

***Schistosoma mansoni*-infected mice produce antibodies that cross-react with plant, insect, and mammalian glycoproteins and recognize the truncated biantennary *N*-glycan Man₃GlcNAc₂-R**

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To reveal the role of cross-reactive carbohydrate determinants in the host immune response in helminth infections and allergenicity, we developed monoclonal antibodies (mAbs) that recognize glycan epitopes present on glycoconjugates from both helminths and plants. An IgM mAb (100-4G11-A) was selected from a panel of anti-glycan mAbs generated from *Schistosoma*-infected or immunized mice because it recognized both a plant glycoprotein horseradish peroxidase and phospholipase A2 from honeybee venom. On further characterization, it was shown that mAb 100-4G11-A recognizes the truncated biantennary *N*-glycan Man₃GlcNAc₂-R. Immunocytochemical analysis and immunoblotting with this mAb demonstrated that Man₃GlcNAc₂-R structures occur on many glycoproteins of schistosomes and other invertebrates. Remarkably, Man₃GlcNAc₂-R is also expressed on a restricted number of vertebrate glycoproteins. Our data indicate that this truncated *N*-glycan is immunogenic in mice during the course of infection. Nevertheless, no elevated antibody levels against this glycan epitope could be detected in sera of individuals infected with *Schistosoma mansoni*.

Key words: anti-carbohydrate monoclonal antibodies/ antigenicity/*N*-glycans/schistosomes

Introduction

Parasites affect millions of people worldwide and cause tremendous suffering and death, especially in developing countries. Glycoconjugates play a crucial role in the pathology of most parasitic helminth infections and are a major focus of the host immune response. Little is known, however, of the molecular basis of these processes, although many helminth carbohydrate structures have been

characterized in recent years (see reviews in Cummings and Nyame, 1996, 1999; Dell *et al.*, 1999; Hokke and Deelder, 2001). To understand the role of helminth carbohydrate structures in the induction of host immune responses and to evaluate their potential application in diagnostics or vaccine development, it is essential to determine the subcellular localization and stage-specific expression of these structures and establish whether these structures are unique for specific helminths.

Recently, several monoclonal antibodies (mAbs) have been identified that bind to carbohydrate structures of schistosomes. These mAbs recognize carbohydrate structures that may be specifically related to schistosomes and/or other helminths (e.g., GalNAc β 1-4[Fuc α 1-2Fuc α 1-3]GlcNAc [LDN-DF] and Fuc α 1-3GalNAcGlcNAc [F-LDN]) (Kantelhardt *et al.*, 2002; Khoo *et al.*, 1995, 1997a; Van Remoortere *et al.*, 2000)) as well as glycan structures that are found on glycoconjugates of both mammals and helminths (e.g., Gal β 1-4[Fuc α 1-3]GlcNAc (Lewis^x, Le^x), GalNAc β 1-4GlcNAc [LDN], and GalNAc β 1-4[Fuc α 1-3]GlcNAc [LDNF]) (Dell *et al.*, 1999; Huang *et al.*, 2001; Nyame *et al.*, 1997, 1998, 2000; Van Dam *et al.*, 1996; Van den Eijnden *et al.*, 1998; Van Remoortere *et al.*, 2000; Wuhler *et al.*, 1999). Most of these mAbs were isolated from *Schistosoma*-infected mice, indicating that the glycan antigens are immunogenic during the course of infection. In addition, humans and primates infected with schistosomes show elevated levels of IgM and/or IgG antibodies against these carbohydrate structures (Van Remoortere *et al.*, 2001; Nyame *et al.*, 1998), establishing the importance of these glycan epitopes in induction of an anti-glycan humoral immune response.

In schistosomes and other helminths, some *N*-glycans contain an α 1 $\rightarrow$ 3-fucose (α 3-Fuc) linked to the proximal core GlcNAc and/or a β 1 $\rightarrow$ 2-xylose (β 2-Xyl) linked to the core β -mannose (Khoo *et al.*, 1997b, 2001; Van Die *et al.*, 1999). These *N*-glycans not only are found on helminth glycoproteins but are typical for glycoproteins derived from plants, insects, and mollusc, but are not found in mammalian glycoproteins (D'Andrea *et al.*, 1988; Kubelka *et al.*, 1993; Lerouge *et al.*, 1998; Van Die *et al.*, 1999; Van Kuik *et al.*, 1985, 1986). Cross-reactive carbohydrate antigens observed in immunoassays of helminth, plant, arthropod, and mollusc extracts have been thought to be due to the presence of these unusual *N*-glycan core structures. Both core α 3-Fuc and/or β 2-Xyl residues are epitopes for the binding of IgE antibodies to plant allergens, horseradish peroxidase (HRP), honeybee venom phospholipase A2 (PLA2), and helminth glycoproteins (Tretter *et al.*, 1993; Van Die *et al.*, 1999; Van Ree *et al.*, 2000).

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This is of particular interest as a Th2 type immune response, and high serum IgE levels are typical for helminth-infected hosts as well as for individuals suffering from atopic diseases (Bell, 1996). To unravel the role of these cross-reactive glycoconjugates in the host immune response in helminth infections and allergenicity, we set out to identify and characterize mAbs that bind to carbohydrate epitopes shared by helminths and plants. Here we report the isolation and characterization of mAb 100-4G11-A that recognizes a truncated high-mannose-type *N*-glycan, Man₃GlcNAc₂-R, which is expressed on many glycoproteins of invertebrate organisms, as well as on a limited amount of mammalian glycoproteins.

Results

Isolation of an mAb that binds HRP and PLA2

To isolate anti-glycan mAbs that show cross-reactivity between helminths and allergenic plant glycoproteins, a panel of 188 anti-carbohydrate mAbs derived from fusions with spleen cells of mice infected with cercariae of *Schistosoma* spp. or immunized with hatching fluid from *Schistosoma* eggs (Van Remoortere *et al.*, 2000), was screened for reactivity with HRP. Six mAbs recognized HRP, and not human transferrin (Tr) or BSA (Table I). To investigate whether the mAbs showed cross-reactivity with other glycoproteins, they were tested in a subsequent screening for binding to several glycoproteins with defined glycan structures. A structural overview of the major *N*-linked glycans of the glycoproteins tested is depicted in Figure 1. Of the six HRP-positive mAbs, five also recognized PLA2, which resembles HRP because it contains truncated *N*-glycans modified with a core α3-Fuc epitope, but in contrast to HRP, PLA2 carries no β2-Xyl epitope (Table I). None of the six mAbs showed binding to a neoglycoprotein carrying a biantennary *N*-glycan modified with a β2-xylose (data not shown). These data demonstrate the occurrence of mAbs derived from *Schistosoma*-infected or immunized mice that cross-react with both HRP and PLA2, indicating

that these mAbs recognize a shared carbohydrate epitope on these glycoproteins.

mAb 100-4G11-A binds Man₃GlcNAc₂-R

Because mAb 100-4G11-A (hereafter designated as 100-4G11-A) showed the strongest binding to both HRP and PLA2, this mAb was selected for further characterization. To identify which glycoform of PLA2 was recognized by 100-4G11-A, PLA2 glycoforms were separated by affinity chromatography with Conacavalin A (ConA), a lectin that specifically recognizes biantennary complex-type, hybrid-type, and high-mannose-type *N*-glycans, but does not recognize either tri-/tetraantennary complex-type *N*-glycans or O-glycans (Figure 2A). Four pools were made from the individual fractions as indicated in Figure 2A and analyzed by western blotting for binding with 100-4G11-A. 100-4G11-A recognized only glycoproteins in fraction 4, which consists of PLA2 proteins that bound to the ConA column (Figure 2B). Because a core α3-Fuc epitope is present on both PLA2 and HRP, we tested whether this core fucose is required for recognition by 100-4G11-A by analyzing the fractions for reactivity with affinity purified polyclonal anti-serum specific for core α3-Fuc residues (Faye *et al.*, 1993). The anti-core α3-Fuc antibodies recognized fractions corresponding with both ConA-binding and nonbinding fractions. These data demonstrate that 100-4G11-A is not specific for the core α3-Fuc epitope but only recognizes PLA2 glycoform(s) that bind to ConA.

Table I. mAbs isolated from *S. mansoni*-infected or immunized mice that react with HRP

mAb	Infection	HRP	PLA2	Human transferrin
36-1F10-A	Infection <i>S. mansoni</i>	0.80	0.43	0.0
39-1B11-A	Infection <i>S. mansoni</i>	1.02	0.34	0.0
39-2A12-A	Infection <i>S. mansoni</i>	0.86	0.19	0.0
54-5F5-A	Infection <i>S. mansoni</i>	0.59	0.00	0.0
99-2B9-A	Infection <i>S. mansoni</i>	0.63	0.13	0.0
100-4G11-A	Infection <i>S. mansoni</i>	2.11	1.03	0.0

Binding of the mAbs to HRP, honeybee PLA2, and human transferrin was determined by ELISA and indicated as mean OD values measured at 405 nm after correction for the blank. mAbs were isolated from mice experimentally infected with *S. mansoni* cercariae.

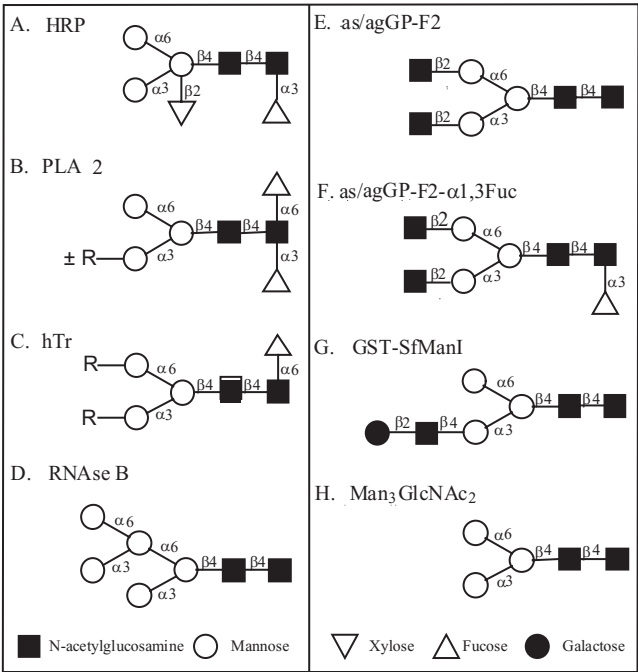


Fig. 1. Overview of carbohydrate structures used in this study. (A–D) Structures of the major *N*-linked glycans from the glycoproteins HRP, PLA2, human transferrin, and RNase B (Man₅GlcNAc₂); (E, F) carbohydrate structures coupled to BSA: as/agGP-F2 (E) and core-α1,3-fucosylated as/agGP-F2 (F); (G) recombinant protein GST-SfManI, produced in insect cells; and (H) Man₃GlcNAc₂, the proposed minimal epitope of 100-4G11-A.

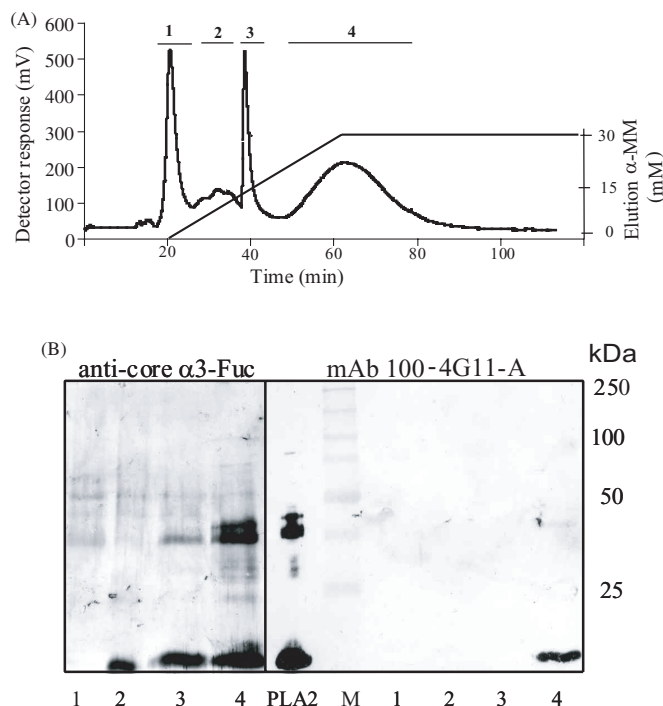


Fig. 2. Analysis of PLA2 glycoforms by ConA affinity chromatography. (A) Honeybee PLA2 glycoforms were separated by FPLC affinity chromatography with the lectin ConA. Elution of the bound glycoforms of PLA2 was performed with an increasing gradient from 0 to 33 mM methyl α -D-mannopyranoside in PBS in 40 min as indicated. (B) Western blot of pooled fractions (as indicated in A) with 100-4G11-A. Approximately 2.5 μ g of each of the four fractions and PLA2 were analyzed by western blot using affinity-purified anti-serum specific for core α 3-Fuc and mAb 100-4G11-A. As secondary antibody, AP-conjugated goat anti-rabbit or mouse antibody, was used, respectively.

In addition, several monosaccharides were tested for their ability to inhibit the binding between 100-4G11-A and PLA2 in enzyme-linked immunosorbent assay (ELISA) (Figure 3). Methyl α -D-mannopyranoside completely blocked the binding of the mAb at a concentration of 50 mM, whereas D-mannose was also effective as an inhibitor. In contrast, D-GlcNAc, L-fucose, or D-xylose did not interfere with the binding even at high concentrations. These results suggest that mannose is an essential constituent of the epitope that is recognized by 100-4G11-A.

To characterize the carbohydrate specificity of 100-4G11-A in more detail, the recognition profile of this mAb was analyzed by ELISA using several (neo)glycoproteins that contain glycan epitopes resembling those found on PLA2 (for structural details, see Figure 1). 100-4G11-A did not bind to RNase B, containing high mannose-type *N*-glycans with the composition Man₅₋₉-GlcNAc₂. No significant binding was observed to glutathione-S-transferase (GST)-SfManI, a soluble GST fusion protein produced in insect cells that carries a monoantennary *N*-glycan containing a single terminal Gal β 1,4GlcNAc (LacNAc) unit on the α 1,3-linked mannose of the *N*-glycan core structure. These data indicate that neither high mannose of the size composition Man₅₋₉-GlcNAc₂- nor

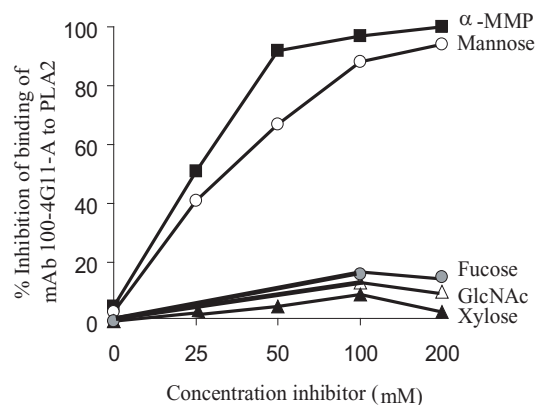


Fig. 3. Inhibition of binding of 100-4G11-A to PLA2 by monosaccharides. The ability of several monosaccharides to inhibit the binding between 100-4G11-A and PLA2 was determined by ELISA. Plates were coated with PLA2. mAb 100-4G11-A was preincubated with monosaccharides in concentrations as indicated; binding of 100-4G11-A was measured using AP-labeled goat-anti mouse IgM.

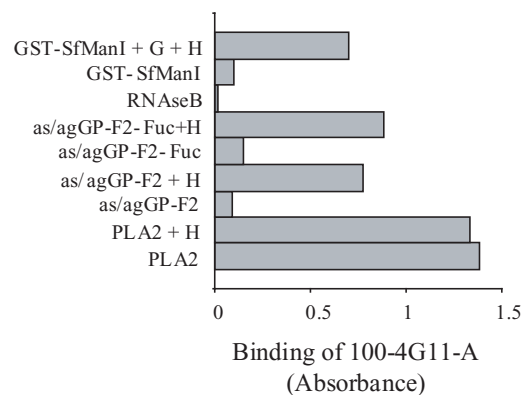


Fig. 4. mAb 100-4G11-A binds to Man₃GlcNAc₂-R. The binding of 100-4G11-A to different glycoproteins and neo-glycoproteins that contain glycan structures that occur on PLA2 was determined by ELISA. Some antigens were pretreated with glycosidases, as indicated by H (*N*-acetylglucosaminidase) or G (β 4-galactosidase). mAb 100-4G11-A only binds to antigens that were pretreated with the glycosidases, resulting in the structure Man₃GlcNAc₂-R.

hybrid-type *N*-glycans are recognized by 100-4G11-A. Similarly, 100-4G11-A did not recognize either neo-glycoproteins carrying the biantennary glycopeptides as/agGP-F2 or as/agGP-F2 modified with a core- α 1,3Fuc or neo-glycoproteins containing the oligosaccharides LDN or LDNF (not shown).

The results suggested the possibility that 100-4G11-A might recognize a truncated high-mannose-type *N*-glycan smaller than Man₅₋₉-GlcNAc₂. To address this, we treated both as/agGP-F2-BSA and as/agGP-F2- α 1,3Fuc-BSA with *N*-acetylglucosaminidase to generate the core *N*-glycan Man₃-GlcNAc₂-R. Likewise, GST-SfManI was treated with both β -galactosidase and *N*-acetylglucosaminidase to generate Man₃-GlcNAc₂-R. The resulting Man₃-GlcNAc₂-R structures on as/agGP-F2-BSA, as/agGP-F2- α 1,3Fuc-BSA, and GST-SfManI were strongly bound by

100-4G11-A (Figure 4). These results demonstrate that 100-4G11-A recognizes the truncated high-mannose-type *N*-glycan $\text{Man}_3\text{-GlcNAc}_2\text{-R}$. Our data strongly suggest that the antibody requires both a free terminal $\alpha 1,3$ -linked and $\alpha 1,6$ -linked mannose in the trimannosyl core structure $\text{Man}\alpha 1-3(\text{Man}\alpha 1-6)\text{Man}\beta$ - to recognize its ligand. 100-4G11-A does not recognize either $\text{Man}_5\text{GlcNAc}_2\text{-R}$, the major *N*-glycan on RNase B that contains a free $\alpha 1,3$ -mannose, or the monoantennary *N*-glycan on GST-SfManI that has a free $\alpha 1,6$ -mannose. The presence of a core $\alpha 1,3$ -Fuc linked to the inner GlcNAc does not affect the binding (Figure 4).

Antibody 100-4G11-A binds to many glycoproteins of schistosomes and other helminths

To determine the occurrence and localization of $\text{Man}_3\text{-GlcNAc}_2\text{-R}$ epitopes in *Schistosoma* and other helminth species, the reactivity of 100-4G11-A to protein-linked glycans of different life-cycle stages of *S. mansoni* and

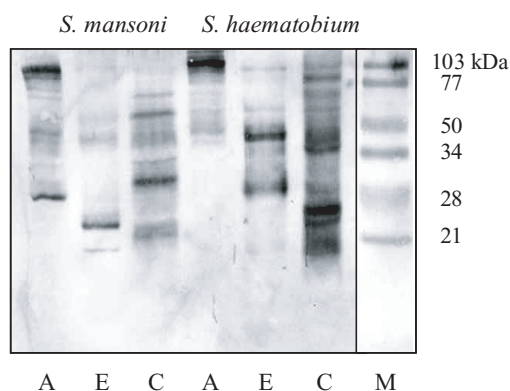


Fig. 5. Western blot analysis of glycoproteins of various life-cycle stages of *Schistosoma*. Proteins within extracts of *Schistosoma* were separated by SDS-PAGE, and the presence of glycoproteins carrying $\text{Man}_3\text{GlcNAc}_2\text{-R}$ was identified by immunoblotting with 100-4G11-A. Extracts were derived from adults (A), eggs (E), and cercariae (C) from *S. mansoni* (lanes 1–3) and from *S. haematobium* (lanes 4–6).

S. haematobium was determined by western blotting. All life-cycle stages of both *Schistosoma* species contain glycoproteins that react strongly with this mAb (Figure 5), whereas no binding was observed to (glyco)lipids extracted from *S. mansoni* eggs and adult worms (data not shown). Immunofluorescence analysis (IFA) of different schistosome life-cycle stages showed that 100-4G11-A predominantly recognizes the tegument and cells in the parenchyma of the adult worm, spots in the eggs, and the oral sucker of cercariae (Figure 6). Several extracts derived from other helminths were analyzed for the presence of glycoproteins binding to 100-4G11-A. Most of the extracts contained several glycoproteins that were recognized by this mAb (Figure 7A). Interestingly, some proteins of the excretory/secretory antigen of *Haemonchus contortus* showed strong binding, whereas no reactivity was observed in the adult stage (Figure 7A, lane 7–8). Western blot of adult *Caenorhabditis elegans* extracts showed little if any staining with 100-4G11-A, whereas staining was observed toward extracts from the eggs and larval stages (Figure 7A, lanes 1–5). Our data demonstrate that the truncated *N*-glycan recognized by 100-4G11-A is present in all helminths investigated and that its expression in some of these helminths is stage-specific.

Antibody 100-4G11-A recognizes many invertebrate glycoproteins and a restricted number of glycoproteins in mammals

Several extracts of different tissues or cell lines from vertebrates and invertebrates were analyzed for the presence of glycoproteins recognized by 100-4G11-A. The mAb showed a strong reactivity with a range of glycoproteins of the albumen gland and prostate gland of the snail *Lymnaea stagnalis* (i.e., Figure 7B, lane 2), the intermediate host for the avian schistosome *Trichobilharzia ocellata*. Remarkably, several glycoproteins in tissues of vertebrate species showed specific binding with the mAb, such as glycoproteins in human liver and the rat macrophage cell line NR 8383 (Figure 7C). Because

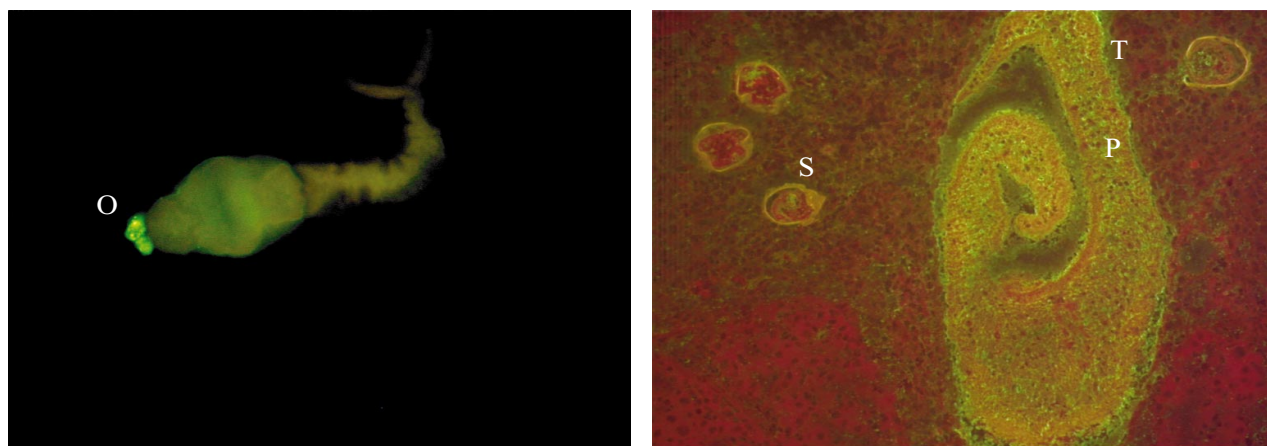


Fig. 6. Immunofluorescence patterns of schistosomes with 100-4G11-A. Cercaria (left) and a frozen section of a liver of *S. mansoni*-infected hamster (right) were incubated with 100-4G11-A, followed by staining with a fluorescein isothiocyanate conjugate of a rabbit anti-mouse Ig antibody. Fluorescence (illustrated as white spots in the photographs) was observed on the tegument (T), in structures in the parenchyma (P) of the adult worm, in structures in the egg (S), and on the oral sucker of the cercaria (O).

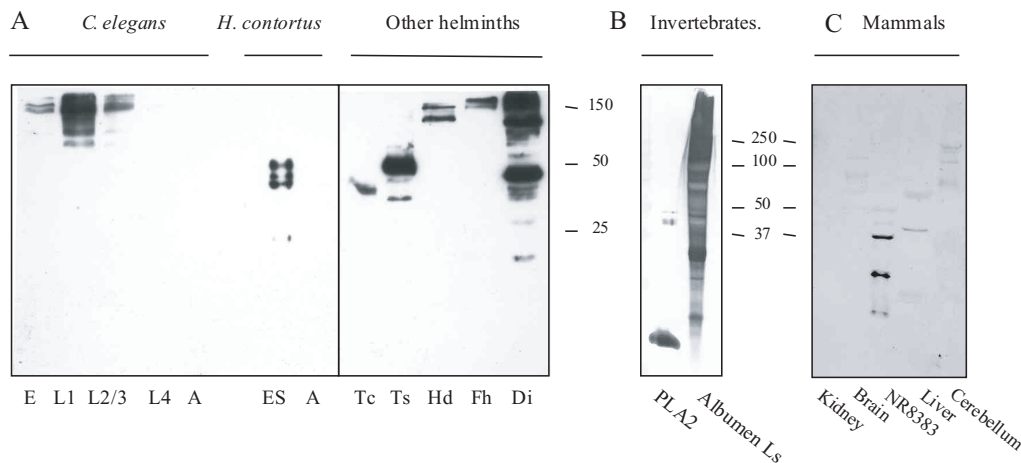


Fig. 7. Binding of mAb 100-4G11-A to glycoproteins within extracts of different tissues or cell lines of invertebrates and mammals. Reactivity of 100-4G11-A with extracts of different helminths (A) and extracts of different tissues or cell lines of invertebrates (B) and mammals (C). Proteins within the extracts were separated by SDS-PAGE and transferred to nitrocellulose by western blotting. The blots were incubated with 100-4G11-A, followed by incubation with goat anti-mouse IgM AP or PO conjugates. (A) Samples tested were various stages of the free living nematode *C. elegans* (egg [E], larval stages L1, L2/3, L4/young adult [L4], and adult worm [A]), the parasitic nematode *Haemonchus contortus* (excretory secretory antigens [ES] and adult worms [A]), and extracts derived from the adult stage from the parasitic nematodes *Toxocara canis* (Tc), *Trichinella spiralis* (Ts), *Dirofilaria immitis* (Di), the trematode *Fasciola hepatica* (Fh), and the cestode *Hymenolepis diminuta* (Hd). (B) The insect glycoprotein PLA2 and glycoproteins of the albumen gland of *Lymnaea stagnalis*. (C) Extracts of veal kidney, veal brain, rat macrophage cell line (NR 8383), human liver, and human cerebellum.

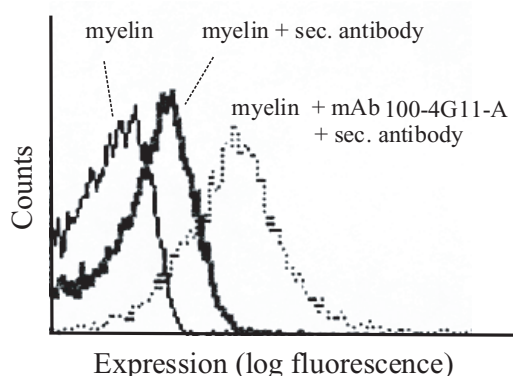


Fig. 8. Binding of 100-4G11-A to myelin. FACS analysis of mouse myelin (continuous line), myelin with R α M-PE (bold continuous line) or with 100-4G11-A followed by incubation with R α M-PE (broken line). Similar results were obtained with rat myelin (not shown).

100-4G11-A recognizes glycoproteins in brain tissues, we were interested in whether myelin contains Man₃GlcNAc₂ N-glycans. Analysis of myelin by FACS analysis demonstrated that this mAb indeed bound to mouse and rat myelin (Figure 8). In contrast, the mAb does not recognize glycoproteins in the white matter of the brain, in astrocytes, or in the cerebral endothelial cell line HBZCZ, nor glycoproteins in veal kidney and heart, or in human placenta (data not shown). In conclusion, the truncated N-glycan recognized by 100-4G11-A is abundantly expressed on glycoproteins of the snail *L. stagnalis* and is found on insect glycoproteins (i.e., honeybee PLA2). Remarkably, this carbohydrate epitope is also found on a restricted number of glycoproteins in some vertebrate tissues.

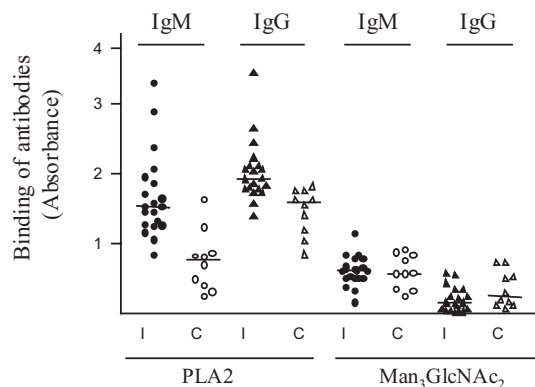


Fig. 9. Levels of IgG and IgM recognizing the neoglycoprotein Man₃GlcNAc₂-BSA and PLA2 in sera of individuals, infected (I) with *S. mansoni*, and uninfected (C). The binding of antibodies to PLA2 and Man₃GlcNAc₂-BSA was measured by ELISA. Glycoproteins were coated at a concentration of 5 μ g/ml.

No significant antibody levels are found against Man₃GlcNAc₂-R in individuals infected with schistosomes

Because Man₃GlcNAc₂-R is a glycan that is expressed on all stages of schistosomes, allowing an early and continuous contact with the host immune system, we investigated whether sera of individuals infected with *S. mansoni* contain antibodies against this epitope by ELISA. Sera of individuals infected with *S. mansoni* contained both significant levels of IgM and IgG antibodies against honeybee PLA2 as compared to the antibody levels in sera of uninfected individuals. However, these antibodies did not recognize the truncated N-glycan Man₃GlcNAc₂-R because no increase in antibody levels (IgG or IgM) could be detected in

N-acetylglucosaminidase treated as/agGP-F2 (Figure 9). In addition, no IgE antibodies could be detected that reacted with Man₃GlcNAc₂-R, whereas an IgE response was observed against PLA2 in several patients (data not shown).

Discussion

In this study we searched for mAbs recognizing glycan epitopes that are shared by antigens of schistosomes and allergenic plants, such as the immunogenic core α 1,3-Fuc or β 1,2-Xyl epitopes that are epitopes for binding of cross-reactive IgE antibodies to plant allergens and helminth glycoproteins (Tretter *et al.*, 1993; Van Die *et al.*, 1999; Van Ree *et al.*, 2000). To select mAbs specific for such cross-reactive glycan epitopes, a panel of anti-carbohydrate mAbs isolated from *Schistosoma*-infected or immunized mice was screened with HRP. Out of six mAbs that recognized HRP, five also showed reactivity with honeybee PLA2, implying that the epitope is a glycan structure present on both glycoproteins. Our results showed that 100-4G11-A recognizes truncated *N*-glycans that contain a trimannosyl unit Man₃GlcNAc₂-R with both a free α 1,3- and α 1,6-linked mannose. The presence of a core α 1,3-Fuc residue on the inner core GlcNAc does not affect the binding.

Relatively large amounts of the truncated *N*-glycan recognized by 100-4G11-A have been found previously on type II variant surface glycoproteins of the parasite protozoan *Trypanosoma brucei* (Zamze *et al.*, 1991), and on immunodominant antigens of the parasite helminth *Trichinella spiralis* (Reason *et al.*, 1994). Here we show that other helminths from different orders of the phyla platyhelminths and nematodes also synthesize glycoproteins containing this truncated *N*-glycan. In some helminths, such as *H. contortus* and the free-living nematode *C. elegans*, expression of this glycan epitope is developmentally regulated. In invertebrates, the truncated *N*-glycan structure is apparently occurring abundantly. Recently, the presence of these structures has been demonstrated on recombinant neuropsin, synthesized in insect cells (Takahashi *et al.*, 1999). 100-4G11-A may therefore be a useful tool in identifying the glycosylation on recombinant glycoproteins produced in insect cells. As far as we know, however, this is the first report that shows the presence of these truncated structures in vertebrates, and this observation poses questions about the role or significance of such *N*-glycans that may be formed by degradation of complex-type *N*-glycans.

Despite the isolation of mAbs from infected mice against the Man₃GlcNAc₂ glycan structure, we could not detect significantly higher levels of antibodies against this epitope in sera of individuals infected with *S. mansoni* in comparison with sera of controls. The Man₃GlcNAc₂-R epitope is found on human endogenous glycoproteins, which may explain why only low antibody levels against this glycan epitope are found in infected individuals. Similarly, antibody levels against the endogenous monomeric Le^x carbohydrate epitope are low in individuals infected with schistosomes, whereas mAbs recognizing this structure have been isolated, as reported previously (Nyame *et al.*, 1998; Van Remoortere *et al.*, 2001). Despite the low

antibody levels against Le^x, however, LNFPIII, which contains Le^x, is a potent inducer of a Th2 response and acts as an adjuvant (Okano *et al.*, 2001), indicating that glycan structures may have indirect effects on the host humoral immune system.

Unexpectedly, none of the 188 mAbs tested recognized neoglycoproteins carrying *N*-glycans with a core β 1,2-Xyl or a core α 1,3-Fuc. Because these core modifications have been demonstrated on both adult worms and the eggs of schistosome glycoproteins (Khoo *et al.*, 1997b, 2001; Van Die *et al.*, 1999) and do not occur on mammalian glycoproteins, they are expected to be immunogenic in mice. The reason for our failure to detect hydridomas secreting such mAbs is not known.

Our data indicate that the generation of antibodies against glycan antigens differs in mouse and human infections. The murine model has been widely used to dissect the immune responses in *S. mansoni* infection. Recently it has become clear that immunological data obtained from schistosome-infected mice and humans may differ profoundly, one of the differences being that a Th2 response in infected mice is associated with pathology, whereas in infected humans the same type of response is thought to be beneficial for the host (Boros, 1994; Cheever *et al.*, 1997; Fallon, 2000). Because glycoconjugates play an important role in the pathogenesis and host immune responses in schistosomiasis, such differences may be partially due to differences in endogenous glycosylation between mice and humans, contributing to differences in the discrimination between self and non-self antigens upon infection.

Characterization of the structure of parasite glycans by conventional analytical methods such as mass spectrometry and nuclear magnetic resonance (NMR), and analysis of their functions is hampered by the small amounts of parasite material that are usually available. Anti-glycan mAbs with a well-defined specificity, such as 100-4G11-A, will be very valuable research tools to provide us with more insight in the expression, distribution, and function of glycan epitopes in the different life-stages of schistosomes and other parasites and, importantly, also in defining interactions between parasite glycans and the host immune system.

Materials and methods

Materials and parasites

HRP, honeybee venom PLA2, human transferrin, methyl α -D-mannopyranoside, D-mannose, L-fucose, D-xylose, *N*-acetylglucosamine (GlcNAc), and jackbean β -galactosidase were purchased from Sigma (St. Louis, MO). All parasitic helminths were obtained as described previously (Van Die *et al.*, 1999). Affinity-purified polyclonal antiserum specific for core α 1-3-fucose was provided by Dr. Veronique Gomord (Faye *et al.*, 1993). GST-SfManI is a soluble, GST-tagged version of an Sf9 class I mannosidase (Kawar *et al.*, 2000) produced in the insect cell line Sf + f4GalT (Hollister *et al.*, 1998) and contains a mono-antennary (α 1,3-mannose elongated) *N*-glycan, terminating in galactose (provided by Dr. Jason Hollister, University of Wyoming).

mAbs

Production of mAbs used in this study has been described previously (Van Dam *et al.*, 1993). From a few thousand hybridomas produced over the years in the Department of Parasitology (LUMC), a panel of 188 mAbs was selected for screening based on putative reactivity with carbohydrate epitopes (Van Remoortere *et al.*, 2000).

Construction of neoglycoproteins

Asialo/agalacto glycopeptides from human fibrinogen (as/agGP-F2) were prepared from asialo-fibrinogen by extensive pronase digestion as described previously (Nemansky and Van den Eijnden, 1993) followed by enzymatic degalactosylation with jackbean β -galactosidase. Core α 3-fucosylated as/agGP-F2 (as/agGP-F2- α 1,3Fuc) was synthesized as described in Van Tetering *et al.* (1999). All products were structurally characterized by ¹H-NMR spectrometry as described (Van Tetering *et al.*, 1999). The glycopeptides as/agGP-F2 and as/agGP-F2- α 1,3Fuc (75 nmol each) were activated with ninhydrin and coupled to bovine serum albumin (BSA) by reductive amination essentially as described in Mencke *et al.* (1987). Subsequently, the resulting neoglycoproteins were separated from unincorporated BSA by ConA lectin affinity chromatography. Matrix-assisted laser desorption and ionization time-of-flight analysis showed the presence of about two *N*-glycans per molecule BSA. The oligosaccharides LDN and LDN-F were enzymatically synthesized and coupled to BSA as described previously (Van Remoortere *et al.*, 2000).

ELISA

Flat-bottom 96-well polystyrene microtitration plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with 50 μ l antigen (5 μ g/ml in 0.035 M phosphate buffered saline [PBS], pH 7.8) for 1 h at 37°C. After each incubation step, the plates were washed with 20-fold diluted PBS. Non-specific binding sites were blocked with 100 μ l 0.3% BSA in PBS. Sera and secondary antibodies were diluted in PBS containing 0.1% BSA and 0.3% Tween 20. For detection of bound antibodies, alkaline phosphatase (AP)- or peroxidase (PO) conjugates of anti-mouse or anti-rabbit immunoglobulin were used. As substrate for AP, 3 mM 4-nitrophenyl phosphate (Boehringer Mannheim, Mannheim, Germany) in 0.1 M diethanolamine containing 0.5 mM MgCl₂, pH 9.6, were used; the absorbance was read after 30 min and overnight at a wavelength of 405 nm. 3,3',5,5'-Tetramethylbenzidine and H₂O₂ in 0.1 M acetate, pH 5.5, was used as a substrate for PO, the reaction was terminated with H₂SO₄, and the absorbance was measured at 450 nm.

In the inhibition study 100-4G11-A was preincubated with α -D-mannopyranoside, D-mannose, L-fucose, D-xylose, and D-GlcNAc in a final concentration range of 2.5 to 500 mM for 1 h at 37°C before use.

When indicated, neoglycoproteins were treated after coating with *N*-acetylglucosaminidase (12 mU/ml) in 50 mM sodium citrate, pH 5.0, at 37°C overnight.

To identify the presence of human antibodies binding to PLA2 and *N*-acetylglucosaminidase-treated as/agGP-F2-

BSA, human sera were diluted 1/200 for IgM and IgG and 1/10 for IgE and biotin-conjugated goat anti-human IgG (1/10,000 dilution; Vector, Burlingame, CA), goat anti-human IgE (1/1000; Vector), or goat anti-human IgM (1/5000, Vector) were used. Bound antibodies were detected after incubation with streptavidin-AP (1/4000) as described.

Fractionation of different glycoforms of honeybee PLA2

Honeybee PLA2 (500 μ g) was subjected to FPLC ConA-affinity chromatography using a flow rate of 0.5 ml/min with detection at 280 nm. Coupling of 30 mg Con A (Sigma) to a 1 ml HiTrap-column (Amersham Biosciences AB, Uppsala, Sweden) was performed according to the manufacturer's instructions. The column was equilibrated with PBS. Elution of ConA binding PLA2 glycoforms was performed by gradient elution with 0–33 mM methyl α -D-mannopyranoside in PBS in 40 min, followed by elution with 33 mM methyl α -D-mannopyranoside in PBS for 20 min.

SDS-PAGE and western blotting

Helminth extracts and control samples were separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 12.5–15% gels using the Mini-Protein II system (Bio-Rad, Veenendaal, the Netherlands). Western blotting and antibody reactions were performed essentially as described previously (Agterberg *et al.*, 1993). For detection of 100-4G11-A and polyclonal anti-core α 3-Fuc antibodies, AP or PO conjugates of anti-mouse or anti-rabbit immunoglobulins, respectively, were used. Bound antibodies were detected using x-phosphate/5-bromo-4-chloro-3-inodyl-phosphate (Boehringer Mannheim) and 4-nitrobluetetrazoliumchloride (Boehringer Mannheim) in the case of AP conjugates or using Enhanced ChemiLuminescence reagent (Amersham) and autoradiography in the case of PO conjugates.

IFA

The IFA was performed on frozen liver sections (5 μ m thick) of *S. mansoni*-infected hamsters and whole cercariae as reported previously (Van Dam *et al.*, 1993) using 100-4G11-A and a fluorescein isothiocyanate conjugate of rabbit anti-mouse immunoglobulin antibody (Nordic Immunological Laboratories, Tilburg, the Netherlands). The slides were observed with a Leica DM-RB fluorescence microscope.

Analysis of binding of the mAb 100-4G11-A to myelin

Rat myelin was incubated with 100-4G11-A for 30 min on ice. Subsequently, the myelin was incubated with rabbit anti-mouse-phycoerythrin (R α M-PE) for 30 min on ice. After each step the myelin was washed with PBS containing 0.1% BSA. As control, myelin was incubated with the second antibody. The binding was analyzed by FACSscan (Becton-Dickinson, Franklin Lakes, NJ).

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Abbreviations

AP, alkaline phosphatase; BSA, bovine serum albumin; Concanavalin A, ConA; ELISA, enzyme-linked immunosorbent assay; F-LDN, Fuc α 1-3GalNAcGlcNAc; GST, glutathione-S-transferase; HRP, horseradish peroxidase; IFA, immunofluorescence analysis; LDN, LactidNAc, GalNAc β 1-4GlcNAc; LDN-DF, GalNAc β 1-4(Fuc α 1-2Fuc α 1-3)GlcNAc; LDN-F, GalNAc(α 1-3Fuc)GlcNAc; Le^x, Lewis^x, Gal β 1-4(Fuc α 1-3)GlcNAc/mAb, monoclonal antibody; MALDI-TOF, matrix-assisted laser desorption ionization/time of flight; NMR, nuclear magnetic resonance; PBS, phosphate buffered saline; PLA2, phospholipase A2; PO, peroxidase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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