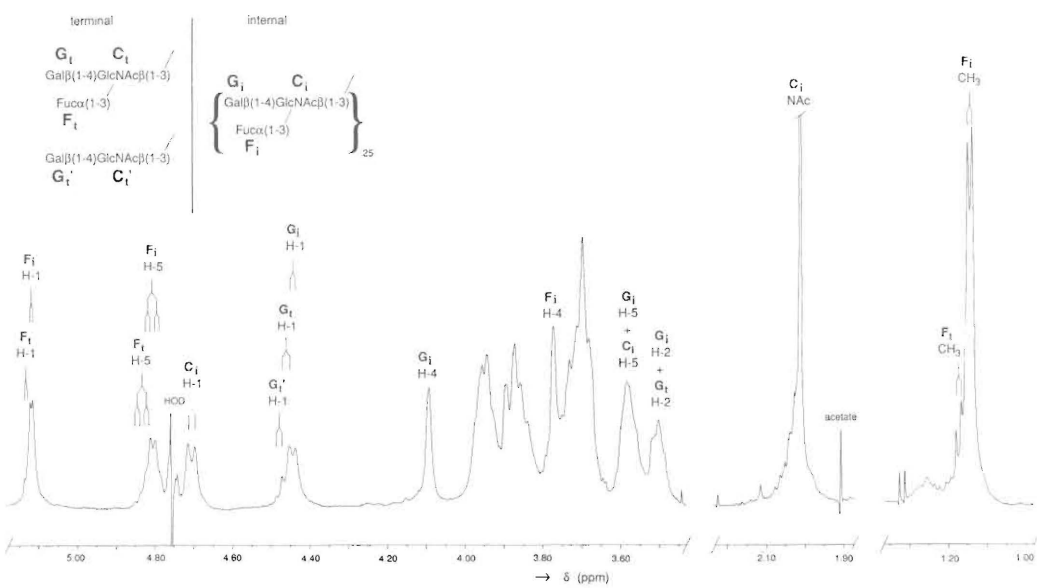


Figure 4. Resolution-enhanced 500-MHz ^{1}H-NMR spectrum at 300 K of fraction P.

Fraction **P** was examined using FAB–MS following permethylation. Mass spectra were obtained in the range *m/z* 50–4000 using an accelerating voltage of 6 kV. In this mass range no ions corresponding to molecular species were observed, although A^+–type fragment ions arising by glycosidic cleavage with charge retention on *N*–acetylhexosamine residues were formed, as previously described [53]. The A^+–type fragment ions (see Table 1) indicate that the polysaccharide consists of more than five repeating units containing the Hex–HexNAc element. Two species are present, one in which all repeating units bear Deoxyhex on C–3 of the HexNAc residue (*m/z* 638, 1261, 1884, 2507) and a second in which the non–reducing terminal repeat is not fucosylated in this way (*m/z* 464, 1087, 1710, 2333, 2956). An additional very minor series of ions is observed corresponding to a species in which the non–reducing terminal two repeats contain no Fuc (*m/z* 913, 1536).

The one–dimensional ^{1}H–NMR spectrum of **P** indicates a polysaccharide, having a repeating unit of –3)Galβ(1–4)[Fucα(1–3)]GlcNAcβ(1–, also known as Le^x (Fig. 4). The spectrum neither showed signals representing residual peptide material, nor signals for the protein–linkage region (carbohydrate core structure). Complete assignment of the ^{1}H–NMR spectrum was accomplished using two–dimensional COSY and HOHAHA ^{1}H–NMR spectroscopy (see Table 3).

The anomeric signals of Gal, GlcNAc and Fuc could be assigned by their unique spin–coupling systems in the HOHAHA spectrum (Gal: H–1—H–4; GlcNAc: H–1—H–6/6'; Fuc: H–1—H–4 and H–5—CH_3) (data not shown). In addition, the HOHAHA spectrum revealed the presence of one major and two minor subspectra for Gal, one major and one minor subspectrum for Fuc and one subspectrum for GlcNAc (Fig. 4). The set of intense signals, corresponding to the major structural element, can be assigned to the internal –3)Galβ(1–4)[Fucα(1–3)]GlcNAcβ(1– repeats of the polymer. The Fucα(1–3)GlcNAc glycosidic linkage can be deduced from the presence of an inter–residual NOE cross–peak between Fuc H–1 and GlcNAc H–3 in the NOESY spectrum (Fig. 5). An Le^x unit between two other Le^x units gives rise to a unique set of structural–reporter–group signals for the αFuc residue, namely Fuc H–1 at δ 5.118, H–5 at δ 4.805, and CH_3 at δ 1.145 [30]. The NOE contact observed between GlcNAc H–1 and Gal H–3, together with the resonance position of GlcNAc H–1 at δ 4.705 and the NAc methyl signal at δ 2.014, reveal that βGlcNAc is (1–3)–linked to Gal. Since the 3–position of GlcNAc is occupied with a Fuc residue, and Gal H–1 shows NOE cross–peaks with GlcNAc H–4 (strong) and GlcNAc H–3 (weak), the βGal residue must be (1–4)–linked to GlcNAc. Additional NOE cross–peaks (Fig. 5) between Fuc H–1 and GlcNAc NAc, between Fuc H–5 and Gal H–2, between Fuc H–5 and Gal H–3, and between Fuc CH_3 and Gal H–2 correspond with those observed for a single Galβ(1–4)[Fucα(1–3)]GlcNAcβ element (unpublished results and [44,82]).

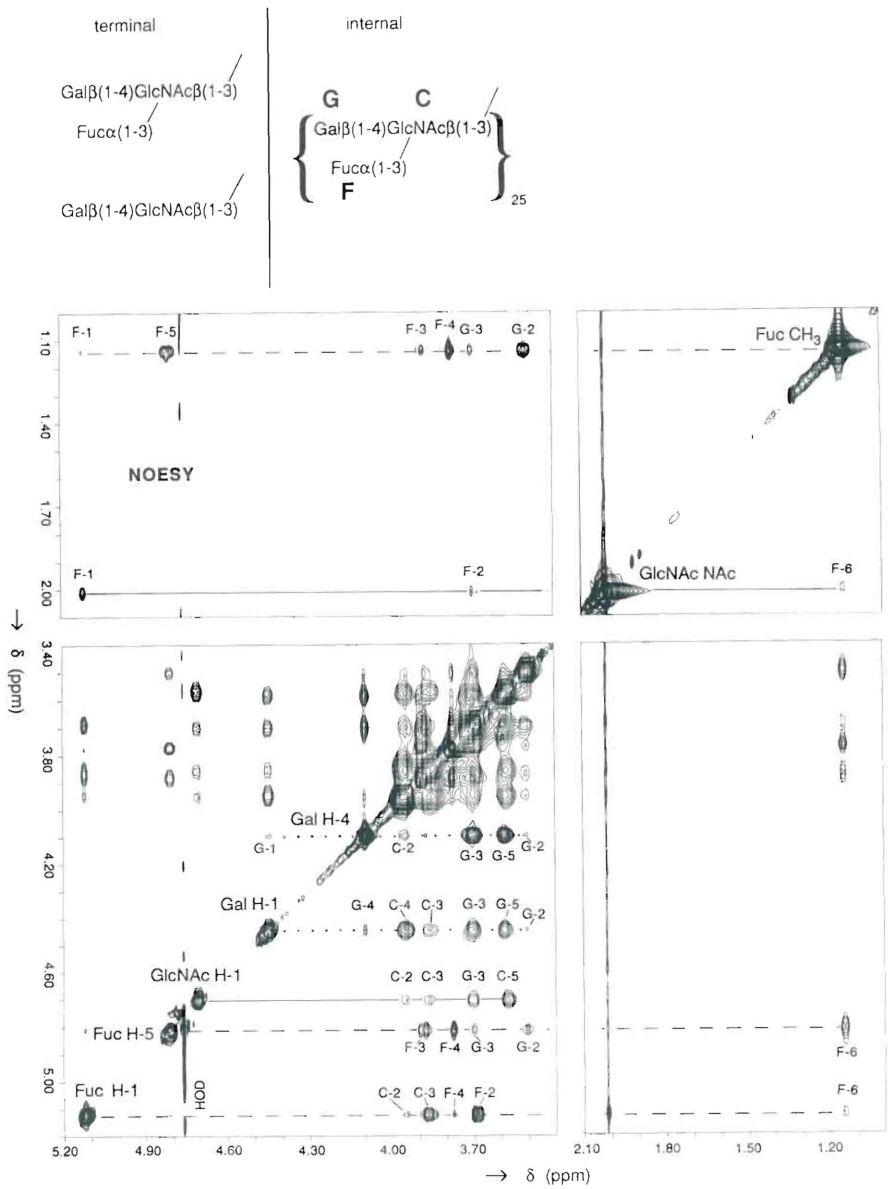


Figure 5. 500-MHz 2D NOESY spectrum of fraction P recorded at 300 K, with a mixing-time of 75 ms. (----), (••••) and (———) are for Fuc, Gal, and GlcNAc, respectively, to show magnetic dipole networks of (from top to bottom) Fuc CH_3, GlcNAc NAc, Gal H-4, Gal H-1, GlcNAc H-1, Fuc H-5 and Fuc H-1. A letter-number combination near the cross-peaks refers to the proton (1–6) of a mono-saccharide residue (C, GlcNAc; F, Fuc; G, Gal), which has a NOE contact with the proton of the monosaccharide residue indicated at the corresponding

In addition to the intense signals arising from the major structural element, the HOHAHA tracks with lower intensity correspond with non-reducing terminal units (Table 3). The set of structural-reporter-group signals, namely Gal H-1 at δ 4.462, Fuc H-1 at δ 5.132, Fuc H-5 at δ 4.832, and Fuc CH_3 at δ 1.174, fit that of a non-reducing terminal Galβ(1-4)[Fucα(1-3)]GlcNAcβ unit in the O-linked reference octasaccharide alditol **164** [30]. Therefore, this set is assigned to a non-reducing terminal Galβ(1-4)[Fucα(1-3)]GlcNAcβ unit in **P**. Non-reducing terminal *N*-acetyllactosamine units without Fuc are reflected by a minor set of signals, namely Gal H-1 at δ 4.480, H-3 at δ 3.664 and H-4 at δ 3.921 (Table 3). These data coincide with those of non-reducing terminal *N*-acetyllactosamine elements in reference compounds **12** and **26** [30]. These elements comprise about 20 mol/100 mol of the total amount of non-reducing terminal units in **P**, as deduced from a comparison of the intensity of the different Gal H-1 signals. Since internal repeating units are nearly completely fucosylated and non-reducing terminal *N*-acetyllactosamine units are 80% fucosylated, it is estimated that the average degree of polymerization is about 25 repeating units/chain, using the intensities of the methyl group protons of Fuc at δ 1.145 (internal units) and at δ 1.174 (terminal unit).

Epitope characterization

To determine the role of the poly-*N*-acetyllactosamine structure in antibody recognition of **P**, human sera containing IgM anti-i or anti-I antibodies were tested in ELISA by incubation with intact immunopurified CCA. The absence of binding (Fig. 6) confirms the immunodominance of Fuc in the poly-α(1-3)-fucosyl-*N*-acetyllactosamine structure.

Mild acid hydrolysis completely destroyed the antigenicity of CCA, as determined in ELISA (Fig. 7), which also indicates the necessity for Fuc in the epitope recognition. To selectively oxidize terminal Fuc or Gal, CCA was subjected to mild periodate treatment. This modified CCA showed a marked reduction in binding of several CCA-specific McAbs (Table 4), again illustrating that the Fuc residues are essential for the expression of antigenicity.

The observation that the detectability of CCA in the antigen-capture ELISA is not reduced after reductive alkaline β-elimination (Fig. 1) demonstrates the presence of multiple epitopes on one **P** polysaccharide chain. The Le^x trisaccharide (Galβ(1-4)[Fucα(1-3)]GlcNAcβ-*O*-Ethyl) was not able to inhibit the binding of anti-CCA McAbs to immobilized CCA in ELISA (Fig. 8), suggesting that the antibodies require for binding a larger epitope than one trisaccharide repeating unit. Neither the modified Le^x trisaccharide (GalNAβ(1-4)[Fucα(1-3)]GlcNAcβ-*O*-Methyl) gave rise to inhibition.

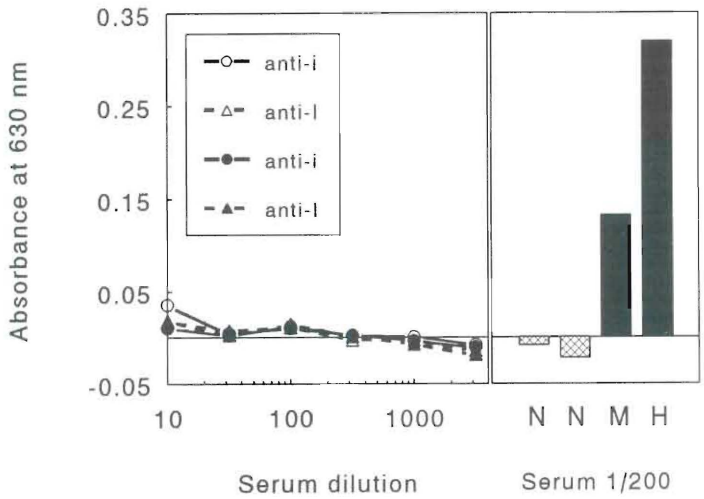


Figure 6. Binding of anti-i and anti-I antibodies to CCA in ELISA. Sera with anti-i or anti-I activity in erythrocyte agglutination assays were tested against CCA in a dilution series (symbols and lines indicated in the legends). As negative controls sera from healthy blood donors (N, hatched bars) were used in a 1/200 dilution, and as positive controls sera from schistosomiasis patients showing moderate (M) or high (H) anti-CCA reactivity (solid bars). Bound IgM antibodies were detected using human IgM-specific peroxidase conjugates and $(Me_2NH_2C_6H_2-)_2$ substrate as described in the text. Absorbances were corrected for background of wells not coated with CCA.

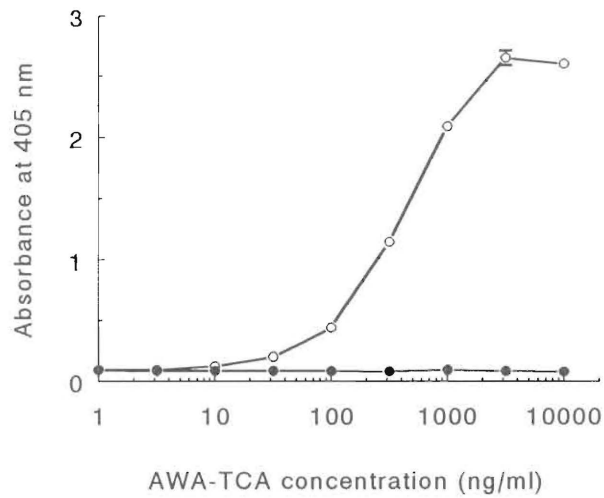


Figure 7. Influence of mild acid hydrolysis of CCA on reactivity in ELISA. AWA-TCA (500 μg/ml) was treated with 0.1 M TCA for 1 h at 100°C and tested in antigen-capture ELISA for CCA (●). As a control untreated AWA-TCA was used (○).

Table 4. Sensitivity to periodate oxidation of epitopes recognized by six different CCA-specific McAbs. Antigen-coated ELISA-plates were treated with different concentrations of $NaIO_4$ and reduction of McAb binding estimated using interpolation. I_{50} is the concentration of $NaIO_4$ at which the McAb showed a 50% decrease in binding to coated antigen.

McAb	Isotype	I_{50}
		mM
8.3C10A	IgM	1.5
24-2E5-A	IgM	0.3
54-5C10-A	IgG3	4
54-6G1-B	IgG1	0.1
114-1H12-A	IgG1	0.1
180-1D9-A	IgG1	1.1

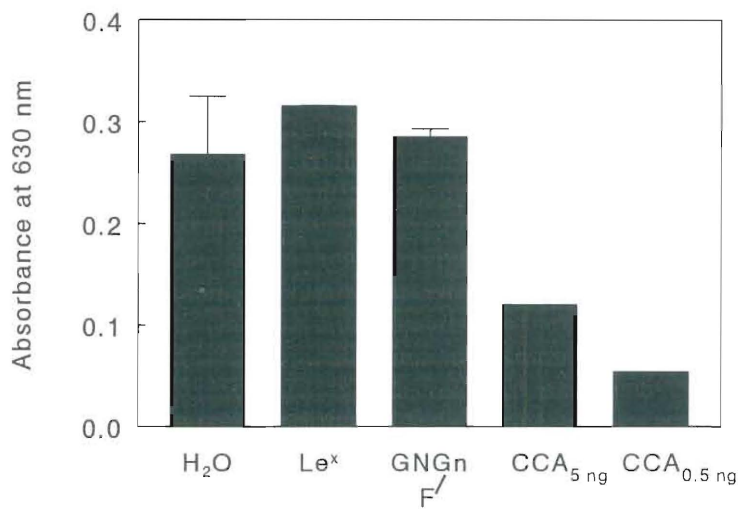


Figure 8. Inhibition of McAb binding to CCA in antigen-coated ELISA. 50 ng of CCA in 100 μl $NaCl/P_i$ were coated, followed after washing by incubation of biotin-labeled McAb 8.3C10, previously incubated with solutions in water containing respectively no trisaccharide, 5 μg Lewis x (Le^x) trisaccharide or 5 μg modified Le^x (labeled 'GNGn', in which Gal is replaced by GalNAc). Bound McAb is detected using streptavidin-peroxidase conjugate and $(Me_2NH_2C_6H_2-)_2$ substrate as described in the text. Specificity is shown by decreased absorbance after coating lower amounts of CCA (5 ng and 0.5 ng, respectively).

The Le^x–specific McAb (anti–CD15, Dako C3D–1) and a lectin (*Lotus*) used for isolation of Le^x structures found in *Schistosoma* [66] bound to CCA coated onto ELISA–plates (Fig. 9), indicating that Le^x units, which are mostly present as internal units, were recognized in CCA. In this experiment, two anti–CCA McAbs (8.3C10, 54–5C10–A) were used as positive controls. Neither a McAb directed against carcinoembryogenic antigen (CEA) nor another Fuc–specific lectin UEA–I bound to CCA.

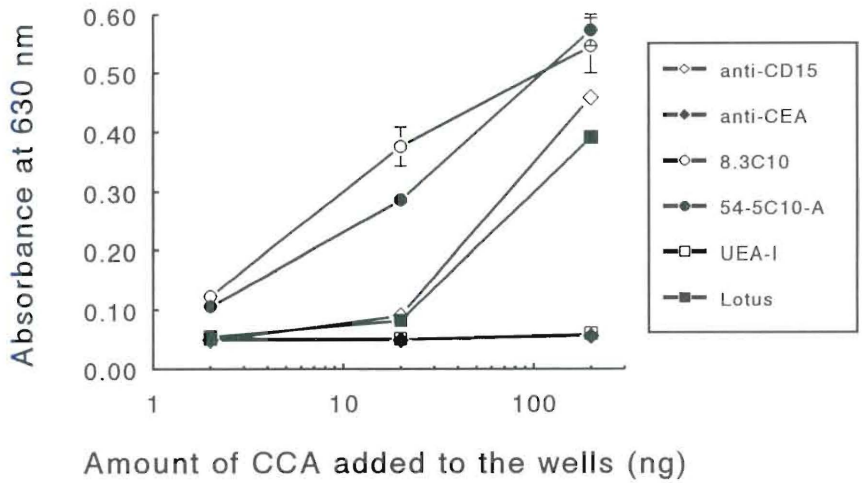


Figure 9. Recognition of CCA in antigen–coated ELISA by different McAbs and lectins. CCA was coated in a concentration series, after which the plate was blocked with BSA, incubated with different McAbs or biotinylated lectins, followed by, respectively, peroxidase conjugated rabbit anti–mouse Ig or streptavidin–peroxidase. Color development was performed using $(Me_2NH_2C_6H_2–)_2$ substrate and absorbance measured at 630 nm. (◇), McAb anti–CD15 (Dako C3D–1, IgM), (◆), McAb anti–carcinoembryogenic antigen (CEA, Dako A5B7, IgG1), (○), McAb 8.3C10 (anti–CCA, IgM), (●), McAb 54–5C10–A (anti–CCA, IgG3), (□), *Ulex europaeus* I–biotin (UEA–I, E–Y Laboratories), (■), *Lotus tetragonolobus* agglutinin–biotin (Lotus, E–Y Laboratories).

Discussion

In this study, the immunopurification of schistosome CCA, by McAb–based immunoaffinity chromatography is reported. Other procedures have been described, based on ion–exchange chromatography [19] or immunoaffinity chromatography using polyclonal antibodies from *Schistosoma*–infected patients [8], which would yield less pure preparations than McAb immunoaffinity

chromatography. The present monosaccharide analyses and carbohydrate content of the glycoprotein as well as the presence of the O-linked chains were in good agreement with the findings of Carlier *et al.* [8]. The presence of Fuc required a mild preparative procedure, omitting the conventional TCA-precipitation step [8,19]. The resulting CCA-preparation was used to elucidate the primary structure of the O-linked carbohydrate chains on which the antigenic determinant was shown to be located. The O-linked structures are predominantly attached via GalNAc-Thr to the glycoprotein and account for approximately 80% of the molecular mass. These characteristics of CCA, in combination with the heterogeneity and the localization within the schistosome gut, allow the antigen to be considered as a mucin-type glycoprotein, which has been proposed to be involved in the protection of the gut epithelium [8].

It is shown that the population of O-linked glycans in CCA comprises for the minor part di- to hexasaccharide and for the major part polysaccharide carbohydrate chains. The oligosaccharide alditols **O** have the Galβ(1-3)GalNAc-OL core in common. This core-structure type 1 can be converted into core type 2 by extension with a βGlcNAc residue (1-6)-linked to GalNAc-OL. Mass spectrometry demonstrated that the oligosaccharide alditols **O** can be fucosylated (Table 1). In addition, CID-MS analysis of a fraction too minor in quantity for NMR analysis showed the presence of an unusual branched core, consisting of a HexNAc-OL substituted with two Hex residues. A core structure identified as Galβ(1-3)[Galβ(1-6)]GalNAc-OL was found in human gastric mucins [62,63] which could be the same as that found in the present study. Analysis of the polysaccharide alditol **P** showed the occurrence of a poly-Le^x carbohydrate chain, containing GalNAc as the reducing terminal monosaccharide. It is tempting to hypothesize that the poly(Le^x) chains are attached to the protein backbone predominantly via core type 1 and/or core type 2 elements.

The monosaccharide analysis of fraction **P** demonstrated that the relative amounts of Fuc, Gal, and GlcNAc are in accordance with the proposed polysaccharide structure. However, the monosaccharide analysis of native CCA shows a larger amount of GlcNAc, probably due to the additional presence of terminal O-linked GlcNAc, as reported for a schistosome glycoprotein pool [49]. It can be assumed that these residues (as GlcNAc-OL) are lost during the chromatographic preparation of the alkaline-borohydride treated CCA.

The single repeating trisaccharide unit identified in **P** is known as the Le^x determinant (also called stage-specific embryonic antigen-1 SSEA-1 or CD15). The presence of this structural element is confirmed by the binding of an Le^x-specific McAb to purified CCA. Therefore, CCA can now be described in terms of an O-linked poly(Le^x) carbohydrate chain with approximately 25 repeating units. This polysaccharide structure is responsible for the antigenic

character, as shown in the indirect and direct ELISA with alkaline–treated and periodate–treated CCA. Recently, in a pool of schistosome glycoproteins which was purified using a completely different method, a similar structure with at least four repeating units of the Le^x determinant as part of N–linked carbohydrate chains was demonstrated [66]. Those authors speculated that the poly(Le^x) structures may be localized at the schistosome surface, while CCA clearly originates from the gut of the parasite [19]. Another major discrepancy is that using immunoblotting procedures CCA can only be visualized as a high–molecular mass smear [8,72], while Srivatsan *et al.* [66] showed a number of distinct bands reactive with anti–Le^x McAb on an immunoblot of their antigen preparation. In addition to these differences, the antigens show structural similarities since anti–Le^x McAbs bound to both antigens.

No indications were found in CCA for the previously described highly immunogenic polyfucosylated structures in *S. mansoni* glycoproteins or glycolipids, consisting of repeating units containing non–reducing terminal and internal Fuc residues: –2)Fuc(1–4)[Fuc(1–3)]GlcNAc(1– [35,80].

Carbohydrate chains containing multiple Le^x determinants have been identified on glycolipids from human colonic and liver adenocarcinomas (two or three Le^x repeating elements [25]). Moreover, circulating granulocytes are enriched in Le^x and carry in relatively high abundance branched N–linked polysaccharides having Le^x repeating units. These structures were hypothesized to be granulocyte–specific antigens [64]. The Le^x sequence and, to a much larger extent, its sialylated form play an important role in granulocyte and monocyte adhesion processes, by serving as ligands for adhesion molecules, *e.g.* P–selectin, present on endothelial cells and platelets [24,34,37,65,68,79]. These adhesion molecules are involved in recruiting granulocytes to sites of inflammation [4,65]. In this context, it has been suggested that inhibition of these adhesion events might have anti–inflammatory and anti–thrombogenic effects [65]. Inflammation reactions as well as blood coagulation are host protection mechanisms which are potentially very harmful to the schistosome, living predominantly in small blood–vessels. It is conceivable that the excretion of relatively large amounts of CCA, subsequently leading to high local CCA concentrations, induces these anti–inflammatory and anti–thrombogenic effects and thus may be one of the parasite's important survival strategies.

Of additional interest is that during schistosomiasis, granulocytes (mostly eosinophils and neutrophils) have been shown to play a major role in the protective response against the parasites [5,6,27,42], in particular in the skin response directly after the penetration of the cercariae [38,43]. The relatively high expression of the poly(Le^x) determinant on CCA would trigger a host immune response, which subsequently would be directed not only to CCA but also against the host's granulocytes. In schistosomiasis patients, high IgM titres

are observed against parasite gut-associated antigens [23,48] and in particular against CCA [18,20]. Ongoing experiments in our laboratory indicate that CCA-specific McAbs of different isotypes also recognize granulocytes isolated from the blood of healthy human donors, confirming that multiple Le^x epitopes are recognized.

Others have found that a murine protective IgM McAb, raised against *S. mansoni* eggs, recognized the Le^x determinant [31]. From this observation it was suggested that such antibodies, which are also directed against host carbohydrate structures, may be involved in affecting the circulating granulocytes [31]. Excretion of gut-associated CCA evokes high titres of IgM anti-CCA [*i.e.* thus also anti-poly(Le^x) antibodies [20]]. It has been therefore suggested to be one of the mechanisms of schistosomes for misleading the host's defense system by raising an antibody response against an excretory antigen. Structural homology of CCA with one of the major granulocyte surface antigens [64] makes it likely that these anti-CCA antibodies also cause complement-dependent granulocyte lysis, thereby reducing the host's cellular immune response activity. This parasite-induced autoimmunity may be balanced by host regulatory mechanisms, since only a mild-to-moderate neutropenia is observed in patients with chronic schistosomiasis [3]. Currently, experiments are being carried out to study whether anti-CCA antibodies in the sera of schistosomiasis patients mediate lysis of granulocytes in the presence of complement.

Acknowledgements

The authors would like to thank H. J. L. Ravestein (Faculty of Biology, Department of Instrumental Analysis, Utrecht University) for performing the amino acid analysis, and Y.E. Fillié, D. Kornelis, and T.M. Falcão Ferreira (Faculty of Medicine, Department of Parasitology, University of Leiden) for excellent technical assistance during the isolation and immunochemical characterization of CCA. This project was financially supported by grants from the Netherlands Foundation for Biological Research (NWO/BION) and the Netherlands Foundation for Chemical Research (NWO/SON).

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