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Various stages of *Schistosoma* express Lewis^x, LacdiNAc, GalNAcβ1–4 (Fucα1–3)GlcNAc and GalNAcβ1–4(Fucα1–2Fucα1–3)GlcNAc carbohydrate epitopes: detection with monoclonal antibodies that are characterized by enzymatically synthesized neoglycoproteins

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We report here that fucosylated epitopes such as Lewis^x, LacdiNAc, fucosylated LacdiNAc (LDN-F) and GalNAcβ1–4(Fucα1–2Fucα1–3)GlcNAc (LDN-DF) are expressed by schistosomes throughout their life cycle. These four epitopes were enzymatically synthesized and coupled to bovine serum albumin to yield neoglycoproteins. Subsequently these neoglycoproteins were used to probe a panel of 188 monoclonal antibodies obtained from infected or immunized mice, in ELISA and surface plasmon resonance analysis. Of these antibodies, 25 recognized one of the fucosylated structures synthesized, indicating that these structures are immunogenic during infection. The MAbs identified could be subdivided in four different groups based on the recognition of either the Lewis^x-, the LacdiNAc-, the LDN-DF-, or both the LDN-F- and LDN-DF epitope. These monoclonal antibodies were then used to investigate the localization of the fucosylated epitopes in various stages of *Schistosoma mansoni* using indirect immunofluorescence. Lewis^x epitopes were mainly found in the gut and on the tegument of adult worms, on egg shells, and on the oral sucker of cercariae. The LacdiNAc epitope was expressed on the tegument of adult worms, on miracidia, and on the oral sucker of cercariae. In contrast, LDN-DF epitopes were mainly present in the excretory system of adult worms, on miracidia and on whole cercariae. These also stained positive with the LDN-F/LDN-DF epitope antibodies, while whole parenchyma reacted characteristically only with the latter antibodies. The identification of different carbohydrate structures in various stages of schistosomes may lead to a better understanding of the function of glycans in the immune response during infection.

Key words: fucose/oligosaccharide/antigenicity/enzymatic synthesis/schistosomiasis

Introduction

Schistosomiasis is a parasitic disease, caused by blood flukes of the genus *Schistosoma* that affects approximately 200 million people worldwide. The life cycle of schistosomes involves a vertebrate definitive host in which sexual reproduction takes place and a fresh water snail in which asexual multiplication occurs. Since the parasites remain in their hosts for extended time periods, they have developed mechanisms to evade or resist the immune system of both hosts. In the various developmental stages, oligosaccharides at the surface of the parasite, in particular fucosylated ones, are proposed to be involved in these processes (Cummings and Nyame, 1996, 1999; Van Dam and Deelder, 1996).

The structures of several schistosomal glycoconjugates have been elucidated. For example, monomeric and polymeric Lewis^x (Le^x) structures have been demonstrated on both membrane-bound and secreted glycoproteins of adult worms (Ko *et al.*, 1990; Srivatsan *et al.*, 1992; Van Dam *et al.*, 1994). In addition to Le^x structures, the adult worms of *Schistosoma mansoni* also synthesize N-linked glycans containing GalNAcβ1–4GlcNAc (LacdiNAc, LDN) and GalNAcβ1–4(Fucα1–3)GlcNAc (LDN-F) (Srivatsan *et al.*, 1992), the LacdiNAc analog of the Le^x blood group antigen. Multifucosylated structures have been found on O-glycans of the cercarial glycocalyx with the following terminal structure: (Fucα1→2)±Fucα1→2Fucα1→3GalNAcβ1→4((Fucα1→2) ±Fucα1→2Fucα1→3)-GlcNAcβ1→3Galα1→ (Khoo *et al.*, 1995) and on glycolipids of *S. mansoni* eggs that consist of a backbone of repeating β1–4 linked GlcNAc residues substituted with Fucα1–2Fucα1–3 side chains (Khoo *et al.*, 1997).

It has long been known that the humoral immune response in schistosomiasis is mainly directed against glycoconjugates (Nash *et al.*, 1981; Aronstein *et al.*, 1983; Omer Ali *et al.*, 1988). More recently, several of these carbohydrates have been identified. In early infections, the most pronounced antibody response (IgM) in humans is directed against the gut-associated circulating cathodic antigen (CCA) (Deelder *et al.*, 1989). Upon infection with *S. mansoni*, humans and primates generate cytolytic IgM and IgG antibodies against the Le^x structure (Nyame *et*

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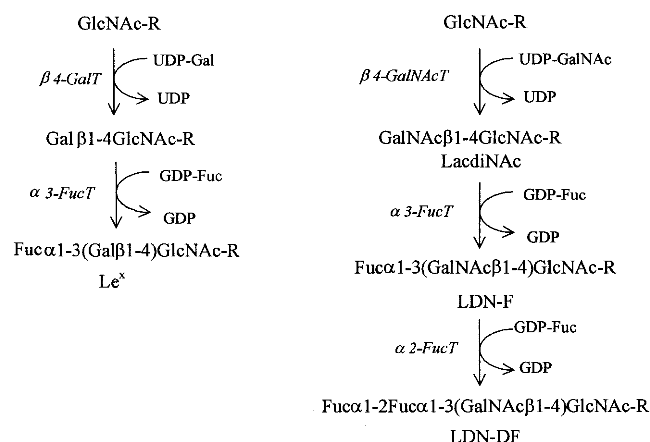

Fig. 1. Outline of the enzymatic synthesis of Le^x (left) and LacdiNAc, LDN-F and LDN-DF (right).

al., 1996; Van Dam *et al.*, 1996). Recent studies of Nyame *et al.* (1999) showed that sera of mice infected with *S.mansoni* show IgM and IgG antibody titers toward LacdiNAc.

The aim of this study was to gain more insight into the expression of fucosylated epitopes such as Le^x, LacdiNAc, LDN-F, and GalNAcβ1–4(Fucα1–2Fucα1–3)GlcNAc (LDN-DF) throughout the schistosome life cycle. To this end, we synthesized these fucosylated epitopes and report here that antibodies recognizing these structures are generated by *Schistosoma* infected mice, indicating that they are immunogenic during infection. MAbs recognizing these structures were used to investigate the localization of these epitopes in various stages of the schistosome by an indirect immunofluorescence assay.

Results

Synthesis of oligosaccharides and preparation of neoglycoproteins

In order to identify MAbs directed against defined fucosylated schistosomal oligosaccharides, different neoglycoproteins were prepared. Spacer-linked oligosaccharides (LacdiNAc, LDN-F, LDN-DF, and Le^x) were synthesized on a 1–5 µmol scale using partially purified glycosyltransferases according to the reaction scheme shown in Figure 1. During the first two steps of the synthesis, the reactions proceeded quantitatively, whereas the conversion of LDN-F to LDN-DF was 70% efficient as deduced from HPAEC analysis. After isolation and purification of the products, the structures of the oligosaccharides were verified by ^{1}H-NMR spectroscopy. The spectral data (not shown) of LacdiNAc and LDN-DF were identical to those of the same compounds published by Hokke *et al.* (1998). The chemical shifts of the structural-reporter-group protons of the monofucosylated product LDN-F were comparable to those of GalNAcβ1–4(Fucα1–3)GlcNAcβ-OCH_3 (compound GN[F]Gn in Bergwerff *et al.*, 1993), with the exception of GlcNAc H-1 which shifted slightly due to the presence of a different aglycon in the reference compound. Similarly, the ^{1}H-NMR data of Le^x are essentially the same as those of Galβ1–4(Fucα1–3)GlcNAcβ-OCH_2CH_3 (compound G[F]Gn in Bergwerff *et al.*, 1993).

Table I. Degree of incorporation of oligosaccharides into BSA

BSA conjugate	Single-charged protein (*m/z*)	Degree of incorporation (mol/mol)
BSA	66.411	0
Le^x-BSA	75.967	14
LacdiNAc-BSA	72.979	12
LDN-F-BSA	68.546	3
LDN-DF-BSA	75.907	11

The spacer-linked oligosaccharides were then converted to their *N*-hydroxysuccinimido-ester complexes and conjugated to the amino groups in BSA. These neoglycoproteins were subjected to MALDI-TOF MS (spectra not shown). The average molecular weight allowed the calculation of the average number of oligosaccharides added to BSA (Table I).

Identification of schistosome specific MAbs that bind the neoglycoproteins

A panel of 188 monoclonal antibodies derived from fusions with spleen cells of mice which either had been infected with cercariae of *S.mansoni*, *S.japonicum*, and *S.haematobium* or had been artificially immunized with hatching fluid, was selected on basis of their expected reactivity with carbohydrate epitopes such as the reactivity with Rossman's fixed parasite sections in IFA and the recognition of repetitive epitopes in immunoelectrophoresis. This panel was used to analyze the binding of MAbs to the different neoglycoproteins in ELISAs. The MAbs that were found to react with the different carbohydrate epitopes on the neoglycoproteins are listed in the first column of Table II. Five MAbs were found to bind the LacdiNAc epitope and 14 MAbs to the Le^x epitope. These MAbs showed no cross-reactivity with the other carbohydrate epitopes. Seven MAbs recognized the LDN-DF epitope, yet three of them cross-reacted with the LDN-F epitope. No MAbs were found that reacted with the LDN-F epitope exclusively. Most MAbs were of the IgM isotype, although several IgG MAbs were identified (Table II, column 3).

Screening of MAbs using neoglycoproteins by surface plasmon resonance

The avidity of binding of the MAbs to the different neoglycoproteins was also determined by SPR analysis, using BSA as a negative control. A summary of the results is given in the fourth column of Table II. MAbs that reacted with LDN or LDN-DF had a response of more than 500 RU. The MAbs that recognized Le^x or LDN-F/LDN-DF had lower and more variable responses from 50–500 to >500 RU. The MAbs that responded negatively in ELISA were also negative in SPR analysis (data not shown). In Figure 2 representative examples of sensorgrams are depicted, illustrating different binding patterns for each group of MAbs.

Localization of fucosylated carbohydrate epitopes in various stages of Schistosoma mansoni

The localization of the different fucosylated carbohydrate epitopes throughout the life-cycle of *S.mansoni* was investigated

Table II. Specificity, isotype and binding avidity of MAbs directed against fucosylated carbohydrate epitopes, and localization of these epitopes in various stages of *S.mansoni*

				Indirect immunofluorescence assay: Adult worm (RM)				Adult worm (FS)					Egg				Cercaria		
MAb	Infection/immunization	Ig Isotype	SPR (RU)	Gut	Parenchyma	Tegument	Excr. Syst.	Gut	Parenchyma	Tegument	Excr. Syst.	Kupffer cells	Free Antigen	Miracidium	Egg Shell	Eggs Spot	Whole cercaria	Partial e.g.head	Oral Sucker
A. directed against LacdiNAc																			
99-2A5-B	Inf. Mans.	M	>500	-	-	+	-	-	-	+	-	-	-	+	+	-	-	-	+
100-2H5-A	Inf. Mans.	M	>500	-	-	+	+	+	-	+	-	-	-	+	-	-	-	-	+
114-2H12-C	Inf. Mans.	M	>500	-	-	+	+	-	+	+	+	-	+	+	+	-	-	-	+
259-2A1	Inf. Haem.	G3	>500	-	+	+/-	-	-	-	+	-	-	-	+	-	-	-	-	-
273-3F2	Inf. Jap.	M	>500	-	-	+	-	-	+	+	-	-	+	+	+	-	-	-	-
B. directed against LDN-DF																			
114-5B1-A	Inf. Mans.	G1	>500	-	-	-	+	-	-	-	+	-	+	+	+	-	+	-	-
176-3A7	Inf. Mans.	G3	>500	-	-	-	+	+	-	+	+	-	-	+	-	-	+	-	-
290-2D9-A	Im. HF	M	>500	-	-	-	+	-	-	-	+	-	+	+	+	-	+	-	-
290-4A8	Im. HF	M	>500	-	-	-	+	-	-	-	+	-	+	+	+	-	+	-	-
C. directed against LDN-DF + LDN-F																			
204-6A1	Inf. Jap.	M	50-500	-	+	+	+	-	+	+	+	-	+	+	+	-	-	+	-
290-2E6	Im. HF	M	50-500	-	+	-	-	+	+	-	+	-	-	+	-	-	-	+	-
294-2A1	Inf. Haem.	M	>500	-	+	-	+	-	+	-	+	-	+	+	+	-	-	+	-
D directed against Le^x																			
22-1B3-A	Inf. Mans.	M	>500	+	-	-	-	+	-	+	+	+	+	-	+	-	+	-	-
22-5G11-A	Inf. Mans.	M	50-500	+	-	-	-	+	-	+	+	+	+	-	+	-	+	-	-
99-1G3-A	Inf. Mans.	M	50-500	+	-	-	-	+	-	-	-	+	-	+	-	+	-	-	+
128-4F9-A	Inf. Mans.	M	>500	+	-	-	-	+	-	+	-	+	-	-	+	+	-	-	-
254-1G9	Inf. Mans	M	50-500	+/-	-	-	-	+	-	+	-	-	-	-	+	-	-	-	+
259-3E11	Inf. Haem.	M	>500	+	-	-	-	+	-	+	-	+	+	-	+	-	nd		
259-3E12-A	Inf. Haem.	M	>500	+	-	-	-	+	-	+	-	-	-	-	+	-	-	-	+
273-3E7-A	Inf. Jap.	M	>500	+	-	-	+	-	-	+	+	-	-	-	+	-	-	-	+
291-2G3-A	Im.HF	G1	>500	-	+	+	+	-	-	+	-	-	-	+	-	-	-	-	+
291-4D10-A	Im.HF	M	>500	+	-	-	+	+	+	+	-	+	+	-	+	-	-	-	+
292-1G3A	Inf. Haem.	M	50-500	+	-	-	-	+	-	+	-	+	-	-	+	-	-	-	+
292-4C1-A	Inf. Haem.	M	>500	+	-	+/-	-	+	-	+	-	+	+	-	+	-	nd		
292-5G4-A	Inf. Haem.	M	>500	+	-	-	-	+	-	+	-	+	-	+	-	-	-	-	+
293-3B5-A	Inf. Haem.	M	50-500	+	-	-	-	+	-	-	-	+	-	+	-	-	-	-	-

MAbs were obtained from fusions of spleen cells of mice infected (Inf.) with *S.mansoni* (Mans), *S.haematobium* (Haem), and *S.japonicum* (Jap) or immunized (Im.) with hatching fluid (HF) of *S.mansoni* eggs. Excr.Syst, excretory system; RM, Rossman's fixative; FS, frozen sections; nd, not determined.

by probing frozen sections of *S.mansoni* infected hamster livers, paraffin sections of *S.mansoni* adult worms fixed in Rossman's fixative (Nash *et al.*, 1977; Nash, 1978) and whole cercariae with the corresponding MAbs in indirect immunofluorescence analysis (IFA). The fluorescence patterns for the MAbs on different life-cycle stages of the parasite are summarized in Table II, and typical examples are given in Figure 3, showing reactivity of several MAbs. A characteristic fluorescence pattern of the anti-Le^x MAbs was found mainly in the gut and on the tegument of adult worms, on the egg shell and on the oral sucker of the cercariae (Figure 3.1, 3.2, 3.6). These MAbs also reacted with Kuppfer cells in the liver, indicating that these antigens were probably released by the parasite into the host circulation (Figure 3.1).

In contrast, the MAbs that specifically recognized the LDN-DF epitopes, showed reactivity with the excretory system including flame cells of adult worms, with miracidia and with whole cercariae (Figure 3.4 and 3.5). Using the MAbs that

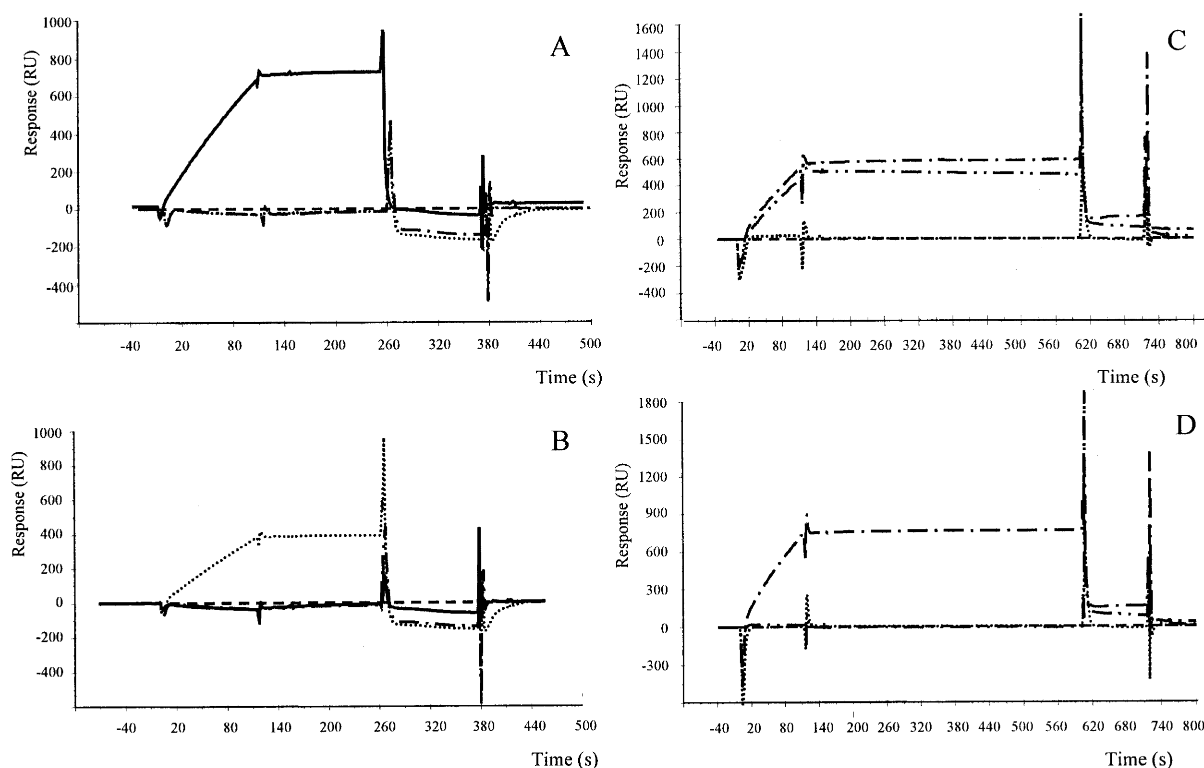

Fig. 2. Composite sensorgrams illustrating binding characteristics representative for each group of MAbs to the immobilized neoglycoproteins. The interaction of MAb 100–2H5-A (1:10 diluted in HBS) (**A**) and MAb 273–3E7-A (1:10 diluted in HBS) (**B**) with LacdiNAc, Le^x and LDN-DF (4000 RU immobilized) is shown. The pattern of both MAbs observed with LDN-F was similar to that found for BSA. The interaction of MAb 294–2A1 (**C**) and MAb 290–4A8 (1:10 diluted in HBS) (**D**) with LDN-F (7000 RU immobilized), Le^x and LDN-DF (2500 RU immobilized) is shown in the right panel. The pattern of both MAbs obtained with LacdiNAc was identical to that found for Le^x. —LacdiNAc-BSA; ·····Le^x-BSA; —·—LDN-F-BSA; —··—LDN-DF-BSA; – –BSA.

bound both the LDN-DF and the LDN-F epitope, the whole parenchyma of the adult worms, miracidia, the upper part of the head of the cercariae, and the tip of the tail showed a positive reaction (Figure 3.3 and 3.7). Finally, MAbs that recognize the LacdiNAc epitope reacted mainly with the tegument of the adult worm, miracidia, and with the oral sucker of the cercariae.

Discussion

Schistosomes are complex organisms shown to contain and to synthesize numerous glycoproteins and glycolipids, some of which include unique carbohydrate structures such as the multi-fucosylated glycans present on O-glycans of the cercarial glycocalyx, on the egg N- and O-glycans and on egg glycosphingolipids (Khoo *et al.*, 1995, 1997). In this study, carbohydrate antigens from *S.mansoni* have been synthesized enzymatically using partially purified glycosyltransferases. Coupled to BSA, these neoglycoproteins were then used both in ELISA and in SPR analysis to identify from an already available library of MAbs directed against schistosomal antigens, MAbs that specifically recognized the oligosaccharides synthesized. Reactivity with a number of carbohydrate specific MAbs indicates that the respective carbohydrate epitopes are indeed present in schistosomes and, at least in the mouse, presented to the immune system during infection leading to the generation of anti-carbohydrate antibodies.

With the identification of several MAbs, tissue specific oligosaccharides throughout the life-cycle stages in *Schistosoma* can be detected. In this study, several MAbs were found that recognize the monomeric Le^x epitope. Previously, a number of MAbs were identified that bind to CCA, the carbohydrate portion of which consists of repeats of the Le^x structure (Van Dam *et al.*, 1994; Deelder *et al.*, 1996). Of the 15 MAbs against CCA tested, only MAbs 22–1B3-A and 22–5G11-A (Deelder *et al.*, 1996) recognized in addition the monomeric Le^x epitope. Both these MAbs bind to the surface of cercariae in contrast to the MAbs that exclusively recognize the monomeric form of Le^x. These data strongly suggest that the staining of the surface of cercariae is due to the presence of polymeric Le^x. The monomeric Le^x epitope is not exposed on the surface of cercariae, but only after their transformation into schistosomula its surface expression is initiated (Koster and Strand, 1994). MAbs that are specific for monomeric Le^x specifically recognize the oral sucker of cercariae, which organ supposedly is involved in attachment of the cercariae. During attachment and penetration of the skin, cercariae produce water insoluble secretions, which can be visualized with fluorescent lectins as “kissing marks.” (Linder, 1990). It was reported that MAb 504–5B1, which recognizes the Le^x epitope, stimulates cercariae to secrete mucous material at the attachment site to which it subsequently can bind (Koster and Strand, 1994).

Several MAbs were isolated that exclusively recognized the LDN-DF epitope. This structure could be detected on miracidia,

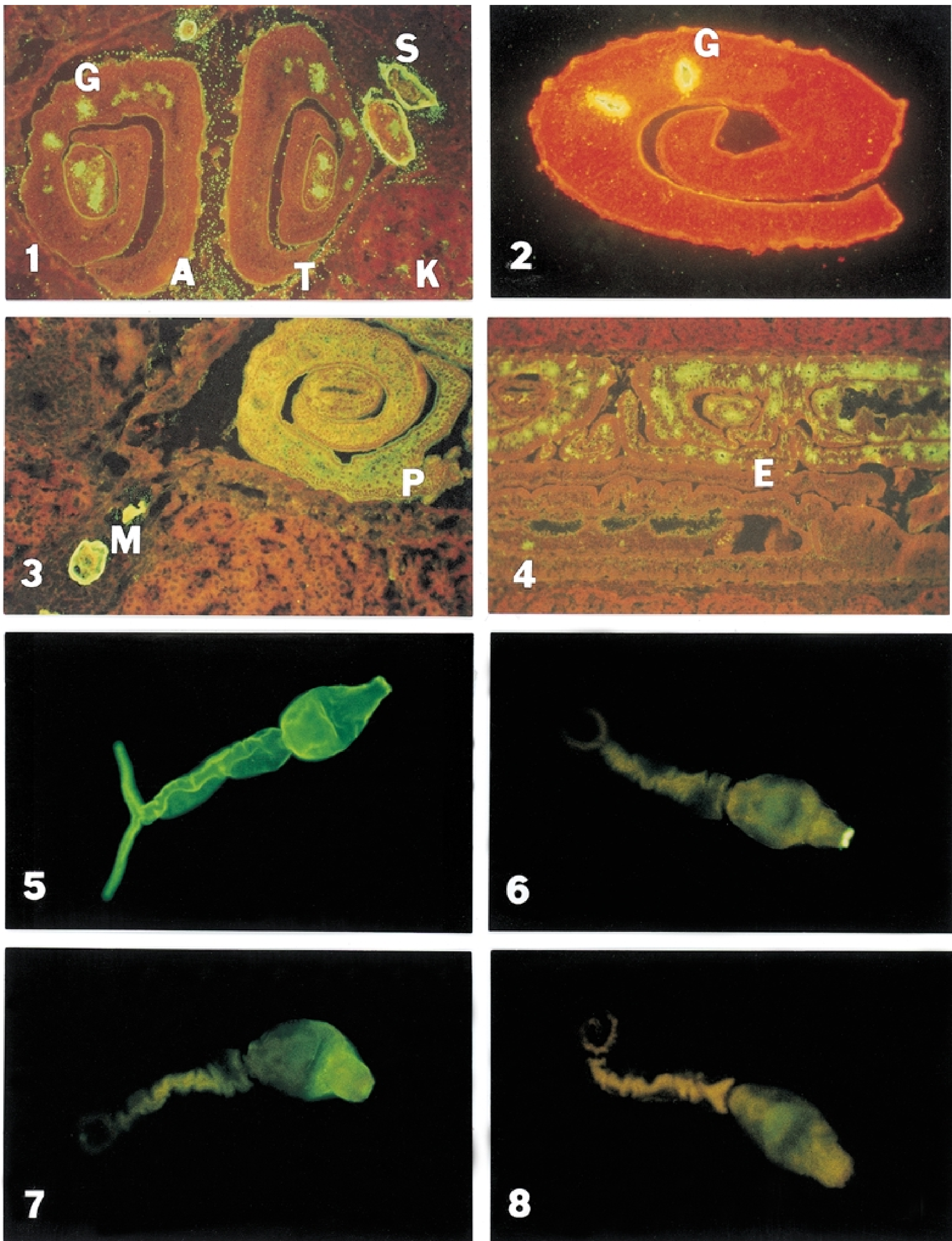

Fig. 3. Immunofluorescence patterns with different Mabs. (**1**, **3**, and **4**) Frozen sections of livers of *S. mansoni* infected hamsters; (**2**) on a section of adult *S.mansoni* worms fixed in Rossman's fixative and (**5–8**) whole cercariae. Sections of cercariae were incubated with (**1**, **2**) MAb 22–1B3-A (anti-Le^x); (**3**) MAb 204–6A1 (anti-LDN-F/LDN-DF); (**4**) MAb 290–2D9-A (anti-LDN-DF); (**5**) MAb 114–5B1-A (anti-LDN-DF); (**6**) MAb 292–1G3-A (anti-Le^x); (**7**) MAb 294–2A1 (anti-LDN-F/LDN-DF); and (**8**) negative medium/supernatant, respectively, followed by staining with a FITC conjugate of a rabbit–anti-mouse Ig antibody. Fluorescence was observed on the tegument (T); in the gut (G), and in Kupffer cells (K); in the parenchyma (P), on the eggshell (S), in the miracidium (M); in the excretory system (E); and with free antigen (A). Magnification: (**1**, **3**) 110×; (**2**) 215×; (**4**) 55×; (**5–8**) 225×.

egg shells, and whole cercariae and in the excretory system of adult worms including the flame cells. It should be mentioned that one of the MAbs that recognize the LDN-DF epitope (MAb 114–5B1-A) was defined in previous studies as a MAb that recognizes repeating carbohydrate epitopes of *S.mansoni* soluble egg and hatching fluid antigens (Nourel Din *et al.*, 1994). Furthermore this antibody has been reported to show a strong immunoreactivity with schistosomulum surface and with germinal cells in miracidia (Bogers *et al.*, 1994, 1995). A very similar fluorescence pattern was observed with MAb 128C3/3 (Koster and Strand, 1994) which recognizes fucose-containing carbohydrate epitopes present on both glycolipids and glycoproteins (Weiss and Strand, 1985; Weiss *et al.*, 1986; Dalton and Strand, 1987). However, while the binding of MAb 128C3/3 could be inhibited with monomeric L-fucose (Levery *et al.*, 1992), such an inhibition could not be shown for MAb 114–5B1-A (H.A.M.Nibbeling, personal communication). Therefore, it is unlikely that MAb 114–5B1-A recognizes exactly the same epitope as MAb 128C3/3. Yet another MAb (485D2/7) has been described that reacts with a part of the antigens reactive with MAb 128C3/3 and appears to be *S.mansoni* specific (Weiss and Strand, 1985). In this respect MAb 485D2/7 differs from MAb 128C3/3, which also is reactive with other schistosome species. It thus seems that the different MAbs each recognize unique epitope sites in fucosylated structures. It would be of interest to know whether the underlying sugars also form part of these epitopes, for instance by comparing the reactivity of LDN-DF with that of Fucα1–2Fucα1–3GalNAc (Kamath and Hindsgaul, 1996).

Of the MAbs tested three reacted with both the LDN-F and LDN-DF structures. Apparently these MAbs recognize a common epitope in LDN-F and LDN-DF. They reacted not only with the excretory system, egg shells, and miracidia, but also with the parenchyma. Comparison of the differences in reactivity of the anti-LDN-DF MAbs and the anti-LDN-F/LDN-DF MAbs (Table II) leads to the conclusion that the LDN-F epitope is probably expressed in the parenchyma and in the head of cercariae. By contrast, whole cercariae were not recognized by these MAbs. It is possible that the third fucose of the multifucosylated side-chain, present on O-glycans of the cercarial glycocalyx (Khoo *et al.*, 1995), blocks the recognition by anti-LDN-F/LDN-DF MAbs. Although LDN-F is present on complex-type N-glycans (Nyame *et al.*, 1989; Srivatsan *et al.*, 1992), no MAb was found that exclusively reacted with the LDN-F epitope. One explanation may be that the LDN-F structure as such is not presented to the immune system, because it is only present inside the parasite. Presentation of LDN-DF might in some instances lead to the generation of antibodies that only recognize the LDN-F part of this structure and hence show reactivity with both LDN-F and LDN-DF.

The advantage of using SPR analysis for screening MAbs against neoglycoproteins is that it gives an indication of the affinity of the MAbs for the neoglycoproteins in terms of association rate and affinity constants. Subsequently, the most promising MAbs can be selected and used for improving the immunodiagnosis of schistosomal infection. Indeed, initial studies have shown that SPR technology can be used to analyze the human antibody response against neoglycoproteins in schistosomiasis (A.van Remoortere, G.J.van Dam, D.H.van den Eijnden, I.van Die, and A.M.Deelder, unpublished observations).

In conclusion, the use of different neoglycoproteins can lead to the identification of MAbs that recognize antigens of *S.mansoni* and may lead to a better understanding of the expression and function of glycans in schistosomes. The identification of the different carbohydrate structures in the life cycle stages of *S.mansoni* provides more insight into the development of the parasite and into the immune response during infection.

Materials and methods

Materials

UDP-[^{3}H]GalNAc (24.0 Ci/mmol) and UDP-[^{3}H]Gal (37.9 Ci/mmol) were purchased from New England Nuclear (Boston, MA). The sugar nucleotide donor was diluted with unlabeled UDP-GalNAc or UDP-Gal (Sigma Chemical Co., St. Louis, MO), respectively, to give the desired specific radioactivity. GDP-Fuc was obtained from BioCarb (Lund, Sweden). GlcNAcβ1-O-$(CH_2)_8COOCH_3$ (GlcNAc-R) was a kind gift of Dr. O.Hindsgaul (University of Alberta, Edmonton, Alberta, Canada). Sep-Pak C-18 cartridges were obtained from Waters. The BIAcore 3000 instrument, BIAevaluation software 3.0, Sensor Chip CM5 and an Amine Coupling Kit were obtained from Biacore AB (Uppsala, Sweden).

Parasites

S.mansoni (Puerto Rico strain) adult worms were collected by perfusion of the hepatic portal system of golden hamsters at seven weeks after infection with 1500 cercariae. Cercariae of *T.ocellata* (Hokke *et al., 1998*) and *S. mansoni* were obtained as described previously (Van Dam *et al.*, 1996).

Monoclonal antibodies

Production of MAbs used in this study has previously been described (Van Dam *et al.*, 1993; Deelder *et al.*, 1996; Nibbeling *et al.*, 1998). From a few thousand hybridomas produced over the years in the department of Parasitology (LUMC), a panel of 188 MAbs was selected for putative probable reactivity with carbohydrate epitopes on basis of their reactivity in IFA on male adult worms fixed with Rossman's fixative and on worms or eggs present in frozen sections of infected hamster livers and/or the recognition of repetitive epitopes in immunoelectrophoresis. Isotypes of the MAbs were generally determined in IFA or Dot Immuno Binding Assay using anti-mouse isotype-specific fluorescein isothiocyanate or peroxidase conjugates (Van Dam *et al.*, 1993).

Enzymatic synthesis of oligosaccharides

An outline of the enzymatic synthesis of LacdiNAc, LDN-F, LDN-DF and Le^x is given in Figure 1 linked to the carboxymethyl octyl group (R). Five micromoles of GlcNAc-R was incubated with 6 μmol of UDP-[^{3}H]GalNAc (0.09 Ci/mol) and partially purified UDP-GalNAc:GlcNAcβ β1→4-*N*-acetylgalactosaminyltransferase (β4-GalNAcT) from the albumen gland of *Lymnea stagnalis* (Mulder *et al.*, 1995) in an incubation mixture containing 100 mM sodium cacodylate pH 6.5, 20 mM $MnCl_2$, 100 mM NaCl, 600 mM GlcNAc, 4 mM ATP, and 0.5% (v/v) Triton X-100 for 18 h at 37°C. Three micromoles of the produced LacdiNAc-R was fucosylated with 4 μmol of GDP-Fuc and partially purified GDP-Fuc:[Galβ1→4]GlcNAcβ

α1→3-fucosyltransferase (α3-FucT) (1.4 mU) from human milk (DeVries *et al.*, 1993) in an incubation mixture containing 100 mM sodium cacodylate pH 7.0, 100 mM NaCl, 20 mM $MnCl_2$, 4 mM ATP and 0.5% (v/v) Triton X-100, for 48 h at 37°C (Bergwerff *et al.*, 1993). Two µmol of LDN-F was further fucosylated with partially purified GDP-Fuc:Fucα α1→2-fucosyltransferase (α2-FucT) from *T.ocellata* (Hokke *et al., 1998*) under the same conditions as described previously, for 72 h at 24°C. During the incubations the progress of the reactions was monitored by HPAEC with pulsed amperometric detection on a CarboPac PA-1 pellicular anion-exchange column (0.9 × 2.5 cm, Dionex) as described previously (Joziasse *et al.*, 1993). Under very similar conditions GlcNAc-R was incubated with UDP-[^{3}H]Gal (0.09 Ci/mol), UDP-Gal:GlcNAcβ β1→4-galactosyltransferase (β4-GalT) from bovine milk (Sigma), GDP-Fuc and α3-FucT to synthesize Le^x-R (Bergwerff *et al.*, 1993).

The products of all incubations were isolated on Sep-Pac C-18 cartridges (1 g) and eluted with methanol (Palcic *et al.*, 1988). The eluate was dissolved in 4 ml 50 mM ammonium acetate pH 5.2 and applied to a column (1.6 × 200 cm) of Bio-Gel P-4 (200–400 mesh) equilibrated and eluted at a flow of 16 ml/h with 50 mM ammonium acetate, pH 5.2 at 25°C. Fractions of 2 ml were collected and monitored for ^{3}H-radioactivity. Fractions containing the products were pooled and lyophilized. The structure of each product was verified by ^{1}H-NMR spectroscopy as described previously (Hokke *et al., 1998*).

Construction of neoglycoproteins

During the enzymatic synthesis, hydrolysis of the carboxymethyl group occurred. To assure total conversion of this group to a carboxylic acid function, the synthesized oligosaccharides were treated with 0.1 M NaOH for 10 min at 80°C. Each of the oligosaccharides LacdiNAc, LDN-F, Le^x, and LDN-DF was coupled to BSA via the carboxyl group of the spacer according to a slightly modified procedure of Andersson *et al.* (1993). Briefly, to a solution of 75 µl dioxane:water (2:1) which contained 1 µmol of oligosaccharide and 1 µmol of triethylamine, 2.4 µmol of *N, N, N′,N″*-tetramethyl-(succinimido)uronium tetrafluoroborate (TSTU) was added. The reaction mixture was rotated head over head for 30 min at room temperature. It was subsequently added to 450 µl of a solution of BSA (2 mg/ml) in 0.1 M sodium borate pH 8.5 and incubated head over head for 4 h at room temperature. By addition of hydroxylamine to a concentration of 25 mM, unreacted succinimido esters were inactivated. Each mixture was dissolved in 4 ml 50 mM ammonium acetate pH 5.2 and applied to a Bio-Gel P-4 column (1.6 × 200 cm, 200–400 mesh) as described above. Two milliliter fractions were collected and monitored for radioactivity. The void volume peak containing the neoglycoprotein, was collected and lyophilized. The degree of coupling of the oligosaccharides to BSA was estimated by matrix assisted laser desorption ionization/time of flight (MALDI-TOF) mass spectrometry (LDI-1700-XP instrument) using the center of the distribution of the singly-charged molecular ion. Unreacted oligosaccharides were collected and lyophilized for re-use. The obtained neoglycoproteins (LacdiNAc-BSA, LDN-F-BSA, LDN-DF-BSA and Le^x-BSA) were used to characterize the MAbs in ELISA and SPR analysis.

Enzyme-linked immunosorbent assay

Flat-bottom 96-well polystyrene microtitration plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 50 µl of LacdiNAc-BSA, LDN-DF-BSA, or Le^x-BSA (1 µg/ml in 0.035 M phosphate-buffered saline (PBS), pH 7.8) or LDN-F-BSA (5 µg/ml), for 15 min at 37 °C while shaking. After each incubation step, the plates were washed with 20-fold diluted PBS. Non-specific binding sites were blocked with 100 µl of 0.3% BSA in PBS. Subsequently the wells were incubated with 50 µl of hybridoma cell supernatant containing 0.3% Tween 20 (Sigma, St. Louis, MO). For detection of bound antibodies, peroxidase conjugates of anti-mouse immunoglobulin were used. After color development using 3,3′,5,5′-tetramethylbenzidine as a substrate, the absorbance was measured at 630 nm with an automated microplate reader EL311 (Biotek Instruments, USA).

Surface plasmon resonance spectroscopy

All experiments were performed at 25°C and all injections were carried out automatically by a BIAcore 3000 instrument with a computer interface for system control, data acquisition and data analysis (Biacore AB, Uppsala, Sweden). All buffers were filtered (0.2 µm) and degassed before use. The neoglycoproteins were immobilized at a flow rate of 5 µl/min in 10 mM sodium acetate (pH 4.0) onto a carboxylmethylated dextran CM5 sensor chip by covalent amine coupling according to the instructions of the manufacturer. In short, 35 µl of a mixture containing 100 mM *N*-hydroxysuccinimide and 400 mM 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide was injected to activate the sensor chip. Initially, the neoglycoproteins (such as BSA, LacdiNAc-BSA, Le^x-BSA, LDN-DF-BSA) to be immobilized were injected at a concentration of 20 µg/ml in 10 mM sodium acetate pH 4.0 until an increase of ~4000 response units (RU) was observed. This chip was used to study the binding of MAbs that recognize the LacdiNAc-, the Le^x-, or the LDN-DF-epitope. For the determination of MAbs that recognize the LDN-F epitope, a new chip was prepared. LDN-F was immobilized to a level of 7000 RU, while for Le^x-BSA, LDN-DF-BSA, or BSA an immobilization level of 2500 RU was accomplished to compensate for differences in carbohydrate density on BSA. Unreacted *N*-hydroxysuccinimide esters were subsequently deactivated by 35 µl 1 M ethanolamine. All analyses were performed at a flow rate of 5 µl/min using HEPES-buffered saline (HBS) as an eluent. Ten microliters of the MAb supernatants was injected, and after each sample the sensor chip was regenerated with 10 µl of 100 mM HCl. Analysis of the data was performed using the BIA evaluation 3.0 software. After subtraction of the blank value (BSA), the binding pattern of each MAb was interpreted as “binding” or “no binding.”

IFA

The IFA was carried out on 5 µm-thick frozen liver sections of *S.mansoni* infected hamsters, on sections of adult worms fixed with Rossman’s fixative (Nash *et al.*, 1977; Nash, 1978). and on whole cercariae fixed according to the method described by Habeeb (Habeeb, 1987). Slides were incubated with MAb culture supernatant in a humid atmosphere at 37°C for 60 min, then washed and incubated for 45 min with a FITC conjugate of rabbit anti-mouse immunoglobulin antibody (Nordic,

Immunological Laboratories, Tilburg, the Netherlands), diluted 1/50 in PBS containing 0.1 mg/ml Evans blue in a humid atmosphere at 37°C. The slides were observed with a Leica DM-RB fluorescence microscope with the appropriate filter combination for FITC fluorescence. The fluorescence was interpreted visually as positive or negative (Table II). As a negative control fresh culture medium was used.

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Abbreviations

BSA, bovine serum albumin; CCA, circulating cathodic antigen; ELISA, enzyme linked immunosorbent assay; IFA, immunofluorescence analysis; α3-FucT, GDP-Fuc:[Galβ1→4]GlcNAcβ α1→3-fucosyltransferase; α2-FucT, GDP-Fuc:Fucα α1→2-fucosyltransferase; β4-GalT, UDP-Gal:GlcNAcβ β1→4-galactosyltransferase; β4-GalNAcT, UDP-GalNAc:GlcNAcβ β1→4-*N*-acetylgalactosaminyltransferase; LacdiNAc, LDN, GalNAcβ1–4GlcNAc; LDN-F, GalNAcβ1–4(Fucα1–3)GlcNAc; LDN-DF, GalNAcβ1–4(Fucα1–2Fucα1–3)GlcNAc; Le^x, $Lewis^x$; MAb, monoclonal antibody; MALDI-TOF, matrix assisted laser desorption ionization/time of flight; PBS, phosphate-buffered saline; SPR, surface plasmon resonance.

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