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Infection of mammals by the parasitic helminth *Schistosoma mansoni* induces antibodies to glycan antigens in worms and eggs, but the differential nature of the immune response among infected mammals is poorly understood. To better define these responses, we used a shotgun glycomics approach in which N-glycans from schistosome egg glycoproteins were prepared, derivatized, separated, and used to generate an egg shotgun glycan microarray. This array was interrogated with sera from infected mice, rhesus monkeys, and humans and with glycan-binding proteins and antibodies to gather information about the structures of antigenic glycans, which also were analyzed by mass spectrometry. A major glycan antigen targeted by IgG from different infected species is the FLDNF epitope [Fucα3GalNAcβ4(Fucα3)GlcNAc-R], which is also recognized by the IgG monoclonal antibody F2D2. The FLDNF antigen is expressed by all life stages of the parasite in mammalian hosts, and F2D2 can kill schistosomulum in vitro in a complement-dependent manner. Different antisera also recognized other glycan determinants, including core β-xylene and highly fucosylated glycans. Thus, the natural shotgun glycan microarray of schistosome eggs is useful in identifying antigenic glycans and in developing new anti-glycan reagents that may have diagnostic applications and contribute to developing new vaccines against schistosomiasis.

Schistosomiasis is a major health problem in tropical and subtropical areas where it is endemic, with more than 200 million people actively infected and 800 million at risk of contracting the disease (1–3). Current treatment for disease is limited to the drug praziquantel (4), but cases of drug resistance have been reported (5). Decades of research on schistosomiasis vaccines have yielded only two candidates for clinical trials, and no encouraging results have been published yet (6–9). Thus, there is an urgent need to develop more sensitive diagnostic methods and to identify new vaccine candidates.

Recent studies have shown that a major part of the host immune response to infection is directed against carbohydrate (glycan) antigens in glycoproteins and glycolipids (10–17). A wide variety of unusual antigenic determinants include glycans containing the LDN, fucosylated LDN sequences (LDNF, LDN-dF, FLDNF), Lewis X (Le³), poly-Le³, core α3 fucose, and core β2 xylene structures (Fig. 1) (11, 14, 17), many of which are expressed by all developmental stages of schistosomules (18). Interestingly, monoclonal antibodies (MABs) specific to these glycans recognize these antigens on the surface of 3-h-old schistosomules, and some anti-glycan antibodies can mediate killing in vitro in a complement-dependent fashion (18–21). *Schistosoma mansoni*-infected rhesus monkeys, which are known to self-cure after infection, have IgG to many glycan antigens, including Le³, LDN, LDNF, core fucose, and core xylene determinants, and their sera are effective in complement-mediated cytolsis of cells expressing Le³ as well as schistosomulum larvae in vitro (22–24). However, it is unknown whether anti-glycan antibodies contribute to parasite killing by animal sera in vivo.

The induced humoral responses in infected animals and people generally are proportional to the severity of the disease and consist of many different antibody classes and antibody titers to glycan antigens (24); reviewed in references 11 and 17). *S. mansoni*-infected patients with either intestinal or hepatosplenic disease generate mainly IgM, but also produce IgG and IgA, to LDN, LDNF, and Le³ (18). Antibodies to LDN-dF and FLDNF, among other glycan structures both known and unknown, also were found in infected individuals, with higher titers observed in children than in adults (25, 26). Therefore, although the role of these glycans and the significance of anti-glycan antibodies in schistosomiasis are not known, glycan antigens may be used as diagnostic tools and/or inform upon new vaccine candidates.

Several studies have focused on defining the schistosome gly-
come by employing high-resolution liquid chromatography and mass spectrometry (MS) techniques (27, 28). These global profiling studies have confirmed the major glycan structures previously identified and provided new information on the diversity of glycans and the heterogeneity found among the life stages of the parasite and their secretions (27, 29–31). Several studies also have demonstrated the feasibility of profiling schistosome glycans using various microarray printing technologies and probing with glycan-binding proteins, antibodies, and antisera, demonstrating that such techniques can be powerful tools for profiling the immune response to this disease (26, 32–34) and the development or characterization of novel anti-glycan reagents.

The combination of these techniques, i.e., glycomics and/or glycan structural profiling, which is guided by the use of immunologically relevant reagents, such as antisera and MAbs, has great potential to improve our understanding of the anti-glycan response and uncover novel, immunologically relevant diagnostic and vaccine candidates. We recently found that there were both similarities and substantial differences in the specificity, titers, and isotype composition of anti-glycan antibodies among rhesus monkeys (naturally protected hosts) as well as humans and mice (chronically infected hosts) based on a small collection of synthetic, schistosome-type biantennary N-glycans (24). In the present study, utilizing methods for fluorescent covalent tagging, separation, and printing of glycans (35, 36), we generated a natural N-glycan microarray from S. mansoni egg glycoproteins. By interrogating these arrays with sera from different species infected with S. mansoni, we identified immunologically relevant fractions for further characterization. Using a combination of mass spectrometry and lectin and antibody binding studies (metadata-assisted glycan sequencing [MAGS]) (37, 38), we have identified several glycan structures that represent the egg N-glycans most prominently recognized by these infected hosts. The shotgun glycan microarray led us to focus on one MAb, F2D2, which targets FLDNF and replicates the binding pattern of infected hosts. This antibody binds all stages of S. mansoni and mediates the killing of schistosomula in vitro. The results demonstrate the power of incorporating glycomics and immunology to identify disease-relevant glycan antigens.

MATERIALS AND METHODS

Materials. All chemicals and glycoproteins were purchased from Sigma-Aldrich (St. Louis, MO) and used without further purification. High-performance liquid chromatography (HPLC) solvents were purchased from Thermo Fisher Scientific (Waltham, MA). PNGase F was purchased from New England Biolabs (Ipswich, MA), and PNGase A was from...
Sigma-Aldrich. Plant lectins were purchased from Vector Laboratories (Burlingame, CA), including Phaseolus vulgaris agglutinin L (PHA-L), Phaseolus vulgaris agglutinin E (PHA-E), peanut agglutinin (PNA), Wisteria floribunda agglutinin (WFA), wheat germ agglutinin (WGA), Lotus tetragonolobus lectin (LTL), Aleuria aurantia lectin (AAL), Sambucus nigra agglutinin (SNA), Ricinus communis agglutinin I (RCA I), concanavalin A (ConA), Ulex europaeus I (UEA I), Maackia amurensis agglutinin (MAA), Bauhinia purpurea lectin (BPL), and Griffonia simplicifolia lectin-I (GSI-B4). The lectin HPA (Helix pomatia agglutinin) was purchased from Sigma-Aldrich. An anti-FLD (IgG, clone Y1H5), an anti-LDNF (IgG, clone L6EB), an anti-Le" (IgM, clone 5F1), two anti-LDNF-DF (IgM, clone 290-29D-9A; IgM, clone 290-4A8), and an anti-Man/GlcNAc2 (M3GN2; IgM, clone 100-4G11-A) MAb, as well as an anti-FNDF antibody (IgG, clone F2D2), were developed as MAbS by the production of hybridomas from spleens of mice that had been infected with S. mansoni by using methods described previously (16, 39–42). The anti-herorashidase peroxidase (HRP) antibody (anti-peroxidase, polyclonal rabbit IgG; P3709) was purchased from Sigma-Aldrich and used to detect core α3 fucose and core xylose (CE/EX) determinants (43, 44). The secondary antibody conjugates goat anti-human, anti-mouse, anti-rabbit (Alexa-488 anti-human IgM, Alexa-555 anti-human IgG, Alexa-488 anti-mouse IgM, Alexa-555 anti-mouse IgG, Alexa-633 anti-mouse IgG, and Alexa-488 anti-rabbit IgG), and Cy5-streptavidin were from Invitrogen (Carlsbad, CA). Goat anti-mouse HRP-conjugated secondary antibody was purchased from KPL (Gaithersburg, MD). SuperSignal West chemiluminescent substrates were purchased from Thermo Scientific (Rockford, IL). N-hydroxysuccinimide (NHS)-activated slides were purchased from Schott (Elmsford, NY). The printing of glycan arrays was performed using a Piezorray printer (PerkinElmer, Waltham, MA), and the analysis of hybridomas from spleens of mice that had been infected with S. mansoni by using methods described previously (16, 39–42). The anti-herorashidase peroxidase (HRP) antibody (anti-peroxidase, polyclonal rabbit IgG; P3709) was purchased from Sigma-Aldrich and used to detect core α3 fucose and core xylose (CE/EX) determinants (43, 44). The secondary antibody conjugates goat anti-human, anti-mouse, anti-rabbit (Alexa-488 anti-human IgM, Alexa-555 anti-human IgG, Alexa-488 anti-mouse IgM, Alexa-555 anti-mouse IgG, Alexa-633 anti-mouse IgG, and Alexa-488 anti-rabbit IgG), and Cy5-streptavidin were from Invitrogen (Carlsbad, CA). Goat anti-mouse HRP-conjugated secondary antibody was purchased from KPL (Gaithersburg, MD), and the analysis of glycan arrays was accomplished by scanning with a ProScanArray Scanner (PerkinElmer) equipped with 4 lasers. Percoll was purchased from GE Healthcare (Piscataway, NJ). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Cellgro (Manassas, VA), Penicillin-streptomycin was purchased from Gibco (Grand Island, NY). Standard guinea pig complement was purchased from Cedarlane (Burlington, NC). Fetal bovine serum was purchased from Atlanta Biologicals (Lawrenceville, GA). 4′,6-Diamidino-2-phenylindole (DAPI) was purchased from Life Technologies (Foster City, CA). Ninety-six-well microplates were purchased from Greiner Bio-One (Frickenhausen, Germany).

**Isolation of Schistosoma mansoni eggs and life stages.** S. mansoni eggs were obtained from livers of infected Swiss Webster female mice (n = 30) 7 weeks after infection with 150 cercariae per mouse. Livers were perfused with perfusion fluid (0.85% NaCl, 0.75% trisodium citrate dihydrate) and homogenized in 1.7% NaCl, and the eggs were collected by filtration over a series of mesh filters as described previously (45). Eggs were washed repeatedly by low-speed centrifugation to remove any liver tissue, and wet pellets of 1 to 2 g were stored frozen at −70°C.

S. mansoni-infected Biomphalaria glabrata snails, strain NMRI NR-21962, were provided by the Schistosome Research Reagent Resource Center through BEI Resources, NIAID, NIH. Snail maintenance, collection of cercariae, transformation to schistosomula, and isolation of adult worms from mice were conducted as previously described (34, 46). The schistosomula were cultured for 2 to 3 days in DMEM with 10% fetal bovine serum (FBS) and penicillin-streptomycin at a density of 500 to 1,000 organisms/ml in tissue culture dishes. All work with B. glabrata and S. mansoni was approved by the Emory University Office of Occupational Health and Safety and conducted in biosafety level II animal surgery facilities and laboratories in compliance with university-approved biosafety and IACUC protocols.

**Infected sera.** Mouse serum was obtained and pooled from Swiss Webster mice (n = 10) infected with ~60 cercariae of S. mansoni Puerto Rican strain after 7 weeks or 8 weeks and 20 weeks of infection under an approved IACUC protocol at Emory University. Rhesus sera were obtained from 4 monkeys (Chinese rhesus macaques, M. mulatta) infected percutaneously with 500 cercariae, and sera were collected during the course of infection at various time points from 8 to 78 weeks postinfection from an IACUC-approved study conducted at the Division of Parasitic Diseases and Malaria at the National Centers for Disease Control and Prevention (CDC). Infections were monitored by the examination of eggs in stool samples. Sera from infected humans, four nutritionally exposed Kenyan individuals (H-19, H-26, H-42, and H-59) who were employed as sand harvesters in the Lake Victoria area and five Peace Corp volunteers in Africa (H1 to H5), were provided by the CDC with approval by the ethical review boards of the Kenya Medical Research Institute.

**Schistosome egg glycan isolation and labeling.** S. mansoni eggs (1.0-g pellet) were resuspended in 4 ml H2O, homogenized using a glass homogenizer, and sonicated (6 pulses/10 s; Misonix 3000) in an ice bath. The glycolipids were extracted by the method of Folch et al. (47). Methanol was added to 2.7 volumes, and the sample was sonicated (2 pulses/10 s). Chloroform was added to 1.33 volumes, bringing the extract to 4:8:3 chloroform–methanol-water, and the extract was sonicated (2 pulses/10 s) and centrifuged at 6,000 × g for 10 min to separate the aqueous phase, containing glycolipids, from the pellet, containing the glycoproteins. The glycolipids were stored for other analyses, while the glycoprotein pellet was resuspended in 2 ml 0.2 M Tris-HCl, pH 8.2, and dried under vacuum to remove residual chloroform. The dried extract was resuspended in 2 ml of denaturing buffer (8 M guanidinium hydrochloride [GnHCl] in 0.2 M Tris-HCl, pH 8.2) and 2 ml of reducing buffer (0.18 M dithiothreitol [DTT] in 0.2 M Tris-HCl, 8 M GnHCl, pH 8.2) and incubated for 1 h at 25°C. Alkylation was done by the addition of 7.75 ml of 0.18 M iodoacetamide in 0.2 M Tris-HCl, 8 M GnHCl, pH 8.2, and incubated for 30 min at 25°C. The extract then was dialyzed against 8 liters of water using a 10,000-Da molecular-mass-limit membrane at 4°C overnight and freeze-dried under vacuum. The dried material then was resuspended in 3 ml of 50 mM phosphate buffer, pH 8.2, and digested with trypsin (tosylsulfonyl phenylalanyl chloromethyl ketone [TPCK] treated; Sigma) at 100 µg/ml for 12 h at 37°C.

N-glycans were released from tryptic glycopeptides by treatment with PNGase F. The 3-mL glycopeptide mixture first was boiled for 5 min to inactivate the trypsin and adjusted to pH 7.5, and 10 µl of PNGase F (500,000 U/ml; NEB) plus NaCNBH3 (0.2%) was added and incubated at 37°C for 24 h. After digestion, the solution was boiled for 5 min and passed through a C18 Sep-Pak cartridge (2 g) with 2 column volumes (CVs) of water, and the flowthrough was collected and passed through a 500-mg Carboxylate cartridge (Grace Discovery Science) to which free glycans bound. The Carboxylate column was washed with water (6 CVs), and the bound glycans were eluted with 3 CVs of 50% acetonitrile in 0.1% trifluoroacetic acid (TFA) and dried under vacuum before labeling. To release potential N-glycans that are resistant to PNGase F, as has been seen for N-glycans containing core α3-fucose residues, the glycopeptides retained in the C18 column were eluted with methanol (2 CVs), dried under vacuum, and digested with the glycosidase PNGase A. Digestion was performed by resuspending the dried glycan in 1.0 ml of 0.1 M citrate-phosphate buffer, pH 5.0, addition of 10 µl of PNGase A (from almonds, 60 U/µl), and incubation at 37°C for 24 h. The PNGase A-released glycans were purified over C18 Sep-Pak and Carboxylate columns as before and dried under vacuum before labeling.

The N-glycan preparations (PNGase A and F digested) were labeled with the bifunctional fluorescent linker 2-amino-N-(2-aminoethyl)-benzamidine (AEAB), which was synthesized in the laboratory and used as described previously (35). The dried glycans were resuspended in 0.1 ml of 0.35 M AEAB plus 0.1 ml of 1 M NaCNBH3 in dimethyl sulfoxide (DMSO)-acetic acid (7:3, vol/vol) and incubated at 65°C for 2 h. After cooling, 2 ml of acetonitrile was added, the sample incubated at −20°C for 30 min, and then it was centrifuged at 6,000 × g for 10 min. The pellets then were dissolved in 0.2 ml water for separation by HPLC (see Fig. S1 in the supplemental material).

**Separation of GAEABs by HPLC and mass spectrometry analysis.** HPLC separations were performed with a Shimadzu HPLC CBM-20A system coupled to a UV detector (set at 330 nm; SPD-20A) and a fluores-
cescence detector (set at 330 nm/420 nm excitation/emission; RF-10AxL). Both UV absorption and fluorescence intensity were used for the quantification of AEAB-labeled glycans (GAEABs) using LFNPIII-AEAB as a standard. The first-dimension (1D) HPLC separation was done by injection of 199 µl of sample into a reverse-phase C18 column (250 mm by 4.6 mm) with mobile phases consisting of acetonitrile and water with 1% TFA. The concentration of acetonitrile increased from 1% to 5% in 40 min and 5% to 20% in 15 min. Each peak fraction was collected, quantified by fluorescence, dried under vacuum, and resuspended to a final concentration of 200 mM before being used for microarray printing. In addition, each fraction was analyzed by matrix-assisted laser desorption ionization—time of flight (MALDI-TOF) mass spectrometry in an Ultraflex II system from Bruker Daltonics (Billerica, MA). GAEAB fractions obtained in the first-dimension HPLC and showing positive results by microarray when probed with lectins and sera were further separated in a second dimension (2D). The second-dimension HPLC was performed using a PGC column (150 mm by 4.6 mm; Thermo Scientific). The mobile phase was acetonitrile and water with 0.1% TFA, with the acetonitrile gradient increasing from 25% to 55% in 40 min. Fractions were collected, quantified by fluorescence, dried under vacuum, and resuspended in water to a final concentration of 100 mM before being used for microarray printing.

**Array printing, binding assays, and scanning.** Noncontact printing was performed using a Piezorray printer. All samples were printed in phosphate buffer (300 mM sodium phosphates, pH 8.5). The average spot volume was within 10% variation (intrapit) of 1/3 nanoliter. Each compound was printed at 100 mM and in replicates of six. After printing, slides were placed in a high-moisture chamber at 50°C and incubated for 1 h. The slides were washed and blocked with 50 mM ethanolamine in 0.1 M Tris base, pH 9.0, for 1 h, subsequently dried by centrifugation, and stored desiccated at −20°C. Before assay, the slides were rehydrated for 5 min in TSM buffer (20 mM Tris-HCl, 150 mM NaCl, 2 mM CaCl₂, and 2 mM MgCl₂). Biotinylated lectins and MAbs were used in binding assays to validate each slide for printing efficiency using control GAEABs and for the characterization of schistosome GAEABs. Microarray slides first were assembled in a 16-chamber cassette and washed for 5 min with 200 µl/well TSM buffer plus 0.05% Tween 20. Lectins were added at 10 µg/ml in TSM binding buffer (TSM buffer plus 0.05% Tween 20, 1% bovine serum albumin [BSA]) and incubated for 1 h at 25°C, and the slides were washed 3 times for 5 min each in TSM wash buffer (TSM plus 0.05% Tween 20). Slides then were washed 4 times in TSM, Cy5-SA was added at 5 µg/ml, and the slides were incubated for 1 h at 25°C in the dark. MAbs and polyclonal antibodies were added at 0.125 to 20 µg/ml in TSM binding buffer or as unlabeled hybridoma supernatant and incubated for 1 h at 25°C, and the slides were washed 3 times for 5 min each in TSM wash buffer. Appropriate fluorescently labeled secondary antibodies (Alexa-488 anti-mouse IgM, Alexa-633 anti-mouse IgG, and Alexa-488 anti-rabbit IgG) were added at 2.5 to 5 µg/ml and incubated for 1 h at 25°C in the dark. After a final incubation, the slides were washed 4 times in TSM wash buffer and once in water, dried by centrifugation, and scanned. For serum screening, 200 µl of serum diluted in TSM binding buffer (1:100 dilution mouse sera, 1:1,000 dilution for rhesus and human sera) was added per well, and the slides were incubated for 1 h at 25°C. Slides were washed 3 times for 5 min each in TSM wash buffer, and fluorescently labeled secondary antibody (Alexa-488 anti-mouse IgM, Alexa-555 anti-mouse IgG, or Alexa-633 anti-mouse IgG) was added at 5 µg/ml in TSM binding buffer for 1 h at 25°C. Following 4 washes in TSM, a rinse in water, and drying by centrifugation, the slides were scanned.

For multipanel experiments on a single slide, the array layout was designed using Piezorray software according to the dimension of a standard 16-chamber adaptor. The adaptor was affixed to the slide to separate a single slide into 16 chambers sealed from each other during the assay. The slides were scanned with a PerkinElmer ProScanArray microarray scanner equipped with 4 lasers spanning an excitation range from 488 to 647 nm. The scanned images were analyzed with ScanArray Express software, and basic statistical methods were applied. Fluorescence scanning for Cy5 was at 649 nm/670 nm (excitation/emission) and as specified for Alexa-488- and Alexa-555-labeled antibodies.

**Parasite lysates, SDS-PAGE, and Western blotting.** Cercariae, schistosomula, adult worms, and eggs were isolated and processed for parasite life-stage lysates or soluble egg antigen (SEA) as previously described (34, 48). For SDS-PAGE and Western blotting, 1 µg of SEA and 5 µg parasite lysates were boiled in 1X NuPAGE SDS sample buffer plus 2.5% β-mercaptoethanol for 10 min and then run in 10-well Mini-Protein-TGX gels at 200 V for 30 min with broad-range protein standards (Spectra multicolor). Protein was transferred to a nitrocellulose membrane using a Trans-Blot Turbo semidyv transfer system. Unless noted otherwise, all subsequent incubations and washes included shaking at ambient temperature. Membranes were blocked for 1 h in 5% (wt/vol) BSA (fraction V) in TBS wash buffer (20 mM Tris, 300 mM NaCl, 0.05% Tween 20). Incubations with F2D2 antibody were performed overnight at 4°C at 1 µg/ml in BSA diluent (0.5 to 1% BSA in TBS wash buffer). The membranes then were washed three times for 10 min each in TBS wash buffer. A secondary detection antibody, HRP-conjugated goat anti-mouse-IgG, was added for 1 h at 1:20,000 in TBS wash buffer. The same wash procedure was repeated, and then SuperSignal West Pico chemiluminescent substrate was added for 30 s. The membranes were dabbed dry and exposed to film.

**Immunofluorescence imaging.** Intact cercariae, schistosomula, and adult worms were fixed in 10% neutral buffered formalin for 24 h at 4°C. All subsequent incubations and washes were performed at ambient temperatures with gentle shaking. Parasites were washed 4 times with phosphate-buffered saline (PBS) and blocked with 3% BSA in PBS for 1 h. F2D2 antibody was added at 10 µg/ml and incubated for an additional 1 h. As controls, a set of the parasites was incubated with an isotype control mouse IgG antibody. Samples were washed four times in PBS and a secondary antibody, Alexa-488-labeled goat anti-mouse IgG, diluted 1:100 in 3% BSA in PBS for 1 h. The parasites were washed 4 times with PBS to remove unbound excess antibodies and set on glass slides in mounting medium. Slides were imaged at ×20 magnification.

**Schistosomulum killing assay.** Schistosomula were isolated as described previously (34, 48). After vortex transformation, separation from tails via Percoll, and washing in DMEM, the newly transformed schistosomula were cultured for 3 h at 37°C in 96-well plates with 50 to 100 parasites in 40 µl of DMEM with penicillin-streptomycin in each well. The specified antibodies then were added (diluted in 12 µl DMEM). At 3.5 h, 15 µl of freshly thawed, active, or heat-inactivated (1 h at 55°C) guinea pig complement was added (final concentration, 1:5), followed by 7.5 µl fetal bovine serum (final concentration 10%) and penicillin-streptomycin to a final concentration of 100 U/ml. Each antibody and killing condition was assayed in duplicate or triplicate wells within each assay. The total number of live schistosomula was counted at 0 h. At approximately 22 h, 1 µl of 100 µg/ml DAPI stain was added to each well. Dead schistosomula were counted as DAPI positive at 24, 48, and 72 h.

**RESULTS**

**Preparation of N-glycans from Schistosoma mansoni eggs.** We prepared N-glycans from schistosome eggs for initial HPLC separation (termed 1D) in order to identify potential immunologically relevant fractions for further study. The overall workflow for the release, conjugation, separation, and glycan microarray analysis of N-glycans from *S. mansoni* eggs is shown in Fig. 2 and is described in Materials and Methods. N-glycans were released by consecutive treatments with PNGase F and PNGase A based on previous studies showing the presence of core α3-fucose in schistosome egg glycans (27–29, 49). PNGase F treatment releases N-glycans that lack α3-fucosylation in the chitobiosyl core, but susceptible glycans may be α6-fucosylated and β-xyllosylated in their cores. Successive treatment with PNGase A can efficiently release
core α3-fucosylated N-glycans (50) and any residual N-glycans if the first digestion is incomplete. The N-glycans released by PNGases F and A were enriched not by binding to C18 Sep-Pak but by binding to Carbograph cartridges and then were conjugated by reductive amination to the bifunctional fluorescent linker AEAB, which permits the direct detection and quantitation of total glycan amounts (35, 36). The first-dimension separation was performed in a C18 reverse-phase column with an acetonitrile gradient, and the elution was monitored by fluorescence.

HPLC purification showed 20 major PNGase F-released glycan peaks, designated fractions F1 to F20 (see Fig. S2A in the supplemental material), and 14 PNGase A-released peaks, designated fractions A1 to A14 (see Fig. S2B). Each collected fraction was quantified by absorbance at 330 nm, and the area under each peak was used for determining the total concentration of glycans released and purified. From 1.1 g of egg pellet processed, the total amount of N-glycans recovered after separation by HPLC was 321 nmol released by PNGase F and 85 nmol released by PNGase A, on the order of 0.01 to 0.1% of the total material by weight. Each peak fraction was printed onto NHS-activated glass slides at 100 μM in the master plate. Since none of the glycans were identified prior to printing on slides at equal concentrations, the microarray was termed a schistosome shotgun glycan microarray (SSGM) and represents the 1st dimension (1D-SSGM).

Microarray slides consisting of the fractions F1 to F20 and A1 to A14 and of negative controls (PBS and biotin) were printed with hexareplicate samples. The 1D-SSGM slides were incubated with a panel of 13 different lectins of known specificity to establish features of the glycan determinants and validate glycan printing. As shown in Fig. S3A in the supplemental material, most PNGase F-released GAEABs were recognized by the fucose-binding lectin AAL (51), with the most prominent binding in fractions F7 to F15. Most fractions also were recognized by the mannose-binding lectin ConA, which binds high mannose-, hybrid-, and biantennary complex-type N-glycans (52, 53), with the strongest binding in fractions F5 to F14. Lectins that bound select fractions at lower levels included WGA, which recognizes GlcNAc and N-acetyl-5-neuraminic acid (F1, F3, F11, F15, and F18), WFA, which recognizes terminal α/β-linked GalNAc (F18), and PHA-E and MAA, which recognize bisedected, terminal galactosylated N-glycans and α2,3-linked sialic acids, respectively (F14). PNGase A-released GAEABs fractions A3 to A9 also were recognized by AAL, and fractions A6 and A8 were moderately recognized by ConA (see Fig. S3B). Fractions F19, F20, and A14, which are the last fractions eluted from the C18 column, showed low levels of binding to all lectins tested. Aside from F19, F20, and A14, the lectin binding studies showed background levels or only weak binding to egg glycans with the lectins PHA-L, PNA, HPA, SNA, UEA-I, LTL, and RCA-I, which recognize various determinants, such as branched and galactosylated N-glycans, blood group H, and terminal sialic acid. This was anticipated, since such epitopes are not characteristic of schistosome glycans but can occur in mouse-derived glycans (37, 54), which is of concern since the eggs are prepared from infected mouse organs. Thus, the results indicate that there was not appreciable contamination of the SSGM with mouse-derived glycans and that the schistosome GAEABs separated by the 1D HPLC were recognized primarily by mannose- and fucose-binding lectins. The minimally purified glycans at this stage represent the PNGase F/A-releasable N-glycome and are consistent with previous studies showing the presence of a variety...
of fucosylated and mannosylated N-glycans in schistosome eggs, miracidia, and egg secretions (27–29, 49). The development of this array indicates that our methods of isolation and tagging of glycans generate a glycan library that is representative of the N-glycome of *S. mansoni*, and its interrogation can point to immunologically relevant fractions.

Identification of antigenic egg N-glycan fractions for further purification. The 1D-SSGM was interrogated with representative sera from acutely and chronically infected mice (pooled), one rhesus monkey, and one human infected with *S. mansoni* to explore whether specific GAEAB fractions might be immunologically relevant (Fig. 3). As shown in Fig. 3A, unexpectedly we found very specific recognition of only a few glycan fractions. For example, only F7 to F13 were recognized by IgG in mouse sera infected for 20 weeks, and only F11 was recognized by IgG in mouse sera infected for 7 weeks. F7 to F13 also were strongly recognized by IgG in serum from an infected rhesus monkey 8 weeks postinfection, and binding declined by 78 weeks after infection (Fig. 3B), at which point the monkeys are considered self-cured, since they showed no detectable eggs in the stool. Serum from an infected human showed a strong IgG response to F7 to F13 and F15, with slightly lower levels of reactivity to A13, F1, F3, and F18. IgG binding to most of these fractions was higher before treatment with praziquantel than after treatment (Fig. 3C). These results demonstrate that F7 to F13 contain natural egg glycan epitopes, predominantly characterized by AAL and ConA binding, which are immunologically relevant during infection in mice, rhesus monkeys, and humans. They could be relevant as antigenic targets for protective antibodies or immune-modulatory molecules or involved in host-pathogen interactions. Based on these data, we chose these fractions for further purification and analysis.

Preparation and characterization of 2D-SSGM. F7 to F13 were further separated in a second dimension by HPLC using a reverse-phase PGC column, and each fraction contained 7 to
11 peaks for a total of 60 fractions (see Fig. S4 in the supplemental material). Fractions appearing to contain one or a few major GAEAs, or coeluting GAEAs, were collected and quantified. Fifteen glycan fractions that contained enough material were brought to H926 and printed on the 2-dimensional SSGM (2D-SSGM). These fractions, along with parent 1D fractions (F7-0, F10-0, F11-0, and F13-0) and 4 controls [LNnT, Galβ4GlcNAcβ3Galβ4Glc-; Man5GlcNAc2, Manα6(Mano3)Manα6(Mano3)Manβ-4GlcNAcβ4GlcNAcβ-; asialo-bGP (NA2), Galβ4GlcNAcβ2Manα3(Galβ4GlcNAcβ2Manα6)Manβ-4GlcNAcβ4GlcNAcβ-; biotin] then were interrogated with a panel of lectins and several previously characterized MAbs and polyclonal antibodies (Fig. 1 and 4). Most glycan fractions were strongly recognized by ConA and AAL (Fig. 4A and B). Several fractions (F9-4, F11-0, F11-4, F11-5, and F11-6) also were recognized by GlcNAc-binding lectin WGA (55, 56) and GalNAc-binding lectin BPL (57), while some (F7-4, F7-5, F9-4, F11-4, and F11-6) were recognized by the β-galactose-binding lectin RCA-I (58, 59). The controls were recognized as predicted by RCA-I, ConA, BPL, and WFA (60–62). The lectins PHA-L, UEA-I, LTL, PNA, GSI-B4, MAA, and HPA showed no significant reactivity in the further separated egg glycans. Therefore, the separation of GAEAs by 2D HPLC allowed the isolation of immunologically relevant glycans with unique lectin binding properties, some of which were not seen in the 1D-separated glycans, probably because the relevant glycans were not highly abundant in the mixture.

The array next was interrogated with defined anti-glycan
MAbs and polyclonal antibodies (Fig. 4C). We screened with six mouse MAbs derived from schistosome-infected mice, referred to as anti-LDNF (clone L6B8), anti-LDN (clone Y1H5), anti-LeX (clone 5F1), anti-M3GN2 (clone 100-4g11-A), and two anti-LDN-dF (clones 29D-2D9-A and 29A-4A8) MAbs (34, 39–42).

Anti-LDNF bound fractions F9-4 and F11-4. Interestingly, the anti-LDN, both anti-LDN-dF, and the anti-LeX MAb, the latter of which identifies both terminal and internal LeX structures, did not bind glycans on this array. Recent glycomics profiling has shown that PNGase F-releasable egg glycans do contain the minimal determinants for LDN, LeX, and LTDN-dF (31). However, our studies show that the anti-LeX, anti-LDN, and anti-LDNF antibodies prefer these determinants on extended chains or noncomplex-type N-glycan cores, which may explain the lack of binding to the 2D-SSGM (34, 42). In addition, some of the glycans recognized by these MAbs may not occur at significant levels in egg glycoproteins. The anti-M3GN2 antibody (clone 100-4g11-A) recognized fractions F11-0, F11-4, F11-6, and F13-6. In addition to the MAbs generated from infected mice, we also screened with a commercial anti-HRP antibody produced in rabbits (P7899), which is directed against core β-xylene and core α3-fucose determinants (43, 44). The specificity of P7899 for core β-xylene and core α3-fucose determinants also was confirmed in our hands using defined glycan microarrays. On the 2D-SSGM, P7899 bound F10-4, F11-0, F11-5, F13-0, F13-4, and F13-7. These data indicate that the 2D-SSGM contains N-glycans containing terminal fucose, galactose, GalNAc, and GlcNAc, likely forming LDNF, core β-xylene, and truncated Man3 determinants, as well as many yet-undefined fucosylated N-glycan antigens. The array therefore represents a useful tool for discovering novel targets of anti-glycan immunity from a natural library of parasite glycans.

Anti-FLDNF antibody shares a common binding pattern with sera from S. mansoni-infected animals and humans on 2D-SSGM. The 2D-SSGM was interrogated with pooled sera from infected mice (acute versus chronic), sera from 4 infected rhesus monkeys (patent versus postpatent period), and sera from 9 infected individuals (Fig. 5A to C). Results shown in Fig. 5A demonstrate that infected mice have IgG at 8 and 20 weeks postinfection that recognized glycan fractions F9-4, F11-4, and F11-5. Notably, F11-5 was highly recognized by serum at 20 weeks postinfection. In rhesus monkeys (Fig. 5B), relatively high titers of IgGs were found against most glycans (F9-4, F9-5, F10-4, F11-0, F11-4, F11-5, and F13-4) with sera obtained during the patent period at 8 weeks postinfection. However, significant variability was observed among the 4 infected monkeys, with rhesus 1 and 3 always showing the highest response to these glycans. In humans, the 9 individuals tested showed generally lower levels of anti-glycan antibodies than rhesus monkeys, with two individuals (H-2 and H-4) showing the strongest binding for IgG in sera with fractions F9-4, F11-4, F11-5, and F13-4 (Fig. 5C). These results show that mice, rhesus monkeys, and humans differentially recognize the various egg glycans in a pattern that is strikingly similar among the three hosts, but importantly, glycans fractions F9-4, F11-4, and F11-5 were the most highly recognized by IgG antibodies in all three hosts. Notably, the F2D2 antibody, which targets FLDNF (N. Dorabawila, M. Mandalasi, M. L. Mickum, Jr., B. Ezeabikwa, D. S. Smith, R. D. Cummings, and A. K. Nyame, unpublished data), also bound robustly to fractions F9-4, F11-0, F11-4, F11-5, and F13-4 and mildly to F10-4 (Fig. 5D), generating a pattern similar to that of the mouse, rhesus monkey, and human antiserum (Fig. 5A to C). The strongest binding occurred with fractions F9-4, F11-4, and F11-5, with F11-5 being the most prominent down to dilutions of 0.125 μg/ml (Fig. 5D). Therefore, we hypothesized that FLDNF is a major immune target of the anti-glycan response during S. mansoni infection.

FLDNF epitope is expressed on S. mansoni intramammalian life stages. Given the similarity of F2D2 specificity with infection antiseras on the 2D-SSGM, we hypothesized that this antibody would show binding to intramammalian developmental stages of the parasite. The localization of the FLDNF epitope on S. mansoni was investigated by probing whole, fixed parasites, including cercariae, 48-h-old cultured schistosomula, and 8-week-old adults. Bound antibody was detected with Alexa-488-conjugated antimouse IgG secondary and imaged by fluorescence microscopy. Fluorescence patterns indicated robust expression on the surface of all life stages investigated (Fig. 6A). The FLDNF motif is potentially expressed by cercariae and then appears to wane slightly after transformation to schistosomula and subsequent culture. In adults, the expression of FLDNF is markedly different in male versus female worms, with females expressing more glycan antigen.

Immune-relevant glycan epitopes have been shown to occur on both glycoproteins and glycolipids. To determine if FLDNF is expressed on glycoproteins, soluble extracts of S. mansoni eggs (SEA) and lysates of cercariae, 3-day-old schistosomula, and adults were separated by SDS-PAGE and probed with F2D2 (Fig. 6B). Five-fold less SEA relative to the other extracts was used in the analysis due to the intense expression of the epitope in the parasite eggs. F2D2 bound diverse and unique glycoprotein bands from each of the developmental stages, including glycoproteins from ~35 kDa to >260 kDa in both cercariae and SEA; however, the banding pattern is markedly different in these two samples. FLDNF expression appears more restricted in schistosomula and mixed-sex adults. In schistosomula, the major glycoprotein is ~40 kDa, with several fainter bands of ~20 kDa, ~30 kDa, and ~200 kDa. Adults also express the F2D2 epitope on few molecular species, primarily a glycoprotein of ~70 kDa with faint expression on proteins of ~45 kDa and ~230 kDa. Given the robust expression via immunostaining in schistosomula and adults (Fig. 6B), it is possible that the glycan epitope also is expressed on glycolipids, which is consistent with previous reports of lipid antigens (13, 63–66).

F2D2 kills schistosomula in vitro. Given the evidence that schistosomula express antigens bound by F2D2, which are thought to be the most vulnerable target of host immunity, we tested whether F2D2 was able to kill in vitro-transformed schistosomula. Three-hour-old schistosomula were cultured for up to 48 h with F2D2 in the presence of active or heat-inactivated guinea pig complement. The antibody was lethal to schistosomula in a dose-dependent manner at concentrations of 10 to 100 μg/ml (Fig. 6C). At 50 μg/ml antibody and at 48 h, 30 to 60% of the schistosomula were dead, as indicated by gross morphology and DAPI uptake. F2D2 killing of schistosomula was significantly higher than the baseline toxicity of mouse IgG and was dependent on the presence of active complement (Fig. 6C). These data show that the target of F2D2 could be important to the host immune response.

Proposed structures for antiserum glycan targets using MAGS. We characterized several of the 2D-GAEAB fractions by MALDI-TOF and compiled the compositional MS data with lec-
tin and antibody binding characteristics in order to tentatively predict their composition and propose possible structures using MAGS (37, 38). MS profiles for each fraction are shown in Fig. S5 and S6 in the supplemental material. Table 1 shows the compiled lectin and antibody binding patterns of each 2D fraction along with compositional predictions from MS. The proposed structures, monosaccharide compositions, sequence, and branching also were based on motifs identified in previous literature (31, 33, 65, 67, 68).
For example, F11-5, which was among the most highly reactive with antisera and F2D2 antibody, represents a mixture of primarily core xylosylated glycans with 3-4 Hex, 4-5 HexNAc, and 1-3 DeoxyHex residues (Fig. 7 and Table 1). In addition to robust AAL and ConA binding, F11-5 also is bound by WGA and BPL lectins and LDNF and anti-HRP antibodies (Fig. 4 and Table 1), suggesting that it contains determinants with terminal GlcNAc, terminal GalNAc, LDNF, and core β-xylose. F11-5 also contained glycan compositions consistent with a multifucosylated truncated N-glycan core and glycans of undetermined composition. MALDI-TOF/TOF of the highest magnitude peak (1,926.564) supported the MAGS data, suggesting a core fucose and LDNF (see Fig. S7 in the supplemental material). Although TOF/TOF data were unavailable for some of the smaller peaks of high molecular weight (2,072.610 and 2,275.669), their compositions were consistent with either multifucosylated LDN epitopes (FLDNF and LDN-dF) or two singly fucosylated GlcNAcs on the antennae (Fig. 7). Recognition of F11-5 by anti-LDN-dF and anti-M3GN2 antibodies, however, was low. This MAGS information led us to hypothesize that antenna in many N-glycans in F11-5 were extended past GlcNAc and that the multifucosylated antennae were FLDNF rather than LDN-dF.

Several other fractions were moderately to strongly reactive with F2D2 (F9-4, 11-4, F11-5, and F13-4) (Fig. 5D and Table 1). These glycan fractions also had compositional and MAGS data consistent with those of fucosylated LDN determinants (F9-4, 1,973; F11-4, 1,626.5; F13-4, 1,626.5 and 2,196). In particular, F13-4, which was not reactive with LDNF or LDN-dF antibodies, demonstrated compositions consistent with those of LDN and 4 Fuc residues, suggesting the presence of fucosylated GalNAc residues (see Fig. S7 in the supplemental material). Serum reactivity with some fractions where F2D2 reactivity was significantly lower or nonexistent (F7-3, F9-5, F10-4, and F13-0) suggested other immunologically relevant epitopes, such as high mannose- and core β-xylose-containing structures on the glycan array.

These results demonstrate that the most immunodominant
egg N-glycans recognized by infected hosts share common determinants containing core β-xylene and 1-4 fucose residues and are consistent with fucosylated LDN structures. Other epitopes likely contained in the serum immunodominant fractions are high mannose, core β-xylene, core β-xylose, core β-6-fucose, Lex, and LDNF. Therefore, using both defined reagents and immunologically relevant samples allowed us to partially characterize the egg glycans printed on the array and predict possible immunodominant glycan targets of the response to *S. mansoni*.

**DISCUSSION**

Our studies show that specific antigenic epitopes within N-glycans in egg glycoproteins from *S. mansoni* are the targets of adaptive immunity in some animal species and people infected by the parasite. While schistosomes synthesize many types of unusual glycans and both monoclonal and polyclonal antibodies have been found to recognize specific glycan determinants, the overall screening of such responses has been difficult due to the lack of parasite-derived glycan reagents and technologies. The studies here, employing total N-glycans from egg glycoproteins, defined glycan microarrays based on schistosome glycan antigens, along with MAbs to glycan antigens, have allowed us to identify key glycan antigens in this parasite and, most notably, the fucosylated glycan determinant FLDNF. These studies have broad implications for understanding the overall animal and human responses upon infection to glycan antigens from this parasite and may lead to new diagnostic and vaccine targets.

It is well established that schistosome infection of humans, primates, and rodents induces strong humoral responses against parasite antigens, and while antibody titers to glycan antigens generally are proportional to the severity of the infection (11), it is not yet clear whether such responses provide protection. Clearly, antibodies to carbohydrate antigens, especially glycoprotein N-glycans, dominate the humoral response, particularly during egg deposition (10, 69), suggesting that egg N-glycans play an important role in the immunopathogenesis of the disease. Antigenic responses to glycan determinants or epitopes, such as Lex, LDN, LDNF, and LDN-dF, give rise to different intensity levels and antibody isotypes (10, 11, 18, 25, 70–72). Recent elegant studies on structural glycomics profiling of schistosomes have shown that schistosome eggs contain a diverse repertoire of high-mannose, truncated, and complex N-glycans with core motifs such as α3 fucose, α6 fucose, and β2 xylene (27–29, 49). There is also growing evidence that worm products have immunomodulatory roles and can alter the functions and activation of dendritic cells, macrophages, and intestinal epithelial cells (73–77). However, the precise structures of the molecules that are bioactive in that regard are not yet clear.

In targeting the N-glycans of egg glycoproteins, we used both PNGase F and A to release N-glycans from these glycans, and we
generated 1D and 2D shotgun glycan microarrays after glycan separation of the fluorescently labeled glycans by HPLC fractionation. The interactions of glycans on these microarrays with a panel of lectins and antibodies indicated that egg N-glycans are characterized by a high content of α-linked fucose and mannose residues and are consistent with previous studies showing an abundance of glycans with these modifications in schistosome eggs (27–29, 49). It also should be noted that in earlier studies on serum-derived anti-glycan antibodies in infected chimpanzees, we found that antibodies to periodate-sensitive epitopes dominate the chimpanzee response to SEA, newly transformed schistosomulum-secreted products, and soluble worm material (10). To help focus our studies, we interrogated the 1D microarrays with sera from mice, rhesus monkeys, and humans to determine which of these glycan fractions were most relevant to the induction of humoral responses during disease progression in these hosts. We found that fractions F7 to F13 were recognized by IgG from all three species (Fig. 3), demonstrating the N-glycans released by PNGase F contain key antigenic determinants. In contrast, we found that PNGase A-released glycans, which were recognized by lectins AAL and ConA, were poorly recognized by infected mouse, rhesus, or human sera. PNGase A can release N-glycans containing unusual core modifications that block PNGase F release. It is possible that the cleavage of glycans with PNGase A results in an alteration in the conformation of antigenic features within the glycan cores that are recognized by antibodies, especially in light of results from Luyai et al. that demonstrated that rhesus and human sera strongly recognized core α3-fucosylated and core β2-xylosylated glycans when presented as glycopeptides on a defined glycan microarray, implying the need for a peptide moiety in the recognition (24). The glycan microarrays we prepared here from SEA were developed by reductive amination with AEAB and free glycans; thus, they lack a peptide component. Alternatively, the subset of schistosome glycans that receive core α3-fucose modifications may contain fewer terminal immunodominant epitopes. Interestingly, a recent glycomics study demonstrated that there were major differences in the PNGase F- and PNGase A-released N-glycans of both eggs and miracidia. PNGase A-released glycans from eggs were primarily multiantennary with singly fucosylated termini (LDN, LDNF, and Lex), while PNGase F-released glycans were primarily biantennary with several multifucosylated LDN motifs (31). Our data suggest that the latter type of glycan is more immunodominant in schistosomiasis-infected hosts.

Screening of 1D microarrays with serum from infected rhesus macaques showed a very strong IgG response (Fig. 3B), primarily to fractions F7 to F13, but only during the patent period at 8 weeks.
postinfection. No significant antibody response to these glycans was obtained with the serum of animals infected for 78 weeks in either this study or a recent one from our group (24). The results suggest that anti-glycan responses to some antigens dissipate by this stage of the infection in rhesus monkeys. A salient feature of the rhesus monkeys is that an infection becomes patent, but above a threshold worm burden, egg output declines over the ensuing weeks and months (78, 79), and the monkey clears the infection and becomes resistant to reinfection (80). Recent studies showed that in rhesus monkeys, an early IgG antibody response is associated with the elimination of worms and that peak titers of IgG to core α3-fucose and core β2-xylose at 8 to 11 weeks coincide with schistosomula killing by rhesus sera in vitro (81). Therefore, it is possible that the strong humoral IgG response to egg glycans induced during the patent phase plays a role in natural worm elimination and acquired resistance in rhesus monkeys.

In agreement with these studies, we also observed rhesus monkey serum reactivity against PNGase A-released egg N-glycan fractions A3 and A6, which was of much lower magnitude than what we observed toward PNGase F-released glycans. Complex type N-glycans possessing core α3-fucose and core β2-xylose, associated with resistance to PNGase F but not PNGase A, have been detected in schistosomes (27–29, 31, 41, 49, 67). Smit et al. (31) showed that the core α3-fucosylated egg N-glycans were primarily complex, in contrast to the truncated trimannose used on the defined array in Luyai et al. (24), to which a robust rhesus response was seen. Therefore, the low reactivity of rhesus antibodies with our PNGase A-released fractions could be explained by a preference for truncated rather than complex core fucosylated N-glycans, which may be expressed earlier than the egg stage. Core α3-fucosylated egg-derived glycans also are known to induce a strong Th2 cytokine response in infected mice (82). Thus, both PNGase F- and A-released glycans from the worm appear to be important immunogens and antigens in the response against schistosomiasis.

IgG within one human serum sample recognized fractions F1, F3, F7 to F13, and F15, with fractions F11 and F15 showing the highest recognition (Fig. 3C). Compared to the reactivity of the rhesus serum, the human sample lacked the relatively lower reactivity to PNGase A-released fractions seen in the rhesus monkeys but did possess low reactivity to A13 before and after treatment. Reactivity to different PNGase F- and A-released fractions and the presence of IgG at disparate stages of disease progression suggest differences in the immune response to egg glycan antigens among these three mammalian hosts (Fig. 3 and 4). Luyai et al. (24) also found that there were both similarities and substantial differences in the specificity, titers, and isotype composition of anti-glycan antibodies among humans, rhesus monkeys, and mice (24). An important issue for future studies will be to determine whether the differentially recognized egg glycan epitopes contained in these fractions represent markers of infection status and/or contribute to productive immune responses in protected hosts.

Further separation of the immunologically relevant GAEAB fractions F7 to F13 in a second dimension resulted in a total of 15 purified fractions (see Fig. S4 in the supplemental material) printed on the 2D microarrays and interrogated with lectins, anti-glycan antibodies, and sera (Fig. 3 and 4). We have shown that although many glycans separated by multidimensional HPLC have similar carbohydrate compositions, their separation indicates structural differences that also cause differences in the binding specificity of lectins and antibodies (35). Similar to the 1D parent fractions, most 2D fractions were highly fucosylated and mannose containing, as demonstrated by their strong binding to the lectins AAL and ConA. Interestingly, further purification by 2D HPLC enriched for N-glycans bound by lectins that only bound weakly to the mixed glycans in the 1D parent fractions, such as the GlcNAc-binding lectin WGA, the GalNAc-binding lectin BPL, and the β-galactosidase binding lectin RCA-1 (Fig. 4A and B and Table 1). Therefore, the serial purification of heterogeneous glycans is important to define the characteristics of distinct structures. One caveat to our approach may be that using serum to prioritize 1D-separated fractions for further analysis could mask important epitopes in the heterogeneous mixture. In any case, robust analyses of more highly purified fractions is always recommended.

The differential responses of infected animal and human sera to the 2D-SSGM microarrays was striking. When tested with pooled infected mouse serum, sera from 4 infected rhesus monkeys, and sera from 9 infected humans, results showed that glycan fractions F9–4, F11–0, F11–4, F11–5, and F13–4 were recognized in all species (Fig. 5). Mice responded prominently to fraction F11–5, composed of Hexα3HexNAc2, DeoxyHexα3Xylα1– (Fig. 5A), while rhesus monkeys (Fig. 5B) and humans (Fig. 5C) showed high reactivity to multiple fractions during the patent phase of disease. In some rhesus monkeys, IgG reactivity against F11–4 and F11–5 also was observed at 26 weeks and 78 weeks postinfection, demonstrating that the humoral response to glycans declines after the egg-laying patent phase but that low levels of IgG against particular glycans can remain after egg deposition ceases, worms are eliminated, and animals become self-cured.

Due to the striking similarity of the F2D2 MAb binding pattern with antisera, in particular that of the acutely infected, naturally resistant rhesus monkey, we explored the expression of the FLDNF epitope and the antibody’s effector abilities. The F2D2 epitope, FLDNF, is highly expressed on cercarial and egg glycoproteins and has more restricted expression in cultured schistosomula and adult worms (Fig. 6B). These data, in combination with immunostaining data depicting vast surface expression (Fig. 6A), are in agreement with several reports profiling the expression of fucosylated LDN determinants (31, 39, 64, 83), particularly FLDN and FLDNF, which are reported to exist on glycoproteins and glycolipids of the cercarial and egg stage and predominantly on glycolipids in adults. Importantly, we found that the F2D2 antibody was lethal to schistosomula in vitro in a complement-dependent manner. Rhesus serum also has schistosomulum-lethal activity from 2 months postinfection (24, 84). It is still unknown whether anti-glycan antibodies contribute to parasite resistance in the context of a host response, but it is tempting to speculate that glycan targets present on schistosomula, such as the F2D2 epitope, could be protective if targeted early in infection.

The predicted structures of glycans contained in F11–5, the fraction most strongly bound by F2D2 and serum samples, suggest they contain a combination of antigenic epitopes such as variably fucosylated LacdiNAc (LDNF, LDN-df, FLDN, and FLDNF), Leα, and those containing core α3-fucose and β2-xylose (Fig. 7 and Table 1). The MAGS data for other F2D2 binding fractions suggested the presence of fucosylated LDN motifs (Fig. 7 and Table 1). Tandem MS data were not available for the highly fucosylated peaks in fractions 11 (2,072.6; X1F3H3N4; Na+ ion) and 13 (2,196.7; X1F4H3N4; H+ ion) due to the lack of abundance, but
the compositions predicted for these peaks matched those of PNGase F-released cercarial and egg glycans recently identified by Smit et al. (31). In that study, the authors performed exoglycosidase digestions on cercarial and egg N-glycans, which suggested that FLDNF and LDN-dF, respectively, were antigenic determinants within these glycans, which was also consistent with findings for the F2D2 epitope.

Several studies in multiple species have attempted to define the various roles that different anti-glycan antibodies to LDN, LDNF, FLDN, LDN-dF, and Le-^e^ may have in disease progression and immunity (10, 24–26, 34, 41, 70). Chimpanzees vaccinated with radiation-attenuated cercariae or naturally infected developed a strong cellular and humoral immune response predominantly directed against glycans associated with both cercariae and eggs, including LDN, LDNF, Le-^e^, and unidentified glycans present on Keyhole limpet hemocyanin. Vaccinated chimps showed a 40% reduction in infection intensity compared to that of infected control chimpanzees (10). Later studies revealed that antibody levels to LDN-dF and FLDN epitopes were strikingly higher than those against LDN, LDNF, and monomeric Le-^e^ in vaccinated or infected chimps and infected human cohorts. Interestingly, the anti-LDN-dF and anti-FLDN antibodies were predominantly IgGs, whereas anti-Le-^e^, anti-LDN, and anti-LDNF were IgMs; however, this was not always consistent in human studies (25, 26, 41, 70). Note that in mice, the acute infection is marked by IgM antibodies to LDNF, LDN, and Le-^e^ (24). Similarly, mice generated predominately IgM and low levels of IgG to LDN and LDNF postimmunization with LDN/LDNF-expressing cells (34). The differential responses in infected animals raise important questions to address in the future, especially in relation to the specific glycan target, isotype responses, time courses of responses, and whether the specificity and titer of anti-glycan antibodies play a role in disease outcome and overall susceptibility to infection in the host animal.

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