Development and characterization of a specific IgG monoclonal antibody toward the Lewis x antigen using splenocytes of *Schistosoma mansoni*-infected mice

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The parasitic blood fluke *Schistosoma mansoni* synthesizes immunogenic glycans containing the human Lewis x antigen (Le\(^x\); Galactose-\(\beta-1-4\)(Fuc\(\alpha-1-3\))N-acetylglucosamine-\(\beta\)-R, also called CD15), but the biological role(s) of this antigen in the parasites and in humans is poorly understood. To develop IgG-based monoclonal antibodies (mAbs) specific for Le\(^x\), we harvested splenocytes from *S. mansoni*-infected Swiss Webster mice at Week 10 post-infection, when peak IgG responses to glycan antigens occur, and generated a panel of hybridomas secreting anti-glycan IgG that recognize periodate-sensitive epitopes in soluble egg antigens of the parasites, and also recognize a neoglycoprotein containing a pentasaccharide with the Le\(^x\) sequence. One murine mAb, an IgG3 designated F8A1.1, bound to glycoproteins and glycolipids from schistosomiasis adults and human promyelocytic leukemic HL-60 cells that express Le\(^x\) antigens, as assessed by a wide variety of approaches including immunofluorescence staining, confocal microscopy, flow cytometry and western blotting, as well as overlay assays of glycolipids after thin-layer chromatography. In contrast, F8A1.1 bound weakly to cercariae, 3-h schistosomula and human Jurkat cells. We also directly compared the glycan specificity of F8A1.1 with commercially available anti-CD15 IgG1 (clone W6D3) using a defined glycan microarray. The results demonstrated that F8A1.1 recognized glycans expressing Le\(^x\) epitopes in a terminal nonreducing position, whereas anti-CD15 bound to glycans with multiple repeats of Le\(^x\) epitopes, but not to glycans with a single, terminal Le\(^x\) epitope. Our results show that F8A1.1 recognizes terminal Le\(^x\) epitopes and can be used for identification, immunolocalization, immunoprecipitation and purification of Le\(^x\)-containing glycoconjugates from schistosomes and mammalian cells.

Keywords: glycans / helminth / Lewis x antigen / monoclonal antibody / Schistosoma mansoni

Introduction

The parasitic blood flukes of the genus *Schistosoma* synthesize glycoconjugates with a variety of complex glycan structures, many of which are immunogenic in infected hosts and have been proposed to play important roles in the immunobiology of infections (Cummings and Nyame 1996; Van Die and Cummings 2010; Meevissen et al. 2012b; Van Diepen et al. 2012). Schistosome-derived glycans have many structural features that are distinct from mammalian cell-derived glycans (Nyame et al. 2004; Hokke et al. 2007), including the lack of sialic acid residues, which are common terminal sugar residues on mammalian cell glycoconjugates (Nyame et al. 1987; Nyame et al. 2004). Furthermore, many schistosome N-glycans have outer branches containing the LacdiNAc- [LDN; GalNAc\(\beta-1-4\)N-acetylglucosamine (GlcNAc)-R] backbone rather than the lactosamine [LN; Galactose (Gal)-\(\beta-1-4\)GlcNAc-R] structure commonly found on mammalian glycans (Srivatsan et al. 1992b; Srivatsan et al. 1994; Nyame et al. 1999; Van Die and Cummings 2010). Such LDN structures may be modified further with fucose (Fuc) residues in very unusual linkages to yield a large assortment of novel fucosylated LDN structures (Cummings and Nyame 1999; Wurher et al. 2002; Jang-Lee et al. 2007; Van Die and Cummings 2010; Van Diepen et al. 2012).

While the lactosamine-type sequences in schistosome glycans are less common, they often occur in outer branches of N-glycans within poly-N-acetyllactosamine (polyLN) sequences. These are typically further modified with Fuc residues in \(\alpha-1-3\) linkages to yield terminal Lewis x antigens (Le\(^x\); Gal\(\beta-1-4\)(Fuc\(\alpha-1-3\))GlcNAc-R) and so-called “poly-Lewis x” (polyLe\(^x\); R-3Gal\(\beta-1-4\)(Fuc\(\alpha-1-3\))GlcNAc\(\beta-1\)-R) structures comprised of both terminal Le\(^x\) and so-called “internal” Le\(^x\)-related glycan determinants (Ko et al. 1990; Srivatsan et al. 1992a; Van Dam et al. 1996). Current evidence using available...
monoclonal antibodies (mAbs) to the Le^x antigen suggests that Le^x expression and localization in *S. mansoni* are developmentally regulated in that the intramolluscan stages, mother and daughter sporocysts, do not express Le^x (Nyame et al. 2002). Le^x appears to be expressed by cercariae, the infective prevertebrate stage larvae, but expression may be restricted to secretions in the acetabular gland of cercariae, and Le^x appears undetectable on the surface of the infective larvae (Van Remoortere et al. 2000). Transformation of cercariae to schistosomula that results from contact with the vertebrate hosts is accompanied by a low expression of Le^x glycans on the surface of the juveniles and the expression increases as the parasites mature from schistosomula to adults, which highly express Le^x glyccan determinants on their surfaces (Koster and Strand 1994; Nyame et al. 2003). Le^x determinants are also present on membrane and secreted glycoconjugates from schistosome eggs and represent a major source of Le^x antigens released into body fluids of infected individuals (Robijn et al. 2007).

Interestingly, Le^x expression appears limited among trematodes and nematodes (Nyame et al. 1998). Thus far, the only other helminth known to express Le^x determinants is the bovine lung nematode, *Dictyocaulus viviparus*, which synthesizes N-glycans with Le^x determinants in outer branches (Haslam et al. 2000). Both schistosomes and *D. viviparus* reside in the lungs of their vertebrate hosts at various periods in their life cycles, but whether there is a biological relationship between worm residence in lungs and Le^x glycan expression is not known. Interestingly, the dendritic cell lectin dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrit (DC-SIGN) can recognize Le^x epitopes on soluble egg antigen (SEA) and this interaction may play an immunoregulatory function in the immunobiology of infections (Van Die et al. 2003). The relationship between the developmental expression of Le^x epitopes by the vertebrate stages of schistosomes and the potential immunoregulatory role of Le^x glycans remains to be elucidated. It is also not understood why the expression of the Le^x antigen is restricted to this small subset of parasitic worms.

The Le^x antigen was first discovered as a stage-specific embryonic antigen-1 (SSEA-1) in mice by Solter and Knowles using an IgM antibody (Solter and Knowles 1978), and SSEA-1 was subsequently shown to contain the trisaccharide determinant now defined as Le^x (Gooi et al. 1981; Hakomori et al. 1981). However, to date, the biological function of Le^x in animals is poorly understood. It is not a precursor to the common adhesion determinant sialyl Lewis x, since the Le^x structure cannot be sialylated by known sialyltransferases. Studies relying on available antibodies to the Le^x antigen indicate its expression on glycoconjugates of human, rat and bovine brain tissues (Dasgupta et al. 1996). Le^x glycans are also present on promyelocytic leukemia HL-60 cells and human neutrophils (McEvers and Cummings 1997; Fukuda et al. 1984; Spooncer et al. 1984). Few glycan-binding proteins in animals appear to specifically recognize Le^x-containing glycans, but recent studies show that Le^x-containing glycans on neutrophil lactoferrin mediate the uptake and clearance of lactoferrin released systemically at the site of inflammation by binding to the scavenger receptor C-type lectin expressed on the surface of endothelial cells (Graham et al. 2011). Le^x antigen is also expressed by various human carcinomas and leukemias, including urinary bladder carcinomas, breast cancer cells and gastrointestinal Hodgkin’s lymphoma (Shirahama et al. 1992; Brooks and Leathem 1995; Von Wasielewski et al. 1997). A recent study suggested that Le^x epitopes on CD98 determinants of Hodgkin’s lymphoma Reed–Sternberg cells bind to DC-SIGN and other lectins to promote interactions of lymphoma cells with other lymphocytes and myeloid cells in lymph nodes (Powlesland et al. 2011). Le^x also is expressed by the pathogenic bacteria, *Helicobacter pylori* (Sherburne and Taylor 1995).

In spite of its common expression in mammalian cells and tissues, *S. mansoni*-infected humans, primates and rodents generate IgM and IgG antibodies to Le^x glycans during the course of infection (Nyame et al. 1996; Nyame et al. 1997). The mechanisms by which infected hosts generate antibodies to self-molecule determinants, such as the Le^x antigen, are not understood. Furthermore, the exact functional role(s) of Le^x and the other schistosome glycans in the biology of the parasites are unclear. Some of the major obstacles to the study of the biological role(s) of Le^x and other schistosome glycans include the lack of the requisite reagents, most importantly the lack of highly defined and specific IgG-based anti-glycan antibodies, that are needed for these studies. Here, we report the identification and characterization of a novel mAb termed F8A1.1 developed using the spleens of *S. mansoni*-infected mice. F8A1.1 recognizes Le^x determinants present in schistosomes and mammalian cells using a variety of immunosassays. The availability of this specific IgG to Le^x will promote future studies in the field to define the expression and function of this epitope in many different biological systems.

**Results**

**Purification and characterization of antibody class and specificity of mAb F8A1.1**

In an earlier study, we determined the kinetics of antibody responses to glycan antigens during the course of *S. mansoni* infections in mice and observed that the peak IgG responses of Swiss Webster mice to glycan antigens of the parasites occur ~8–10-week postinfection (Nyame et al. 1997). Based on this finding, we harvested splenocytes from *S. mansoni*-infected Swiss Webster mice at Week 10 postinfection and used them to generate hybridomas. The hybridomas were screened to identify clones that secrete IgM antibodies, that are needed for these studies. Here, we report the expression and function of a novel mAb termed F8A1.1 during the course of infection (Nyame et al. 1996; Nyame et al. 1997). A recent study suggested that Le^x epitopes on CD98 determinants of Hodgkin’s lymphoma Reed–Sternberg cells bind to DC-SIGN and other lectins to promote interactions of lymphoma cells with other lymphocytes and myeloid cells in lymph nodes (Powlesland et al. 2011). Le^x also is expressed by the pathogenic bacteria, *Helicobacter pylori* (Sherburne and Taylor 1995).

**Material and methods**

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To produce purified mAb for detailed characterization of the class and binding specificity, hybridoma cells secreting F8A1.1 were adapted for growth in serum free media (SFM) as described in the “Material and methods” section. The IgG mAb was purified from SFM following centrifugation and chromatography on a MEP HyperCel column (Figure 1A). Approximately 40 mg of F8A1.1 was obtained from 500 mL
Fig. 1. F8A1.1 is an IgG3 that binds specifically to Le\(^x\) epitope. (A) Purification of F8A1.1 over MEP HyperCel. Hybridoma secreting F8A1.1 generated from splenocytes of \textit{S. mansoni}-infected mice were grown and adapted in SFM. The culture media was recovered and applied to MEP HyperCel column to affinity purify the secreted antibodies. Bound monoclonal antibodies were eluted with 50 mM sodium acetate, pH 4.0 buffer and neutralized with 1 M MOPS buffer pH 7.5, 0.15 M NaCl. Fractions with absorbance >1 were pooled and dialyzed against 100mM MOPS buffer, pH 7.5, 0.15 M NaCl and used for the characterization of binding specificity of the F8A1.1. (B) Purity of the purified F8A1.1 determined by SDS–PAGE and staining with Coomassie blue. (C) Determination of IgG subclass of F8A1.1. Microtiter plates coated with LNFPIII-BSA or SEA were incubated with 10 \(\mu\)g/mL F8A1.1 and detected with anti-mouse IgM, IgG, IgG1, IgG2a, IgG2b or IgG3. Error bars represent means ± 1 SD from three replicate readings within one experiment; representative of three experiments. (D and E) Determination of the specificity of F8A1.1 by ELISA using neoglycoconjugates. Microtiter wells coated with LNFPIII-BSA (Le\(^x\) epitope), LNFPII-BSA (Lewis \(a\) epitope), LNFPI-BSA (Blood group H, type 1 epitope), LDNFHI-BSA (Lewis b epitope), LNnT-BSA (lactosamine epitope) and were incubated with F8A1.1 as in (C). (E) The presence of Fuc on the antigens used in the ELISA in (D). Antigens were coated as in (D) and incubated with biotinylated AAL or AAL + Fuc and detected with streptavidin. Error bars represent means ± 1 SD from three replicate readings within one experiment; representative of three experiments. (F–H) Determination of the specificity of mAb F8A1.1 by ELISA using defined schistosome antigen (LDNF epitope), \textit{S. mansoni} SEA and glycoproteins. (F) Wells were coated with LDNFP-BSA, SEA, KLH, HRP or PLA2 and incubated with F8A1.1 and detected with anti-mouse IgG. (G) Cross-reactivity of antigens in (F) against 1 : 100 dilution of 10 weeks sera from mice infected with \textit{S. mansoni}. (H) The presence of Fuc in antigens. Wells were coated as in (F) and incubated with biotinylated AAL as in (E). Error bars represent means ± 1 SD from three replicate readings within one experiment; representative of three experiments.
fucosylated glycan antigens (Nyame et al. 2000). The neoglyco-panel of neoglycoproteins expressing a variety of different N\textsubscript{ac}diNAc fucopentaose-BSA (LDNFP-BSA; Gal\textsubscript{NAc}β1-3Gal\textsubscript{β1}-4Glc-BSA) were obtained for both the purified F8A1.1 and the control IgG indicating that a high degree of purity of F8A1.1 was obtained (Figure 1B). The isotype of the purified F8A1.1 was determined by enzyme-linked immunosorbent assay (ELISA) using SEA and LNFPIII-BSA as antigenic targets and anti-mouse IgM, IgG, IgG2a, IgG2b or IgG3 for detection of bound antibodies. F8A1.1 bound to both SEA and LNFPIII-BSA and was detectable by both anti-mouse IgG and IgG3, but not by anti-mouse IgM, IgG1, IgG2a or IgG2b (Figure 1C), indicating that F8A1.1 is an IgG3 that is potentially able to recognize Le\textsuperscript{e} epitopes.

We further tested the binding of F8A1.1 in ELISA toward a panel of neoglycoproteins expressing a variety of different fucosylated glycan antigens (Nyame et al. 2000). The neoglycoproteins used included lacto-\textsubscript{N}-fucopentaose II-BSA (LNFPII-BSA; Gal\textsubscript{β1}-3(Fucol-4)GlcNAcβ1-3-Gal\textsubscript{β1}-4Glc-BSA), which bears the Lewis a trisaccharide epitope; lacto-\textsubscript{N}-difucohexaose I-BSA (LNDHFI-BSA; Fucol-2Gal\textsubscript{β1}-3(Fucol-4)GlcNAcβ1-3-Gal\textsubscript{β1}-4Glc-BSA), which expresses the Lewis b tetrasaccharide epitope; lacto-\textsubscript{N}-fucopentaose I-BSA (LNFPI-BSA; Fucol-2Gal\textsubscript{β1}-3Gal\textsubscript{β1}-4Glc-BSA), which expresses blood group H (type I) epitope; and lacto-\textsubscript{N}-neo-tetraose-BSA (LNNtT-BSA; Gal\textsubscript{β1}-4GlcNAcβ1-3-Gal\textsubscript{β1}-4Glc-BSA), which bears the LN glycan backbone. F8A1.1 bound to LNFPIII-BSA but not to other fucosylated glycan antigens tested (Figure 1D). To rule out the possibility that the observed result might be due to differences in antigen coating efficiencies, neoglycoconjugate coated wells were incubated with biotinylated \textit{Aleuria aurantia} lectin (AAL) and probed with peroxidase conjugated streptavidin to estimate the densities of the coated neoglycoproteins by the density of their Fuc residues (Kochibe and Furukawa 1980). AAL bound to all of the fucosylated neoglycoproteins with equivalent intensities and the binding was completely inhibited with free Fuc, thus confirming the specificity of the AAL binding. This result indicates that the observed binding of F8A1.1 to LNFPIII-BSA was not due to differences in antigen coating efficiency (Figure 1E) but due to the apparent specificity of the F8A1.1 for the Le\textsuperscript{e} epitope.

To further characterize the specificity of F8A1.1, we tested its binding in ELISA against a number of antigenic targets that are bound by antibodies in sera of schistosome infected humans and animals. The antigens analyzed included lacto\textsubscript{N}Ac fucopentaose-BSA (LDNFP-BSA; Gal\textsubscript{NAc}β1-4(Fucol-3)GlcNAcβ1-3Gal\textsubscript{β1}-4Glc-BSA), which bears the defined fucosylated lacto\textsubscript{N}Ac (LDNF; Gal\textsubscript{NAc}β1-4(Fucol-3)GlcNAcβ-R) antigens of schistosomes, horseradish peroxidase (HRP), phospholipase A2 (PLA2) and keyhole limpet hemo-cyanin (KLH), which are glycoproteins reported to share serologically cross-reactive fucosylated and nonfucosylated glycan epitopes with \textit{S. mansoni} (Van Remoortere et al. 2003; Geyer et al. 2005). SEA from \textit{S. mansoni} was also tested as a positive control. Bound antibodies were detected with alkaline phosphatase-conjugated anti-mouse IgG and p-nitrophenyl phosphate substrate rather than HRP-conjugated secondary antibody, since HRP was tested as an antigen. As a control, the antigens were also incubated with pooled sera from mice infected for 10 weeks with \textit{S. mansoni} and bound antibodies were also detected by the same method. F8A1.1 bound to SEA as expected, but it did not bind to LDNFP-BSA, HRP, PLA2 or KLH (Figure 1F), thus indicating that the mAb recognizes a specific fucosylated glycan. All five antigens were bound to some extent by IgG antibodies in sera of mice infected with \textit{S. mansoni}, indicating that they contained immunogenic glycan antigens recognized by various antibodies in sera of infected individuals (Figure 1G). In control studies, all of the glycoprotein targets were bound to some extent by biotinylated-AAL, and binding was inhibited by free Fuc, indicating the presence of Fuc in those glycoproteins as expected (Figure 1H). These results further indicate that F8A1.1 recognizes LNFPIII-BSA containing the Le\textsuperscript{e} epitope and show that the mAb does not cross-react to other immunogenic fucosylated schistosome glycan antigens.

Comparison of the specificities of F8A1.1 and anti-CD15 by analysis on a defined glycan microarray

To further define the fine specificity of F8A1.1, we examined its binding to a panel of 610 glycan structures on the glycan microarray of the Consortium for Functional Glycomics (CFG). We also compared its binding with that of the commercially available anti-CD15 IgG1, that is thought to specifically recognize the Le\textsuperscript{e} epitope. However, the two mAbs showed significant differences in their glycan-binding specificities (Figure 2A–C; complete glycan array data presented in Supplementary Tables S1 and S2). F8A1.1 bound well to several glycans containing the Le\textsuperscript{e} determinant, where the Le\textsuperscript{e} moiety was expressed in a terminal, nonreducing position. For example, F8A1.1 bound to glycans with a simple trisaccharide Le\textsuperscript{e} structure (Glycans #151 and #152) as well as to glycans containing the terminal Le\textsuperscript{e} determinant in polyLe\textsuperscript{e} structures (Glycans #153 and #154). In contrast, anti-CD15 did not bind to glycans with a single, terminal Le\textsuperscript{e} structure (Glycans #151 and #152), but bound glycans expressing multiple Le\textsuperscript{e} determinants (Glycans #153 and #154), and also bound to a glycan with terminal Le\textsuperscript{e} linked to internal Le\textsuperscript{e} (Glycan #292). F8A1.1 showed no binding to glycans with only the internal Le\textsuperscript{e}-like sequence and was unable to bind Glycan #292 with terminal Le\textsuperscript{e} linked to internal Le\textsuperscript{e}. This is likely due to the conformation that results in the presence of the internal Le\textsuperscript{e} with the Gal\textsubscript{β1}-3 linkage, similar to the lack of binding seen when the GlcNAc is linked to a mannose (Man) residue. Interestingly, neither of these mAbs bound to single Le\textsuperscript{e} trisaccharides present on core-2 type O-glycan structures or on biantennary N-glycans (Glycans #447 and #419), but in those cases the trisaccharide is linked β6 to GalNAc or β2 to Man residues, respectively, and such linkage may be important in conformational presentation of the epitope (Figure 2C). Taken together, the results from the glycan array analysis show that F8A1.1 binds to some but not all glycans with terminal and single Le\textsuperscript{e} trisaccharide motif, and that its binding is restricted to a subset of those glycans.
with that structural motif, whereas anti-CD15 binds to some glycans with the terminal Lex trisaccharide motif but not the simple trisaccharide. The data also indicate the subtle but important differences between two closely related antibodies in terms of their specific glycan recognition.

**Fig. 2.** F8A1.1 binds to Le^a^ epitopes with high specificity compared with that of anti-CD15. (A) mAb F8A1.1 (50 μg/mL) and (B) mAb anti-CD15 (50 μg/mL) were incubated with an array of 610 glycan structures (version 5.1 CFG glycan microarray) and detected with Alexa fluor-488 labeled anti-mouse IgG. The RFU of bound antibody are plotted. (C) RFU of binding of anti-CD15 compared with F8A1.1 to selected glycan structures with Le^a^ epitopes present on version 5.1 of the CFG glycan microarray. ND = not detectable (<200 RFU), Sp = spacer group, error bars represent means ± 1 SD Complete glycan array data are given in Supplementary Tables S1 and S2, including structures of spacer groups.

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F8A1.1 binds to Le^a^ epitopes on intact schistosomes and HL-60 cells

To investigate the utility of F8A1.1 to study expression of Le^a^ epitopes on glycoconjugates on cell surfaces, we examined its binding to *S. mansoni* cercariae, 3-h old mechanically
transformed schistosomula, and 6-week old adults. Bound antibody was detected with Alexa fluor 488-conjugated antimouse IgG and imaged by confocal microscopy. Consistent with the reported developmental regulation of expression of Le\(^{x}\) epitopes by *S. mansoni* (Nyame et al. 2003), F8A1.1 bound intensely to the surface of adult *S. mansoni* but by comparison we observed relatively little binding to the body surfaces of 3-h old schistosomula or cercariae (Figure 3A and C), although in some experiments we observed some binding to apparent secretions from the oral sucker. To confirm the specificity of F8A1.1 binding, cercariae, 3-h old schistosomula and adult schistosomes were also incubated with biotinylated AAL and probed for bound lectin with Alexa fluor 488-conjugated streptavidin to determine the density of fucosylated glycans on the surface of the three life cycle stages. As a positive control, AAL stained the surface of all three stages with equivalent intensities, indicating that these stages express fucosylated glycans on their surfaces (Figure 3B and D). Thus, F8A1.1 binds selectively to specific Fuc-containing epitopes among the fucosylated glycans on the surfaces of the parasites.

Because F8A1.1 was generated from Le\(^{x}\) antigens on schistosome glycoconjugates, we tested whether F8A1.1 would bind to Le\(^{x}\) epitopes on mammalian cell glycoconjugates. For this study, we chose the human promyelocytic leukemic HL-60 cells, which expresses Le\(^{x}\) and sialyl Le\(^{x}\) glycans on cell surface glycoconjugates (Spooncer et al. 1984, Fukuda et al. 1984). As controls, we also examined binding to human T leukemic Jurkat cells, which are known to have minimal expression of CD15 determinants (Nakayama et al. 2001), as well as minimal levels of sialyl Le\(^{x}\) antigen (Knibbs et al. 1996). HL-60 and Jurkat cells were also treated with neuraminidase to remove sialic acid residues and expose underlying Le\(^{x}\) epitopes, or mock treated by omitting the glycosidase. Untreated and neuraminidase-treated HL-60 and Jurkat cells were incubated with F8A1.1 or the commercially available anti-Le\(^{x}\) IgG1 mAb anti-CD15 (clone W6D3). The cells were subsequently incubated with Alexa fluor 488-conjugated antimouse IgG and analyzed by flow cytometry. Both mAb F8A1.1 and anti-CD15 bound to HL-60 cells but weak staining was observed toward Jurkat cells; desialylation with neuraminidase enhanced the binding of both F8A1.1 and anti-CD15 to HL-60 cells, as expected, but not the binding to Jurkat cells (Figure 3E–H). These results are consistent with the expected expression of Le\(^{x}\) epitopes on HL-60 cell glycoconjugates and show that mAb F8A1.1 gives similar results to that for anti-CD15 IgG1 (Stocks et al. 1990; Kerr and Stocks 1992).

**F8A1.1 binds specifically to Le\(^{x}\) epitopes on glycoproteins from *S. mansoni* and HL-60 cells**

Le\(^{x}\) epitopes have been shown to occur on both glycoproteins and glycolipids of both *S. mansoni* and HL-60 cells (Symington et al. 1985; Wuhrer et al. 2002). Thus, we sought to characterize the nature of the glycoconjugates from schistosomes and HL-60 cells that bear the Le\(^{x}\) epitopes bound by

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**Fig. 3.** F8A1.1 binds specifically to Le\(^{x}\) epitopes on the surface of intact schistosomes and intact HL-60 cells. Projected images from confocal microscopy showing presence of (A) Le\(^{x}\) epitopes and (B) fucosylated glycan epitopes on the surface of intact cercariae, schistosomula (3 h) and adult schistosomes. Parasites were incubated with 10 μg/mL F8A1.1 (A) or 5 μg/mL AAL-biotin (B). Transmitted light images of parasites incubated with mAb F8A1.1 (C) and AAL-biotin (D). Flow cytometric analysis of desialylated (+neuraminidase) or control HL-60 cells (+neuraminidase) (E and F) and desialylated and control Jurkat cells (G and H). Cells were incubated with 10 μg/mL F8A1.1 (E and G) or 2.5 μg/mL anti-CD15 (F and H) and stained cells were analyzed by flow cytometry. The results are representative of two experiments.
F8A1.1. Soluble and detergent extracts of *S. mansoni* adults and eggs, along with detergent extracts of Jurkat cells, and HL-60 cells, were separated by SDS–PAGE under reducing conditions, blotted onto nitrocellulose membranes and probed with F8A1.1. The detergent extract of *Ascaris suum* was analyzed as a control. F8A1.1 bound to numerous glycoproteins from *S. mansoni* eggs, adults and HL-60 cells (Figure 4A–C), but not to glycoproteins in extracts of Jurkat cells, which lack expression of Le^a^ in addition, F8A1.1 did not bind to extracts of *A. suum*, which is known to express many fucosylated antigens, but do not appear to contain glycans with the Le^a^ antigen structure (Pollt et al. 2007).

A key utility of having an IgG such as F8A1.1 that recognizes the Le^a^ antigen is to use it to immunoprecipitate glycoproteins carrying this antigen. To this end, we immunoprecipitated the native glycoproteins from detergents extracts of biotinylated cercariae and HL-60 cells. To facilitate detection of minor cell surface glycoproteins, the HL-60 cells were first biotinylated with membrane-impermeable sulfo-NHS-Biotin prior to solubilization and immunoprecipitation. Extracts of the biotinylated cercariae and HL-60 cells were immunoprecipitated with F8A1.1, separated by SDS–PAGE under reducing conditions, blotted onto nitrocellulose, and the immunoprecipitated glycoproteins were visualized by incubations with peroxidase-conjugated streptavidin and chemiluminescence substrate and imaging. Numerous glycoprotein bands were immunoprecipitated by F8A1.1 (Figure 5A and B). The banding patterns of HL-60 cells were similar in many respects to those observed for F8A1.1 western blot analysis (Figure 4A–C). No bands were observed in control experiments in which the immunoprecipitation was carried out in the absence of antibody or in experiments in which the immunoprecipitation was performed with nonspecific murine IgG. Furthermore, no bands were observed in experiments in which nonbiotinylated extracts were immunoprecipitated with F8A1.1 (Figure 5A and B). These results further demonstrate the specificity of the immunoprecipitation with F8A1.1, as well as document that some of the antigens recognized by F8A1.1 are accessible to biotinylation at the surfaces of HL-60 cells. Taken together, the western blot and immunoprecipitation results show that glycoproteins carrying Le^a^ determinants can be recognized by F8A1.1 in both denatured and native glycoproteins from schistosomes and mammalian cells and that F8A1.1 can detect Le^a^ epitopes by solid phase and fluid phase assays.

F8A1.1 binds Le^a^ epitopes on glycolipids of schistosomes and HL-60 cells

To explore whether F8A1.1 could also recognize Le^a^ antigens in glycolipids, total glycolipids were isolated from schistosome eggs and adults, as well as HL-60 and Jurkat cells using standard techniques (Makaaru et al. 1992). The total lipids were subsequently fractionated into upper and lower phase Folch fractions and separated by high performance TLC. The TLC plates were blocked and overlinked with F8A1.1. Bound antibodies were detected by incubations with peroxidase-conjugated anti-mouse IgG and chemiluminescence substrate and reactive bands were visualized by exposure to X-ray film.
While orcinol staining revealed a similar overall pattern for glycolipids from both eggs and adults (Supplementary Figure S1A, left), F8A1.1 bound only to slowly migrating glycolipids (relatively large in size) in the upper phase extract from schistosome eggs, but did not bind significantly to glycolipids in the upper phase fraction from adult schistosomes (Supplementary Figure S1A, right). In contrast, while the pattern of orcinol staining of glycolipids in lower phase extracts of both eggs and adult was relatively similar, F8A1.1 bound to a subset of slowly migrating glycolipids in the lower phase extracts from both schistosome egg and adult glycolipids (Supplementary Figure S1B). The results reveal that mAb F8A1.1 binds to Le\(^e\) epitopes on glycolipids of schistosome eggs and adults and that there are differences in the complexity of glycolipids expressing Le\(^e\) from eggs and adult schistosomes. In control studies, we examined binding of mAb F8A1.1 to glycolipids of HL-60 and Jurkat cells. Interestingly, orcinol stained the standard gangliosides, showed equivalent staining of glycolipids in upper phase extracts from both Jurkat and HL-60 cells, and little glycolipid was detectable in the lower phase extracts of either cell type (Supplementary Figure S1C, left and D, left). However, we found that F8A1.1 bound to some slowly migrating glycolipids in both the upper and lower phase extracts of HL-60 cells, but not to glycolipids in either upper phase or lower phase extracts from Jurkat cells, nor to brain gangliosides (Supplementary Figure S1C, right and D, right). We note that there was unavoidable background and nonspecific binding in the higher regions of the TLC plate (Supplementary Figure S1C) that may have obscured antibody binding to faster migrating glycolipids. Overall, the key results demonstrate that F8A1.1 can also recognize Le\(^e\) epitopes on glycolipids of schistosomes and mammalian cells, and does not significantly cross-react to gangliosides.

**Comparison of the binding specificities of mAbs F8A1.1 and anti-CD15 toward Le\(^e\) epitopes on schistosome and mammalian cell glycoproteins by western blotting**

The observation from the glycan array analyses that mAb F8A1.1 and anti-CD15 differ in their specificities toward different presentations of Le\(^e\) glycan structures led us to compare the specificity of the two antibodies toward Le\(^e\) epitopes on natural glycoproteins from schistosomes and mammalian cells by western blot analyses. SEA or detergent extracts of *S. mansoni* eggs, soluble or detergent extracts of adult *S. mansoni*, and detergent extracts of HL-60 cells, were separated by SDS–PAGE, transferred onto nitrocellulose membranes and analyzed by western blots as described in the “Material and methods” section using 20 μg/mL solutions of F8A1.1 or anti-CD15. Detergent extracts of the nematode *A. suum* and Jurkat cells were also analyzed as controls. Anti-CD15 showed marked limitations in its binding interactions with Le\(^e\) epitopes on egg glycoproteins compared with F8A1.1 (Figure 6A and B). Specifically, anti-CD15 poorly bound to a set of high-molecular-weight glycoproteins

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**Fig. 6.** Comparative western blot analyses of schistosome and mammalian cell extracts using anti-CD15 and F8A1.1. Soluble (15 μg) and detergent (2.5 μg) extracts of *S. mansoni* eggs (A and B), soluble (45 μg) and detergent (45 μg) extracts of adult *S. mansoni* and detergent extracts (45 μg) of adult *A. suum* (C and D), and detergent extracts of HL-60 cells (150 μg) and Jurkat (150 μg) cells (E and F) were separated by SDS–PAGE on 4–20% polyacrylamide gradient gel and blotted onto nitrocellulose membrane. The membranes were blocked and incubated with 20 μg/mL of mAb F8A1.1 (A, C and E) or anti-CD15 (B, D and F) and reactive bands were revealed by incubation with goat anti-mouse IgG-HRP conjugate and SuperSignal west pico chemiluminescence substrate followed by imaging on UVP EC-4 imager.
(250–98 kDa) in SEA and low-molecular-weight glycoproteins (50–16 kDa) in detergent extracts of eggs, respectively, which were bound by F8A1.1 (Figure 6A and B). Anti-CD15 and F8A1.1 showed similar binding patterns toward Le^a epitopes on glycoproteins from detergent and soluble extracts of adult S. mansoni, but the bands in the F8A1.1 blot were sharper and more distinctive than the blots using anti-CD15 (Figure 6C and D). F8A1.1 and anti-CD15 recognized similar glycoprotein bands from HL-60 cells and, as expected, both antibodies did not show discernible binding to Jurkat cells (Figure 6E and F). Interestingly, anti-CD15 was much less specific in its binding compared with F8A1.1. Unlike F8A1.1, anti-CD15 consistently bound intensely to two molecular weight marker bands (96 and 36 kDa) and a high-molecular-weight protein from A. suum, which was analyzed as a negative control (Figure 6C and D). Taken together, the comparative western blot analyses employing anti-CD15 and F8A1.1 suggest that glycoproteins from HL-60 cells and detergent extracts of adult schistosomes share similar complexities in their Le^a structures, which are likely to be poly-Le^a structures, whereas glycoproteins from schistosome eggs bear some poly-Le^a structures that are bound by both antibodies, and single Le^a structures that, as observed from the glycan array data, are bound by F8A1.1 but not anti-CD15. Furthermore, F8A1.1 appears to have superior capacity to detect Le^a epitopes as demonstrated by the sharpness of the bands detected with the antibody and minimal non-specific binding.

Discussion

Our results show that F8A1.1 binds to glycans expressing terminal Le^a determinants from intact schistosomes and mammalian cells and can recognize this terminal Le^a antigen on both glycoproteins and glycolipids. Analysis of the binding specificity of mAb F8A1.1 on the glycan microarray of the CFG reveals that unlike the commercially available anti-CD15 (clone W6D3), F8A1.1 binds exclusively to glycans with terminal Le^a residues and does not require expression of internal Le^a epitopes (Figure 2). The specificity of F8A1.1 and its ability to bind glycoconjugees containing Le^a epitopes in schistosomes and mammalian cells by diverse immunoassays makes the mAb a novel and very useful reagent for the identification and study of expression of Le^a in both schistosome and mammalian systems.

Our finding that the commercially available anti-CD15 does not bind to glycans expressing only terminal Le^a epitopes raises questions about the specificity of anti-CD15 reagents in the field. Our studies here indicate the need to systematically analyze the available IgM and IgG mAbs to Le^a using glycan microarray approaches coupled with information from flow cytometry and western blotting to define their anti-glycan specificities. This is important in view of the fact that many of these anti-Le^a mAbs are being employed for the purpose of identifying cancer cells and for the study of the biology of Le^a glycans (Golijanin et al. 1995; Friedrich et al. 2002). In a previous study in which we used commercially available IgM anti-CD15 mAb to determine the expression of Le^a epitopes on intact S. mansoni life cycle stages, we observed slight staining of cercarial surface glycoconjugees in addition to a strong staining of secretions from the oral sucker (Nyame et al. 2002).

In our study, mAb F8A1.1 occasionally stained secretions from the oral sucker, but we did not observe strong binding to the body surfaces of cercariae (Figure 3A). This binding specificity of F8A1.1 to intact cercariae is consistent with previous studies that show that Le^a antigen expression in cercariae is limited to glycoconjugees in secretions from the acetabular gland (Van Remoortere et al. 2000). The IgM anti-CD15 probably has other binding specificities to account for the staining of cercarial surfaces. Future analyses of this IgM anti-CD15 and other anti-CD15 reagents on the CFG glycan microarray could help to identify the specificities of such mAbs.

The binding of F8A1.1 to Le^a epitopes on mammalian cell glycoconjugees indicates the utility of the mAb for use in cancer biology. Numerous cancer cells, including exfoliated bladder cancer cells, breast cancer cells and Hodgkin's lymphoma Reed–Sternberg cells, express Le^a epitopes (Shirahama et al. 1992; Brooks and Leathem 1995; Von Wasielewski et al. 1997). The presence of Le^a epitopes on membrane glycoconjugees of exfoliated bladder cancer cells recovered from urine has been proposed as an accurate method for the diagnosis of the oncogenic transformation of bladder cancer cells and provides a noninvasive test for detection of bladder cancer (Golijanin et al. 1995; Friedrich et al. 2002). Based on its exclusive specificity for Le^a epitopes, mAb F8A1.1 could serve as a useful mAb for the identification of bladder cancer cells in urine samples. Additionally, F8A1.1 could be useful in studying interactions between ligands that utilize Le^a epitopes to bind their cognate C-type lectin receptors. For example, CD98hc and ICAM-1 from Hodgkin’s Reed–Sternberg cells bear Le^a epitopes, which are proposed to interact with DC-SIGN on dendritic cells to facilitate their migration to lymph nodes (Powlisland et al. 2011). F8A1.1 will be a very useful mAb in the study of these interactions, as shown for studies on the interactions between Le^a epitopes on glycoconjugees of SEA and C-type lectin DC-SIGN of human dendritic cells (Van Die et al. 2003). Interestingly, whereas the Le^a antigen on biantennary N-glycans is not well recognized by mAb F8A1.1, it was recently shown that DC-SIGN can recognize the schistosome SEA glycoprotein Interleukin-4-inducing principle from Schistosoma mansoni eggs/α1, which carries the Le^a antigen on biantennary N-glycans (Meevissen et al. 2012a). F8A1.1 could also be useful in studying the recently reported clearance of Le^a-bearing neutrophil granule glycoproteins by interactions with the scavenger receptor C-type lectin (SRCL) on endothelial cells (Graham et al. 2011).

We found in our study using western blots and immunoprecipitation that F8A1.1 binds to numerous glycoproteins from the promyelocytic HL-60 cells, and thus binds to a wide range of Le^a epitopes on myeloid cell glycoconjugees. However, it is noteworthy and unexpected that neither F8A1.1 nor the anti-CD15 tested here recognize a complex-type biantennary N-glycan expressing the Le^a determinant linked to α-linked Man residues, nor do they bind to a core 2 O-glycan expressing the Le^a determinant linked to β-linked GlcNAc. Thus, for specific detection of Le^a in such glycans, other mAbs will need to be developed. It is also noteworthy that we previously reported on a specific murine mAb that recognized sialyl-Le^a antigen only in the context of a core 2 O-glycan (Walchek et al. 19885).
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et al. 2002). Thus, it is likely that the context in which a glycan antigen is expressed is critical to its recognition by specific mAbs, and a glycan structural determinant may be necessary but not sufficient for recognition.

The results of the comparative western blot analyses support the possibility of existence of differences in the complexity of the Le^e structures present on glycoproteins from extracts of schistosome egg and extracts of adult schistosomes and HL-60 cells. The similar western blot patterns of anti-CD15 and F8A1.1 binding toward Le^e epitopes on glycoproteins from extracts of adult schistosomes and HL-60 cells are very consistent with the fact that both adult schistosomes and HL-60 cells synthesize N-glycans with poly-Le^e structures (Figure 2) (Spooner et al. 1984; Srivatsan et al. 1992a), which are recognized and bound by both anti-CD15 and F8A1.1, based on glycan microarray data. The complexity of the Le^e structures on glycoproteins from eggs has not been studied in detail. However, the comparative western blot analyses suggest that egg glycoproteins contain a mixture of both poly-Le^e and single Le^e structures. It can be deduced based on the glycan array data that those glycoproteins bearing poly-Le^e structures are the ones bound by both anti-CD15 and F8A1.1, while those bearing single Le^e structures are the glycoproteins bound by F8A1.1, but not bound by anti-CD15. These results raise the possibility of the existence of variations in the complexity of the structures of Le^e glycans synthesized by different developmental stages of schistosomes. Thus, the observed developmentally regulated expression of Le^e epitopes by the parasites may not be limited to the absence of Le^e epitopes in the larval stages, but it may also involve differences in the complexity of the structures of the Le^e epitopes synthesized by different developmental stages of the parasites. The availability of mAb F8A1.1 should now make it possible to specifically capture released Le^e glycans from eggs, cercariae and schistosomula for evaluation of the complexities of the Le^e structures synthesized by these developmental stages. Structural differences in Le^e epitopes may have implications in host-schistosome interactions and the survival of the parasites in their hosts. The two antibodies could also be employed to monitor changes in the complexity of Le^e epitopes of schistosome glycoconjugates during parasite development.

The role of the regulated expression of Le^e glycans in the immunobiology of schistosomes is not well understood. However, recent studies suggest that glycans containing Le^e may play an immunoregulatory function. The free sugar LNFPIII is reported to stimulate macrophages in vitro to express CD69 and secrete IFN-γ and the activated macrophages are able to activate NK cells (Atochina and Harn 2005). In another study, Le^e glycans on SEA were shown to interact with the human dendritic cell lectin DC-SIGN (Van Die et al. 2003). These observations suggest that Le^e and other fucosylated schistosome glycans may be the molecular epitopes responsible for the immunoregulatory activities associated with SEA, as well as the potential immunoregulatory activity reported for glycoconjugates released from cercarial glycoalxyn upon infection (Van Liempt et al. 2007).

Using defined F8A1.1 it should now be possible to immunoaffinity purify Le^e-bearing glycoproteins and glycolipids from cercariae, schistosomula, adults and eggs of schistosomes and directly evaluate their ability to bind and activate dendritic cells and macrophages. Such affinity-purified glycoconjugates should allow the assessment of the contributions, if any, of the lipid and protein backbones of the glycoconjugates in the activation process. These studies can be strengthened by using F8A1.1 to purify Le^e-bearing glycoconjugates from mammalian sources for use as controls to verify the roles of the lipid and protein backbones of glycoconjugates in the induction of activation of myeloid cells and the concomitant immunoregulatory activities.

F8A1.1 will also be useful in cloning and studying the schistosome fucosyltransferase(s) responsible for the biosynthesis of Le^e epitopes. Schistosomes synthesize a large assortment of fucosylated glycans on both LDN and LN backbones (Wuhrer et al. 2002; Jang-Lee et al. 2007). The fucosyltransferases responsible for the synthesis of fucosylated glycans have not been identified. Many of the glycosyltransferases reported in the schistosome genome database have been annotated as fucosyltransferases (Berriman et al. 2009). The fucosyltransferase(s) responsible for synthesizing Le^e epitopes can now be expression cloned in CHO or COS7 cells using mAb F8A1.1 for detection of transfected cells expressing the glycan epitope. The identification of the fucosyltransferase gene responsible for Le^e biosynthesis in schistosomes should allow the expression of the enzyme in the snail stage parasites, which do not express Le^e glycans (Nyame et al. 2002), to ascertain the effect of expressing Le^e epitopes in those stages. Conversely, RNAi techniques can be used to knockdown the fucosyltransferase gene in the vertebrate stages, which express Le^a (Bhardwaj et al. 2011), to determine the relevance of Le^e epitope in the survival of schistosome in the vertebrate hosts. F8A1.1 would be very useful in examining the expression and deletion of the genes by immunostaining the snail and vertebrate stage parasites with the mAb to determine expression or loss of Le^e epitopes.

Materials and methods

Chemicals and reagents

Chemicals used in this study were purchased from Fisher Scientific (Pittsburgh, PA), unless otherwise stated. KLH and high-performance thin-layer chromatography (TLC) plates were obtained from Calbiochem (San Diego, CA), peroxidase conjugated goat anti-mouse IgG (γ chain-specific), peroxidase conjugated goat anti-mouse IgM (μ chain-specific) and ABTS/peroxidase substrate were purchased from Kirkegaard and Perry (Gaithersburg, MD). Peroxidase-conjugated goat anti-mouse IgG isotyping kit was obtained from Southern Biotechnology Associates, Inc. (Birmingham, AL). Precast polyacrylamide gels, SFM media, Alexa fluor-488, Alexa fluor 488-conjugated streptavidin and Protein A-conjugated Dyna beads were from Invitrogen (Carlsbad, CA). Sypro Ruby, silver staining kit, nitrocellulose membrane, Immun-Star chemiluminescence substrate were obtained from Bio-Rad Laboratories (Hercules, CA). Bicinechonic acid (BCA) protein assay kit, Supersignal picomol peroxidase chemiluminescence substrate and sulfo-NHS-biotin were purchased from Thermo Fisher Scientific (Rockford, IL). PLA2 from honeybee.
Protease inhibitor cocktail tablets, *Arthrobacter* neuminidase (which cleaves α2-3, α2-6 and α2-8 linked sialic acid), peroxidase-conjugated streptavidin and alkaline phosphatase-conjugated streptavidin were purchased from Roche Applied Science (Indianapolis, IN). MEP HyperCel was from Pall Life Sciences (Ann Arbor, MI). Biotinylated AAL was purchased from Vector Labs (Burlingame, CA). Microtiter ELISA plates (Immulon 4HBX) were from Thermo Electron Corp. (Milford, MA). Iscoves modified Dulbecco’s modified Eagle’s medium (DMEM), RPMI-1640 tissue culture media, Hanks buffer, L-glutamine and fetal bovine serum (FBS) were purchased from MediaTech (Manassas, VA). Tissue culture flasks and plates were from Corning Life Sciences (Lowell, MA). Tissue culture roller bottles were obtained from Nalgene Nunc International (Rochester, NY). Bovine serum albumin (BSA, protease-free) was purchased from Boval Company (Cleburne, TX) and used at 1% in glycan microarray studies. BSA for western blots was obtained from Fisher Scientific (Heat-shock Fraction IV) and used as described below.

### Glycoconjugates

Neoglycoconjugates, protein to which sugar has been chemically linked, including LNFPII-BSA, LNFPIII-BSA, LNFP-I-BSA, LDNFH-I-BSA, lacto-N-neolactohexaose I, were purchased from V labs, (Covington, LA). LactoN-Ac-tetraose and LDNF glycan antigens were synthesized from the human milk oligosaccharide LNnT and conjugated to BSA as described previously (Nyame *et al*. 1999). Protein content was determined by the BCA assay. Bovine brain gangliosides were kindly provided by Dr. Xuezheng Song, Emory University (Atlanta, GA).

### Purification of F8A1.1

We selected the hybridoma clone secreting IgG anti-Le x mAb F8A1.1 by ELISA screening against periodate sensitive epitopes of SEA; its specificity for Le x was subsequently identified by ELISA using LNFPIII-BSA as the antigenic target (Nyame *et al*. 1996). The hybridomas were grown to 80% confluence density in Iscove’s DMEM media containing 20% FBS, 8 mM L-glutamine (2×) at 37°C in 5% CO₂ atmosphere in T-150 flasks and split into SFM to obtain a culture containing 10% FBS. The cells were grown to 80% confluence density and split once again into SFM to yield a culture containing 5% FBS and grown to 80% confluence density. Cells from 16 T-150 flasks were harvested by centrifugation at 1200 rpm for 2 min and suspended in 500 mL SFM supplemented with 8 mM L-glutamine (2×). The cell suspension was transferred into roller bottles and grown in a Wheaton roller incubator model IO57606 (Millville, NJ) at 37°C in 5% CO₂ atmosphere over a period of 1 week. The cell culture was centrifuged at 1200 rpm at 4°C to remove residual cells and the media was recovered and centrifuged further at 14,000 rpm at 4°C to remove particulate debris. The media was applied to a 5 mL column of MEP HyperCel and 5 mL fractions were collected. Unbound material was washed from the column with 10 mM 3-(N-morpholino)propanesulfonic acid (MOPS) pH 7.5/150 mM NaCl buffer, and 5 mL fractions were collected. The column run-through and wash fractions were monitored for protein by absorbance at 280 nm. The washing was continued until no protein was detected in the washes by absorption at 280 nm. Bound mAbs were batch eluted by applying 5 mL of 50 mM sodium acetate buffer, pH 4.0 to the column and applying pressure at the top of the column with a pipette bulb. The eluted material was neutralized immediately with 1 mL of 1.0 M MOPS pH 7.5/150 mM NaCl buffer. The neutralized eluted column fractions were monitored for protein by absorbance at 280 nm and fractions containing protein were pooled and dialyzed against 0.1 M MOPS buffer, pH 7.5 containing 150 mM NaCl, 0.02% thimerisol and 1mM benzamidine. The dialyzed was recovered and the protein content was determined by BCA protein assay using instructions provided by the manufacturer and BSA as standard. The mAb was stored at 4°C until use.

### Parasites

Adult schistosomes were recovered by portal perfusion of Swiss Webster mice infected for 6 weeks with ∼200 *S. mansoni* cercariae (Puerto Rican strain). The recovered parasites were washed 4× with cold PBS (Mediatech, Manassas, VA) and used immediately or stored at −80°C. Cercariae were shed into snail water from Biomphalaria glabrata snails infected for 4 weeks with *S. mansoni* miracidia. The cercariae were kept on ice to cause them to slow down and settle at the bottom of the tube. The water was removed and the wet parasites were used immediately or stored at −80°C. *S. mansoni* eggs were recovered from perfused livers of Swiss Webster mice infected for 8 weeks with 200 cercariae using standard protocols (Lewis 1998). Briefly, the livers were kept in ∼1 L of 1.7% saline solution overnight at room temperature and blended to release the eggs from liver tissue. The homogenate was transferred into a glass measuring cylinder and the eggs were allowed to settle by gravity. The supernatant containing liver tissue was removed by suction. The egg sediment was suspended in 1.7% saline and transferred into 50 mL Falcon tubes and centrifuged at 1200 rpm for 5 min and the supernatant containing residual liver tissue was removed carefully by suction. The egg pellet was suspended in 1.7% saline and the eggs were separated from residual liver tissue by centrifugation over Percoll gradient (Lewis 1998). The eggs were recovered in the pellet fraction and washed 4× with 1.7% saline and stored frozen as a wet pellet at −20°C. *Ascaris suum* was a kind gift from Dr. Irma van Die, VU Medical Center, Amsterdam, Netherlands.

### Cell culture and desialylation

HL-60 and Jurkat cells were grown in RPMI supplemented with 2 mM L-glutamine and 10% FBS at 37°C in 5% CO₂ atmosphere to 80% confluence density. The cells were harvested at their highest density after log-phase growth and washed 4× with cold PBS and processed immediately or stored at −80°C. For desialylation, HL-60 and Jurkat cells were grown to 80% confluent density in RPMI as described above and washed 4× with Hanks buffer. Approximately 1 × 10⁷ cells were incubated with 15 μL of neuraminidase in 1 mL of Hanks buffer at 37°C for 30 min. The cells were washed 4× with Hanks buffer and used for analysis. As controls, HL-60 and Jurkat cells were also mock treated by incubation in Hanks buffer without neuraminidase.
Preparation of extracts
To prepare SEA, *S. mansoni* eggs were suspended in PBS supplemented with 5× protease inhibitor cocktail (Roche, Indianapolis, IN) and sonicated on ice using a Branson sonifier (Branson Ultrasonic Corp., Danbury, CT). The homogenate was centrifuged at 16,000×g for 30 min at 4°C and the supernatant fraction was recovered as SEA. The pellet fraction was suspended in PBS containing 5× protease inhibitor cocktail and resuspended by sonication on ice. Triton X-100 was added to a final concentration of 0.5% and the homogenate was kept on ice for 30 min to solubilize membrane proteins. The homogenate was centrifuged at 16,000×g for 30 min at 4°C and the supernatant fraction was recovered as detergent extracted egg antigen. The protein content of the egg extracts were determined by BCA assay and the extracts were aliquoted and stored at −20°C. To prepare adult *S. mansoni* extracts, the adult worms were sonicated in PBS supplemented with 5× protease inhibitor cocktail similar to that as described for SEA extract above. Triton X-100 was subsequently added to the homogenate to a final concentration of 0.5% detergent and the mixture was incubated on ice for 30 min. The homogenate was centrifuged at 16,000×g for 30 min at 4°C and the supernatant fraction was recovered as adult schistosome extract. The protein content of the extract was determined by BCA assay and the samples were aliquoted and stored at −20°C. To prepare extracts of HL-60 and Jurkat cells, ~1×10^8 HL-60 or Jurkat cells were suspended in 1 mL PBS supplemented with 1× protease inhibitor cocktail and sonicated as described above. Triton X-100 was added to the homogenates to a final concentration of 0.2% Triton X-100 and kept on ice for 30 min to solubilize proteins. The homogenates were centrifuged at 16,000×g at 4°C for 30 min to pellet insoluble materials. The supernatant fractions were recovered as cell extracts and the protein contents were determined by BCA protein assay.

Enzyme-linked immunosorbent assay
Microtiter wells were coated with 50 µL/well of either 5 µg/mL of SEA or 5 µg/mL of neoglycoconjugates in PBS and blocked with 3% solution of BSA in PBS. The wells were incubated with 50 µL of tissue culture media or 10 µg/mL solution of mAb F8A1.1 in dilution solution and bound antibodies were detected by incubation with 50 µL of 1:10,000 dilution peroxidase-conjugated goat anti-mouse IgM or IgG in dilution solution, followed by incubation with ABTS/peroxidase substrate for 5 min. The absorbance of the wells was determined on a Perkin-Elmer microtiter plate reader model 1420 (Shelton, CT) at 405 nm. The microtiter wells were washed 4× after antigen coating, BSA blocking and antibody incubations with a wash buffer of PBS/0.3% Tween-20 using a microtiter plate washer (Dynatech, Chantilly, VA). Antibody dilutions were carried out using a dilution solution of PBS/1% BSA/0.3% Tween-20. Antigen coating, BSA blocking and antibody incubations were carried out for 30 min. The ELISAs were performed in triplicates and the results represent averages of the three assays. In some ELISA assays, wells were coated with 50 µL of 5 µg/mL LDNFP-BSA or SEA, 40 µg/mL keyhole limpet KLH, 20 µg/mL HRP or PLA2 from honeybee venom and incubated with 10 µg/mL of mAb F8A1.1 in PBS-Tween-20/1% BSA. Bound antibodies were probed by incubations with phosphatase-labeled goat anti-mouse IgG and p-nitrophenol phosphate substrate.

SDS–PAGE and western blot analysis
SDS–PAGE was performed under reducing conditions essentially as described previously (Nyame et al. 1999) using standard procedures (Laemmli 1970). Briefly, extracts of parasites, cells and protein samples were boiled in reducing SDS–PAGE sample buffer and applied to 4–20% precast polyacrylamide gradient gels (Invitrogen, Carlsbad, CA) under reducing conditions. The antibody gels were stained directly with Sypro Ruby or Coomassie blue and visualized by imaging on UVP EC-3 imager (UVP Bioimaging Systems, Upland, CA), while the gels of parasite and cell extracts were blotted on to nitrocellulose membranes for western blot analysis. After verifying efficiency of the blotting by Ponceau S staining, the membranes were washed in tris-buffered saline (TBS) buffer (10 mM Tris, pH 7.4, 150 mM NaCl) and blocked by incubation in 5% BSA solution in TBS for 1 h at room temperature. The membranes were washed 4× for 10 min/wash with Tween 20-TBS buffer (TBS; 10 mM Tris, pH 8.0, 300 mM NaCl, 0.3% Tween-20) and incubated at room temperature with 20 µg/mL of mAb F8A1.1 or anti-CD15 in dilution solution (20 mM Tris, pH 7.4, 150 mM NaCl, 1% BSA, 0.3% Tween-20) for 30 min. The membranes were washed 4× with TBS containing 0.5% normal goat serum and incubated with 1:20,000 dilution of horseradish-conjugated goat anti-mouse IgG in dilution solution containing 5% normal goat serum and 1% BSA for 1 h at room temperature. The membranes were washed 3× with TTBS as described above and the reactive bands were visualized by incubating the nitrocellulose membrane in SuperSignal chemiluminescence substrate (Pierce, Rockford, IL) for 10 min followed by imaging on a UVP EC-3 imager (UVP Bioimaging systems, Upland, CA).

Biotinylation and immunoprecipitation of glycoproteins from extracts of HL-60 cells and *S. mansoni* cercariae using mAb F8A1.1
To prepare biotinylated cells, intact HL-60 cells were biotinylated with sulfo-NHS-biotin according to the manufacturer’s instructions. Briefly 2×10^7 HL-60 cells were suspended in 1 mL of PBS, mixed with 1.0 mg of sulfo-NHS-Biotin and incubated at room temperature for 30 min. The cells were washed 3× with PBS and excess biotin was quenched by a final wash with PBS containing 100 mM glycine. The HL-60 cells were once again washed 3× with PBS and suspended in PBS. Protease inhibitor cocktail was added to the cell suspension to 1× concentration and detergent extract of the biotinylated HL-60 cells was prepared as described above. Protein content of the biotinylated HL-60 extract was determined by BCA assay. Biotinylation of *S. mansoni* cercariae was carried out by treating 2 mg of detergent extract of cercariae in PBS with 27 µmol of sulfo-NHS-Biotin using instructions provided by the manufacturer. The parasite extract was dialyzed against PBS containing 1mM benzamidine to remove excess biotin and the protein content was quantified by BCA assay. For immunoprecipitation, biotinylated HL-60 cell and cercariae extracts were immunoprecipitated with protein A conjugated-Dynabeads (Invitrogen, Carlsbad, CA).
according to instructions provided by the manufacturer. mAb F8A1.1 (~20 µg) in PBS was incubated with protein A coated magnetic beads at room temperature for 15 min to capture the antibodies. The beads were pulled with a magnet and unbound antibody was removed. The beads were washed 3× with PBS/0.02% Tween-20 to remove residual unbound antibody and incubated with 250 µg of biotinylated or non-biotinylated HL-60 cell extract or 80 µg of biotinylated or nonbiotinylated cercariae extracts at room temperature for 15 min. The resulting immune complexes were pulled down with a magnet and unbound extracts were removed. The beads were washed 3× with PBS to remove any traces of extract. Reducing SDS-PAGE sample buffer was added and the beads were boiled for 10 min to dissociate the immune complexes. The released glycoproteins were recovered for analysis. As controls, immunoprecipitations were also carried out using total mouse IgG (Sigma-Aldrich, St. Louis, MO). For panels in Figure 5, detergent extracts of S. mansoni cercariae were biotinylated or mock biotinylated and dialyzed against PBS to remove free biotin. Approximately 20 µg of F8A1.1 was incubated with protein A-coated magnetic beads to capture the antibody. The beads were separated and washed to remove unbound antibody and incubated with 80 µg of biotinylated or nonbiotinylated cercariae extracts and the immune complexes were pulled down with a magnet according to the manufacturer’s instructions. The beads were washed and boiled in SDS–PAGE sample buffer and the released glycoproteins were recovered for analysis. As controls, immunoprecipitations were carried out with mouse IgG.

Analysis of immunoprecipitated glycoproteins

The recovered immunoprecipitated glycoproteins were separated by SDS–PAGE on 4–20% acrylamide gradient gel and transferred onto nitrocellulose membrane as described above. The membrane was blocked in 3% BSA/TBS for 1 h at room temperature, washed 3× with TBBS wash buffer and incubated with peroxidase-conjugated streptavidin in dilution solution of TBS/0.3% Tween-20/1% BSA for 30 min at room temperature. The membrane was washed 3× with TTBS and the glycoprotein bands were visualized by incubation with Immun-Star chemiluminescent substrate for 2 min and imaging on a UVP EC-3 imager.

Immunocytological staining with mAbs

Untreated, neuraminidase treated or mock treated HL-60 or Jurkat Cells were incubated on ice with 10 µg/mL solution of mAb F8A1.1 or 2.5 µg/mL solution of anti-CD15 (Becton-Dickinson, Franklin Lake, NJ) diluted in Hanks buffer/1% BSA for 1 h. The cells were washed 4× with cold Hanks buffer remove unbound mAbs and incubated on ice with 1: 1000 dilution of Alexa fluor 488-labeled goat anti-mouse IgG in Hanks/BSA for 1 h. The cells were washed 4× with cold Hanks buffer and analyzed on a flow cytometer (Becton-Dickinson, Franklin Lake, NJ). As controls, cells were incubated in Hanks/1% BSA solution without mAbs followed by incubation with Alexa fluor 488-labeled goat anti-mouse IgG and analysis by flow cytometry. Adult S. mansoni used for immunostaining were incubated in Iscoves modified DMEM supplemented with 20% FBS at 37°C for 24 h in 5% CO₂ atmosphere after recovery from mice and washed 4× with Hanks buffer before being used for histological staining. Cercariae were chilled on ice to cause them to sediment and washed 4× with cold water before staining. Freshly transformed schistosomula (3-h old) were prepared by repeated passage through two needles connected by a 3-way stopper in Hanks buffer as described previously (Nyame et al. 2000). The intact cercariae, schistosomula or adult S. mansoni were incubated with 10 µg/mL mAb F8A1.1 in Hanks/1% BSA for 1 h at 4°C and washed 4× with cold Hanks buffer. As controls, a set of the parasites were incubated in Hanks/1% BSA solution without mAb F8A1.1, washed as described above and incubated with 10 µg/mL of Alexa 488-labeled goat anti-mouse IgG in Hanks/1% BSA for 1 h at 4°C. The parasites were washed 4× with cold Hanks buffer to remove unbound excess antibodies and imaged on a Zeiss 710 confocal microscope.

Lectin staining

Intact cercariae, schistosomula and adult S. mansoni were stained with AAL by incubation in a solution of 5 µg/mL biotinylated AAL in Hanks buffer/1% BSA for 1 h and at 4°C and washed 4× with cold Hanks buffer to remove unbound lectin. Bound lectin was detected by incubation in a 5 µg/mL solution of Alexa-488 conjugated streptavidin in Hanks buffer/1% BSA at 4°C for 30 min. The parasites were washed 4× with cold Hanks buffer and imaged on a Zeiss 710 confocal microscope.

Glycolipid extraction and separation by TLC

Total glycolipids from schistosome eggs, adult S. mansoni, HL-60 and Jurkat cells were extracted using a modification of published protocols (Makaaru et al. 1992; Smith and Prieto 2001). Briefly, schistosome eggs, and adult parasites (1 g wet weight) or 0.5 mL packed cell volume of HL-60 or Jurkat cells were suspended in minimal volume of water and homogenized using a Branson sonifier. Methanol and chloroform were added sequentially to the homogenate to a final composition of 4: 8: 3 chloroform:methanol:water. The solvents were added sequentially and the samples were sonicated for 1 min each time a solvent was added. The samples were centrifuged at 8000 × g for 10 min and the supernatant fraction containing total glycolipids was collected. Folch extraction was conducted by mixing the total glycolipid fraction with 0.17 volumes of water followed by centrifugation at 8000 × g for 10 min. The upper phase and lower phase glycolipids were collected into separate tubes. The upper phase fractions were desalted over C18 columns (4 g) essentially as described (Makaaru et al. 1992). The upper and lower phase fractions were transferred into conical bottom Pyrex tubes and dried in centrifuge evaporator. The approximate weight of the dried glycolipids was determined by subtracting the weight of the empty Pyrex tube from the weight of the tube with the dried glycolipid residue. The residues containing total unfractionated upper and lower glycolipids were dissolved in chloroform:methanol (2:1) and analyzed by TLC. Approximately 40 µg of total upper phase and lower phase glycolipids from eggs and adult schistosomes and 20 µg of total upper and lower phase glycolipids of HL-60 and Jurkat cells were spotted on high performance TLC plates (Calbiochem, San
Diego, CA) and subjected to separation by TLC. The lower phase glycolipid fractions were developed in a solvent system of chloroform/methanol/0.2M KCl (60:35:8). The upper phase glycolipids fraction from schistosomes was analyzed in solvent system of chloroform/methanol/0.2 M KCl (50:40:10), while the upper phase fractions from cells were analyzed in a solvent system of chloroform/methanol/0.2 M KCl (60:35:8). Bovine brain gangliosides were analyzed as standards. The chromatograms were ran in duplicate over a period of 30 min and one set of TLC plates were stained with 0.1% orcinol in 5% sulfuric acid to visualize the glycolipid bands and the other set were immunostained with mAb F8A1.1 as described below.

Immuno staining of glycolipids on TLC plates

The TLC plates were dried after the chromatographic separation and soaked in 0.5% polyisobutylmethacrylate in acetone for 1 min to coat the plate with the resin. The plate was dried completely in air and blocked by incubation with a solution of 3% BSA in PBS for 1 h and incubated with 10 µg/mL solution of mAb F8A1.1 in BSA in PBS/1% for 2 h. The plates were washed five times with PBS and incubated with 1:10,000 dilution of goat anti-mouse IgG-HRP conjugate secondary antibody for 1 h. The plate was incubated with SuperSignal chemiluminescence substrate (Pierce, Rockford, IL) for 30 s and the plate was exposed to X-ray film (Fisher Scientific) to reveal reactive glycolipid bands.

Analysis of specificity of mAbs on the defined glycan array of the CFG

mAbs F8A1.1 (50 µg/mL) or anti-CD15 IgG1 mAb (50 µg/mL) were diluted in TSM binding buffer containing 1% BSA (Boval Co.) and incubated with an array of 610 glycan structures (version 5.1) printed on activated glass slides and after washing away any unbound antibody, the bound antibody was detected with Alexa fluor-488 labeled goat anti-mouse IgG, as described previously (Heimburg-Molinaro et al. 2011) and by the CFG (www.functionalglycomics.org). The relative fluorescent units (RFU) of the bound antibody-glycan complexes were detected on Perkin-Elmer ProScanArray 4 laser scanner and quantified using ImaGene software (Biodiscovery, El Segundo, CA).

Supplementary data

Supplementary data for this article is available online at http://glycob.oxfordjournals.org/.

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Conflict of interest

None declared.

Abbreviations

AAL, Aleuria aurantia lectin; BCA, Bicinchoninic acid; CFG, Consortium for Functional Glycomics; DC-SIGN, dendritic cell-specific intercellular adhesion molecule-3 (ICAM-3)-grabbing nonintegrin; DMEM, Dulbecco’s modified Eagle’s medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; Fuc, fucose; Galβ, Galactose-β; GlcNAcβ, N-acetylgalactosamine-β; HRP, horseradish peroxidase; KLH, keyhole limpet hemocyanin; LDN, LacdiNAc; LDNF, fucosylated LacdiNAc; LDNFPII-BSA, lacdiNAc fucopentaose-BSA; LDNT, LacdiNAc-tetraose; Le², Lewis x antigen; LN, N-acetyllactosamine, Galβ1-4GlcNAc-R; LNDFHII-BSA, lacto-N-difucohexaose I-BSA; LNFPIII-BSA, lacto-N-fucopentaose II-BSA; LNFPIII-BSA, lacto-N-fucopentaose III-BSA; LNNT-BSA, lacto-N-neotetraose-BSA; mAbs, monoclonal antibodies; M, Melanoma; MOPS, 3-(N-morpholino)propanesulfonic acid; polyLe², poly-Lewis x; polyLN, poly-N-acetyllactosamine; RFU, relative fluorescent units; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; SFFM, serum free media; SSA-1, stage-specific embryonic antigen-1; TBS, Tris-buffered saline; TTBS, Tween20-TBS.

References


