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Stage-specific expression and antigenicity of glycoprotein glycans isolated from the human liver fluke, *Opisthorchis viverrini*

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Abstract

Infection by *Opisthorchis viverrini* (liver fluke) is a major public health problem in southeastern Asia, resulting in hepatobiliary disease and cholangiocarcinoma. Fluke surface glycoconjugates are prominently presented to the host, thereby constituting a crucial immunological interface that can determine the parasite's success in establishing infection. Therefore, *N*- and *O*-linked glycoprotein glycan profiles of the infective metacercarial stage and of the mature adult were investigated by nanospray ionization-linear ion trap mass spectrometry (NSI-MSⁿ). Glycan immunogenicity was investigated by immunoblotting with serum from infected humans. Metacercariae and adult parasites exhibit similar glycan diversity, although the prevalence of individual glycans and glycan classes varies by stage. The *N*-glycans of the metacercaria are mostly high mannose and monofucosylated, truncated-type oligosaccharides (62.7%), with the remainder processed to complex and hybrid type glycans (37.3%). The *N*-linked glycan profile of the adult is also dominated by high mannose and monofucosylated, truncated-type oligosaccharides (80.0%), with a smaller contribution from complex and hybrid type glycans (20.0%). At both stages, complex and hybrid type glycans are detected as mono-, bi-, tri-, or tetra-antennary structures. In metacercariae and adults, *O*-linked glycans are detected as mono- to pentasaccharides. The mucin type core 1 structure, Galβ1-3GalNAc, predominates in both stages but is less prevalent in the adult than in the metacercaria. Immunogenic recognition of liver fluke glycoproteins is reduced after deglycosylation but infected human serum was unable to recognize

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glycans released from peptides. Therefore, the most potent liver fluke antigenic epitopes are mixed determinants, comprised of glycan and polypeptide elements.

Keywords

Liver fluke; *Opisthorchis viverrini* glycoproteins; *N*-linked glycans; *O*-linked glycans; Antigenic epitopes

1. Introduction

The liver flukes that commonly parasitize humans are members of the family Opisthorchiidae including *Clonorchis sinensis*, *Opisthorchis felinus* and *Opisthorchis viverrini*. These parasites are endemic in many countries in Asia and eastern Europe. Northeastern Thailand is an endemic area for *O. viverrini* infection, where an estimated six million people are infected (Sripa, 2003). The infection is associated mainly with hepatobiliary diseases including cholangitis, obstructive jaundice, hepatomegaly, cholecystitis and cholelithiasis. Both experimental and epidemiological evidence strongly implicate liver fluke infection in the etiology of the fatal bile duct cancer, cholangiocarcinoma (Sripa et al., 2007).

To understand the host-parasite interaction, the cell surface molecules and excretory-secretory (ES) antigens of the parasites have been investigated. These molecules, which are primarily glycoconjugates, are involved in parasite survival, infectivity, host-cell recognition and immune stimulation or protection (Nyame et al., 2004). The relationship between parasite-derived glycoconjugates and host responses has been studied in many parasitic diseases such as schistosomiasis (Hokke et al., 2007), echinostomiasis (Fujino et al., 1996a,b) and fascioliasis (Dalton et al., 1985). In *O. viverrini* infection, secreted and cell surface tegumental components of the parasite are detected along the host biliary epithelium and activate immune/inflammatory responses (Sripa et al., 2000). A few studies of *O. viverrini* glycoconjugates have been reported, but glycan characterization was limited to detection by antibody or lectin staining (Akai et al., 1992; Apinhasmit et al., 2000). To gain a better understanding of parasite biology and host-parasite interactions, the glycans found on *O. viverrini* glycoconjugates need greater characterization.

We previously reported the *N*-linked glycan profiles of adult *O. viverrini* using MALDI-TOF and HPLC mapping methods (Talabnin et al., 2006). In the current study, the analysis is extended to include previously undetected, minor *N*-linked glycans and full characterization of the *O*-linked glycans isolated from both metacercarial and adult *O. viverrini* glycoproteins. The contribution of glycans to the antigenic response elicited by this parasitic infection is also investigated.

2. Materials and methods

2.1. Materials

PNGaseF (*N*-glycanase) was obtained from Prozyme (San Leandro, CA, USA). Trypsin and chymotrypsin were obtained from Sigma (USA) and Calbiochem (USA). Other glycoprotein standards and fine chemicals were from standard sources. Human serum was obtained from infected and non-infected individuals who gave informed consent under an institutionally approved protocol. Hamsters were infected with *O. viverrini* and rabbit serum was harvested under institutionally approved protocols. Animal care and maintenance was in accordance with government and institutional guidelines.

2.2. Preparation of *O. viverrini* metacercaria and adult stages

The metacercariae of *O. viverrini* were obtained from naturally infected cyprinoid fish captured from an endemic area in Khon Kaen province, northeastern Thailand. The fish were digested by pepsin-HCl. After several washings with normal saline, the metacercariae were collected and identified under a dissecting microscope. Viable metacercariae were used to infect hamsters to produce adult parasites. Adult *O. viverrini* were obtained from the liver, gallbladders and extrahepatic bile ducts of hamsters infected for 2-3 months.

2.3. Preparation of *O. viverrini* protein powder

The metacercariae and adult worms of *O. viverrini* were homogenized on ice in cold 50% methanol (MeOH). The homogenate was then adjusted to a ratio of chloroform, CHCl₃:MeOH:water (4:8:3) and extracted for 2 h at room temperature. The extract was centrifuged at 2,500 *g* for 15 min. and the resulting pellets were dried under nitrogen to yield protein powder, which was stored dessicated at -20 °C until use.

2.4. Preparation of glycopeptides and release of N-linked glycans

Opisthorchis viverrini powder, 250 µg for metacercariae or 5 mg for adults, was digested with trypsin and chymotrypsin for 18 h at 37 °C in 0.1 M Tris-HCl, pH 8.2, containing 1 mM CaCl₂. The digestion products were enriched and freed of contaminants by a Sep-Pak C18 cartridge column as described previously (Aoki et al., 2007). After enrichment, the glycopeptides were digested with 2 µl of PNGaseF (7.5 U/mL) in 50 µL of 20 mM sodium phosphate buffer, pH 7.5, for 18 h at 37 °C. Released oligosaccharides were separated from peptides and enzymes by passage through a Sep-Pak C18 cartridge (Aoki et al., 2007).

2.5. Preparation of O-glycans by reductive and non-reductive β- elimination

Opisthorchis viverrini powder, 250 µg for metacercariae or 5 mg for adults, was subjected to alkaline reductive elimination in 100 mM NaOH containing 1.0 M sodium borohydride at 45 °C for 18 h. The reaction mixture was neutralized with 10% acetic acid and desalted on a column of AG50W-X8 (H⁺) (Aoki et al., 2008). The material eluting with 5 % acetic acid was lyophilized and boric acid was removed by evaporation with MeOH. Released O-glycans were purified by a Sep-Pak C18 cartridge column (Aoki et al., 2007). O-linked glycans were also released by non-reductive β-elimination in order to retain an intact reducing terminal for the production of neoglycolipids (see Section 2.13) by treatment with aqueous ammonium hydroxide (28%) in saturated ammonium carbonate as previously described (Huang et al., 2002; Aoki et al., 2008).

2.6. Permethylation of glycans

To facilitate analysis by MS, portions of released oligosaccharide mixtures were permethylated according to the method of Ciucanu and Kerek (1984).

2.7. Nanospray ionization-linear ion trap mass spectrometry (NSI-MSⁿ)

Mass analysis by NSI-MSⁿ was performed as described previously (Aoki et al., 2007). Briefly, permethylated glycans were dissolved in 1 mM NaOH in 50% MeOH and infused directly into a linear ion trap mass spectrometer (LTQ; Thermo Fisher, USA) using a nanospray source at a syringe flow rate of 0.40–0.60 µL/min. The capillary temperature was set to 210 °C, and MS analysis was performed in positive ion mode. MS and MS/MS spectra (at 28% collision energy) were obtained using the total ion mapping (TIM) functionality of the Xcalibur software package (version 2.0). The nomenclature of Domon and Costello (1988) was used to guide the depiction of fragmentation derived from MS/MS spectra.

2.8. General strategy for glycomic analysis of metacercaria and adult *O. viverrini*

This study characterizes and compares the *N*- and *O*-linked glycan profiles of the infective metacercaria and the adult parasite. Glycan structures were assigned based on: (i) parent ion mass determined by NSI-MS; (ii) fragmentation of permethylated glycans by automated TIM (NSI-MS/MS) and by further manual fragmentation (NSI-MSⁿ); (iii) exoglycosidase treatment; (iv) similarity to known structures of previously characterized glycans, and (v) known biosynthetic limitations. The prevalence of each individual glycan in a profile was quantified by comparing its signal intensity with the sum of the signal intensities for all identified glycans in the profile to give “% Total Profile” for each glycan.

2.9. Glycan detection and localization by lectin staining

For visualization by lectin fluorescence, the *in vitro* excysted metacercariae were fixed in neutral buffered 10% formalin and washed with PBS. After washing, FITC-conjugated lectin was applied to the parasite for 1 h at room temperature. The parasites were then washed in PBS and mounted for microscopy in glycerol. Mounted parasites were viewed and photographed under a fluorescence microscope (Nikon Epifluorescence, Model ES600). For detection of lectin binding by conjugated peroxidase histochemistry, paraffin sections of *O. viverrini* were used. Sections were deparaffinized in xylene, hydrated in downgraded ethanol (EtOH) and distilled water, respectively. The endogenous peroxidase was eliminated by treating sections with absolute MeOH containing 5% hydrogen peroxide for 30 min. Sections were then washed in water and PBS. Non-specific staining was blocked by treating slides with 5% normal serum in PBS for 30 min. Biotinylated lectins (Vector, USA) in TBS (20 mM Tris-HCL, pH 7.4, containing 0.15 M NaCl, 10mM CaCl₂ and 1mM MnCl₂) were applied to the sections and incubated for 2 h at room temperature. After rinsing with PBS three times for 10 min, the sections were incubated with streptavidin-peroxidase (Zymed, USA) at a dilution of 1:300 for 30 min. Color development using diaminobenzidine (DAB) reagent and hydrogen peroxide was done after rinsing with PBS twice for 10 min. Sections were counterstained with Mayer's hematoxylin, dehydrated, cleared in xylene and mounted with Permount[®].

2.10. Enzymatic deglycosylation

Enzymatic *N*-linked deglycosylation was carried out using an enzymatic deglycosylation kit (Prozyme, USA). Briefly, adult *O. viverrini* protein powder (100 µg) was dissolved in 50 µL of 50 mM sodium phosphate containing 0.1% SDS, 50 mM β-mercaptoethanol, pH 7.0, and boiled for 5 min. After cooling, 2.5 µL of 15% NP-40 was added and the mixtures were digested with PNGase F (5 U/mL) for 24 h, at 37 °C. After digestion, the deglycosylated proteins were subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining or immunoblotting.

2.11. Chemical deglycosylation by acid hydrolysis of glycosidic bonds

Adult *O. viverrini* protein powder (100 µg) was lyophilized to dryness in a screw-top, teflon-lined glass tube. Trifluoromethanesulfonic acid (TFMS; Sigma Fluka, USA) was mixed with toluene (Sigma Fluka), 9:1 by volume, 200 µL was added to each dried sample, and a stream of N₂ was applied over the surface before capping. Samples were put on ice for 2 h then at -20 °C for an additional 2 h. The reaction was stopped by adding 10 µL of cold 2 M Tris base followed by 300 µL of ice-cold water. Total protein was immediately precipitated by adding 500 µL of ice-cold 10 mM deoxycholate in 50% trichloroacetic acid (TCA). Samples were put on ice for 20 min. The precipitated protein was pelleted by centrifugation at 10,000 *g* for 10 min. The resulting pellet was washed twice with ice-cold acetone and dried by vacuum centrifugation. Fetuin (Sigma) was treated in an identical manner and served as a control to assess the extent of deglycosylation and protein recovery.

2.12. Immunoblotting

Intact and deglycosylated proteins of adult *O. viverrini* were separated in a 4-20% SDS-PAGE gradient gel (Laemmli, 1970). After electrophoresis, the resolved proteins were transferred onto polyvinylidene difluoride (PVDF) membranes for immunodetection with human serum from patients infected naturally with *O. viverrini*. Peroxidase-conjugated goat anti-human IgG+IgM, at dilution 1:10,000 and Enhanced Chemiluminescence (ECL) plus Western Blotting Detection Reagents (Amersham, USA) were used to detect and quantify recognized glycoproteins. Quantification was achieved by scanning densitometry of the ECL films using NIH ImageJ software. Coomassie stained gels were similarly quantified by scanning densitometry. The total densitometry signal for each of the immunoblotted lanes was divided by the total densitometry signal for the Coomassie stain of the corresponding sample lanes in order to determine the relative increase or decrease in immune serum recognition normalized to protein recovery.

2.13. TLC overlay

In order to probe for direct recognition of released *N*-linked and *O*-linked glycans by immune serum from naturally infected humans (and intentionally immunized rabbit serum), glycans were coupled to dipalmitoylphosphatidylethanolamine (DPPE) by reductive amination using standard methods to produce neoglycolipids (Tang et al., 1985; Childs et al., 1989). Briefly, 100 μ L of DPPE (5 mg/mL in $\text{CHCl}_3/\text{EtOH}$ 1:1, v/v) was added to an aqueous solution of *N*-linked oligosaccharides (20 μ g). After evaporating the mixture to dryness under a nitrogen stream, the material was resuspended by the addition of water (5 μ L) and $\text{CHCl}_3/\text{EtOH}$ (95 μ L, 1:1) followed by sonication for 5 min and incubation at 60 °C for 2 h. Reducing agent (sodium cyanoborohydride, 2.5 μ L at 10 mg/mL in EtOH) was then added and the reaction mixture was incubated at 60 °C for a further 18 h. The solvent was then evaporated and the mixture of neoglycolipids was dissolved in $\text{CHCl}_3/\text{MeOH}/\text{water}$ (25:25:8, by volume) and stored at -20 °C until use. For TLC overlay analysis, neoglycolipid preparations were resolved on high performance thin layer chromatography (HPTLC) silica plates using $\text{CHCl}_3/\text{MeOH}/\text{water}$ (60:35:8) as the mobile phase. Once developed, the HPTLC plates were stabilized by coating with plastic (polyisobutylmethacrylate) and then probed with immune or control serum from human or rabbit using standard procedures (Magnani et al., 1982). Binding of serum antibodies to TLC-resolved neoglycolipids was detected using Peroxidase-conjugated goat anti-human IgG+IgM or anti-rabbit IgG+IgM and subsequent precipitation of DAB in the presence of hydrogen peroxide as substrate.

3. Results

3.1. The prevalence of complex and hybrid type N-glycan structures is greater in the metacercaria than in the adult

The *N*-linked glycan profiles of the metacercariae and adult *O. viverrini* are shown in Figs. 1, 2 and Tables 1 and 2. *N*-glycan structures were assigned as high mannose type ($\text{Glc}_{0-1}\text{M}_{5-9}\text{N}_2$) (Structures 7, 9, 10-14), truncated, paucimannose type ($\text{M}_{1-4}\text{N}_2\text{F}_{0-1}$) (Structures 1-6 and 8), or complex and hybrid type ($\text{Hex}_{0-3}\text{HexNAc}_{1-4} + \text{M}_3\text{N}_2\text{F}_{0-1}$) (Structures 15-33). The core fucose was assigned as an α 1-6 linked residue on the reducing end GlcNAc based on sensitivity to PNGaseF digestion and on the detection of a diagnostic MS/MS fragment at $\Delta m/z = 451$ ($474 + \text{Na}^+$), which corresponds to loss of reducing end HexNAc-deoxyHex structure (Fig. 3A). *Opisthorchis viverrini*, both metacercariae and adult parasites, generated similar glycan profiles; however, the profiles differ in the prevalence of individual glycans (Fig. 2). The metacercariae consisted of high mannose and monofucosylated, truncated-type oligosaccharides (62.7%), complex and hybrid type glycans (37.3%) while the adult consisted of high mannose and monofucosylated, truncated-type oligosaccharides (80.0%), complex and hybrid type glycans (20.0%) (Fig. 4). The adult

parasite is significantly enriched for the largest high-mannose glycans (M9N2; Structures 13 and M8N2; Structures 12), indicating reduced *N*-linked glycan processing through the first mannosidase trimming steps that take place in the endoplasmic reticulum. Conversely, the metacercaria is enriched for all of the truncated high-mannose structures and for the most complex, processed glycans.

The minor *N*-linked glycans of *O. viverrini* include complex and hybrid structures. No individual complex or hybrid structure accounts for more than 6% of the total profile in metacercariae or adult parasites. Complex and hybrid type structures were detected with or without core fucose (M3N2F₀₋₁) and with various extensions. Substitutions were assigned based on fragmentation at MS² and MS³ (Fig. 3). Based on known characteristics of glycan biosynthesis and on previously reported structures, the reducing terminal HexNAc, Hex-HexNAc and HexNAc-HexNAc of the complex and hybrid type glycans was assigned as GlcNAc, Galβ1-4GlcNAc (LacNAc) and GalNAcβ1-4GlcNAc (LacdiNAc), respectively. Another possible assignment for HexNAc-HexNAc would be the chitobiose structure, GlcNAcβ1-4GlcNAc, but this motif has only been identified in filarial parasites where it is found within oligomeric repeats of up to 15 GlcNAc residues (Haslam et al., 1999; Khoo et al., 2001). Such polymeric HexNAc extensions were not detected on *N*-linked or *O*-linked cores of *O. viverrini* at either stage of development nor have they been described in any other trematode parasite. Several isobaric glycan sets (glycans sharing the same molecular weight) were detected to include glycans bearing the LacdiNAc determinant (Structures 18, 19, 20, 22, 23, 24, 30 and 32). The relative signal intensity of diagnostic MS/MS fragments demonstrated that these LacdiNAc determinants are more highly expressed in the adult parasite than in the metacercaria (Fig. 5).

3.2. The prevalence of complex, extended O-linked glycan structures is higher in the adult parasite than in the metacercaria

O-glycans were released by reductive β-elimination, which allows easy identification of the reducing terminal monosaccharide through its conversion to an alditol. The detected *O*-glycans of metacercariae and adult *O. viverrini* are shown in Figs. 6, 7 and Table 3. The *O*-glycan profile of *O. viverrini* metacercariae and adults contain short chain oligosaccharides, ranging in size from monosaccharides to pentasaccharides. The Hex-HexNAc-ol, assigned as Galβ1-3GalNAc based on specificity of enzymatic deglycosylation and lectin (peanut agglutinin, PNA) staining, is the most common structure in both metacercariae and adults. The Galβ1-3GalNAc (core 1) disaccharide accounts for almost 80% of the metacercaria total profile, but closer to half of the adult profile, with the adult enriched in extended forms of the core 1 glycan. The *O*-glycan structures that are expressed in metacercaria at higher levels than in the adult are the mucin type core 1 Galβ1-3GalNAc (structure 35) and a hexose-extended core 2 glycan (structure 40). In the adult, GalNAc (structure 34), Hex-extended core 1 (Gal-Galβ1-3GalNAc, structure 37), core 2 and Hex-extended core 3 (structures 38) *O*-glycans are more prevalent than in the metacercaria. Fragmentation revealed that four MS signals, at *m/z* 738, 779, 984 and 1230, arise from isobaric mixtures of two structures (Table 3, Fig. 7). Fragmentation (MS/MS) detects changes in the relative amounts of individual isobaric components in the two stages of *O. viverrini* (Fig. 8). For the glycans that comprise the MS signal at *m/z* = 738, linear structures are in higher prevalence relative to branched isobaric structures in metacercariae, while the opposite is true in adults (Fig. 8A). However, at *m/z* = 779, the branched component is enriched in metacercariae, while the linear isobars are enriched in adults (Fig. 8B).

3.3. Detection and localization of *O. viverrini* glycans by lectin staining

The spatial distribution of glycoconjugates in metacercariae (excysted in vitro) was examined using FITC-conjugated lectin staining. The lectins and their carbohydrate

recognition preferences are listed in Table 4. The metacercarial tegumental surface was intensely stained by concanavalin A (ConA), PNA and wheat germ agglutinin (WGA) lectins (Fig. 9A), indicating that α Man, Gal β 1-3GalNAc and GlcNAc are present. The glycans of the adult parasites were detected histochemically in paraffin sections of liver harvested from *O. viverrini* infected hamsters. Four biotinylated lectins, PNA, *Pisum sativum* agglutinin (PSA), *Ulex europaeus* agglutinin (UEA)-I, and WGA, were used for detection. The tegumental surface, tegument cells and eggs of the adult parasite were positively stained by lectin PNA, PSA and WGA. The testis was stained by PSA and WGA while the ovary was stained only with PNA. The gut surface was stained by PSA, UEA-I and WGA (Fig 9B). These results indicate that the tegumental surface and tegument cells and eggs of adult parasite express Gal β 1-3GalNAc, α Man and GlcNAc. The testis displays α Man and GlcNAc, but the ovary is enriched in Gal β 1-3GalNAc. Gut glycans differ from those of other tissues in that the gut displays a relative lack of Gal β 1-3GalNAc but enrichment in α Fuc. The lectin staining localizes and correlates the existence of glycan structures assigned by mass spectrometry. Lectin staining in both stages is consistent with the predominance of high-mannose *N*-linked structures and of the *O*-linked core 1 disaccharide, as detected by ConA/PSA or PNA, respectively. WGA staining indicates the presence of terminal GlcNAc residues, which were detected by MS analysis on both *N*- and *O*-linked structures. While the lectin histochemistry results are generally consistent with the MS-based detection of glycans that bear the dominant determinants previously characterized for each of these carbohydrate binding proteins, the presence of highly valent, but structurally divergent, low affinity glycan ligands might also contribute to positive lectin binding in the complex trematode tissue matrix. Such a result cannot be ruled out without further in-depth glycomic and glycoproteomic analyses. For instance, UEA-I optimally binds Fuc in α 2 linkage to Gal and UEA-1 binding was clearly detected in the adult gut. However, we did not detect this structure on *N*-linked or *O*-linked glycoprotein glycans of adult parasites, but did not address whether it may be present on glycosphingolipid glycans. Therefore, lectin histochemistry provides a complementary approach to MS-based glycomic analysis in as far as it localizes and detects structural possibilities that can generate new hypotheses.

3.4. Characterization of immunogenicity

Intact and deglycosylated glycoproteins isolated from adult *O. viverrini* were subjected to quantitative immunoblotting using serum harvested from humans naturally infected with the parasite. Antibody binding was quantified by chemiluminescence densitometry and the signal intensity was normalized for protein recovery, based on Coomassie blue densitometry. Protein deglycosylation by TFMS, which removes both *N*- and *O*-linked glycans, partially decreased human antibody binding to parasite proteins (Fig. 10A). This result indicates that a glycan-stimulated immune response is mounted in humans during parasitic infection, but it does not distinguish whether *N*- or *O*-linked glycans are dominant determinants. To dissect the relative contribution of these two classes of glycans, the parasite proteins were treated with PNGaseF to remove *N*-linked glycans. This treatment also partially attenuated antibody binding (Fig. 10B). To determine whether glycans were recognized by immune serum in the absence of peptide, *N*- and *O*-linked glycans released from parasite proteins were conjugated to DPPE to generate neoglycolipids (Feizi et al., 1994). The resulting neoglycolipids were resolved on TLC plates and probed with *O. viverrini*-infected human serum and *O. viverrini*-immunized rabbit serum. The neoglycolipids made from *N*-linked glycans were only detected when probed with immunized rabbit serum (Fig. 10C). A similar result was obtained for neoglycolipids produced with *O. viverrini* *O*-linked glycans (data not shown). The inability of human serum to recognize glycans removed from proteins and the incomplete loss of immunoblot staining

after enzymatic or chemical treatments, both indicate that the most potent epitopes are probably mixed determinants, composed of glycan and associated polypeptide backbones.

4. Discussion

To our knowledge, the data presented in this report represents the first comparative structural glycomics analysis of metacercariae and adult stages of the liver fluke *O. viverrini*. The diversity of the carbohydrate part of the parasite's glycoproteins was demonstrated by mass spectrometry and lectin staining. In addition, the antigenicity of glycoprotein glycans which may contribute to immunogenic recognition during infection, was characterized. The results indicate that *N*-glycosylation of *O. viverrini* is dominated by high mannose and truncated type structures, which can be core-fucosylated in both metacercariae and adults. Furthermore, complex and hybrid type glycans are detected in very small amounts and NSI-MSⁿ fragmentation is consistent with the presence of LacNAc (Galβ1-4GlcNAc) and LacdiNAc (GalNAcβ1-4GlcNAc) as non-reducing caps on some antennae. The profile of *O*-linked glycans in *O. viverrini* metacercaria and adults is dominated by the mucin type core 1 disaccharide (Galβ1-3GalNAc). Adults, more than metacercariae, extend the core 1 disaccharide by addition of a branching or linear hexose. Metacercariae, more than adults, convert their core 1 disaccharide into a core 2 structure by addition of a branching HexNAc. This divergence in *O*-glycan processing indicates stage-specific expression of glycosyltransferases that act on the same core glycan acceptor substrates.

Compared with other parasitic helminths, *O. viverrini* glycan profiles are both similar and distinct. Similar to *Schistosoma* (Wuhrer et al., 2006a; Hokke et al., 2007), *Haemonchus contortus* (Haslam et al., 1996, 1998), and *Taenia solium* (Haslam et al., 2003), *O. viverrini* generates high mannose and truncated type *N*-glycans as a major component of the total glycan pool. In addition, both the *N*- and *O*-linked glycans of *O. viverrini* can be terminated by Gal or GlcNAc, as has been described for other helminths. However, some common structures reported in several parasitic helminths, such as the Le^X epitope and multi-fucosylated termini (Wisniewski et al., 1993; Haslam et al., 1996, 1998, 2003; Wuhrer et al., 2006b; Hokke et al., 2007), were not found in *O. viverrini*. This result suggests that *O. viverrini* lacks the glycosyltransferases that synthesize the longer glycan structures and that this parasite does not need the longer side chain structures for survival. Fucose was detected at the core of *N*-linked glycans only as Fucα1-6 GlcNAc in *O. Viverrini*. Two types of fucosylation of the proximal GlcNAc of *N*-linked core structures have been described; fucose α1-6 and fucose α1-3. The fucose α1-6 GlcNAc has been found broadly distributed throughout the animal kingdom, whereas Fucα1-3 GlcNAc has only been described in plants, arthropods and nematodes. Fucose-substituted core structures (fucose α1-6 to core GlcNAc residue) are commonly found in *Schistosoma* (Hokke et al., 2007) and nematodes such as *H. contortus* (Haslam et al., 1996, 1998), *Acanthocheilonema viteae*, *Onchocerca volvulus* and *Onchocerca gibsoni* (Haslam et al., 1997; Houston et al., 2004). However, α1-3 linked core fucose and extended fucose branches at the reducing terminal, as have been described in *Caenorhabditis elegans* as well as in *Schistosoma* and *H. contortus* (Hanneman et al., 2006), are PNGaseF-resistant and were not accessible by the methods that we employed. While further analysis of *N*-glycans released by PNGaseA or by hydrazinolysis will eventually provide a more comprehensive *N*-glycan profile, it is important to note that PNGaseF digestion reduces the intensity of the immune recognition of *O. viverrini* glycoproteins by human serum (Fig. 10), indicating that the structural features which impart PNGaseF resistance are not essential for immunogenicity. In *O*-glycosylation, the mucin type core structures are commonly found in parasitic helminths, but their substitution and extensions vary by species (Khoo et al., 2001). Compared with other parasites, *O. viverrini*

generates simple *O*-linked substitutions, including short termini, i.e. HexNAc and Hex-HexNAc, while other parasites generally elaborate longer side chains (Nyame et al., 2004).

Comparing the glycan profiles of the *O. viverrini* metacercaria with the adult, the difference in glycan prevalence most likely reflects stage-specific changes in glycosyltransferase expression. Glycosylation by these enzymes is important in many aspects of cellular and tissue biology including development, reproduction, protein trafficking and folding (Hokke et al., 2007). The parasitic infection begins by metacercarial colonization of the biliary epithelium. Interactions between the biliary epithelium and microbial pathogens can be disrupted by soluble Gal/GalNAc monosaccharide and by highly glycosylated mucin glycopeptides, indicating an important role for appropriate glycan expression during colonization of the biliary duct (Chen et al., 2000). A major morphological change during development from metacercaria to adult is the maturation of the sex organs and reproductive systems. Therefore, some of the differences in glycan expression, comparing adult with metacercaria, may reflect the relative increase in the mass of this specific tissue and the requirement for specific glycans in their reproductive system, sex organ development and gamete production. Similar findings on proteomics of juvenile and adult *O. viverrini* have been reported. At least 35 protein spots are more highly expressed in adult worms than in juveniles (Boonmee et al., 2003).

Comparing the glycan profiles of *O. viverrini* with the mammalian host, differences and similarities are apparent. For the two major differences, first, the parasite generates complex structures as a minor component of the total glycan pool, while in the host, complex structures are the major component (Hart, 1992; Gagneux et al., 1999). Second, a common terminal modification of mammalian glycans, sialic acid, was not detected in *O. viverrini*. These differences may play a role in host-parasite interactions, for example, the high mannose and trimmed mannose structures which are the major *N*-linked glycans of *O. viverrini* are ligands for the mammalian host mannose binding lectin (MBL), which may activate complement (antibody-independent pathway) and promote opsonophagocytosis (Turner, 2003). This process can lead to severe inflammation of the biliary epithelium in contact with the parasite in infected hosts (Sripa, 2003). On the other hand, *O. viverrini* shares several glycosylation features in common with the mammalian host, such as terminal Gal, GlcNAc, Gal β 1-4GlcNAc or GalNAc β 1-4GlcNAc structures (Haslam et al., 2001). The similarity between these simple *O. viverrini* glycan termini to some termini of host glycans may offer protection from host immune surveillance through “molecular mimicry,” in which molecules are either derived from the pathogen or acquired from the host to evade recognition by the host immune system (Damian, 1965, 1997), or through “glycan gimmickry,” in which parasites use their glycans to neutralize glycan binding proteins (GBPs) of the host immune system (van Die et al., 2010).

The glycoproteins of *O. viverrini* commonly found in both metacercaria and adult parasites are immunogenic (Amornpant et al., 1991; Akai et al., 1992). Anti-glycan antibody, such as the anti-tyvelose containing structure of *Trichinella spiralis* (Wisniewski et al., 1993; Ellis et al., 1997) and anti-Lewis X, LDN, LDNF of *Schistosoma* (Nyame et al., 1998, 2003) are potential targets for immunodiagnosis. However, no report of anti-carbohydrate antibody in *O. viverrini* infection has been described to date. This study has demonstrated that the most potent immunogenic epitopes are likely to be mixed determinants, composed of both glycans and their associated polypeptide backbones. The proposal that the antigenic epitopes are mixed protein-glycan determinants is based on the following observations: i) both chemical removal of *N*- and *O*-linked glycans (Fig. 10A) as well as PNGaseF removal of *N*-linked glycans (Fig. 10B) resulted in only a partial reduction of human immune serum binding to *O. viverrini* proteins, indicating the presence of resistant glycans or residual peptide-based recognition; ii) when the released *N*- or *O*-linked glycans were subsequently attached to

DPPE and probed with immune serum by TLC overlay (Fig. 10C), human immune serum did not recognize the neoglycolipid glycan structures that when removed from protein resulted in decreased antibody binding. On the other hand, rabbit immune serum did recognize *N*-linked and *O*-linked glycans coupled to DPPE, indicating the success of the neoglycolipid preparation and, perhaps, species specificity in the immune response. Alternatively, the ability of rabbit serum antibodies to recognize *O. viverrini* glycans may simply reflect differences between induced immunity in rabbits and naturally acquired immune responses in humans. Therefore, identifying specific glycoprotein or glycopeptide epitopes as markers for *O. viverrini* infection might provide valuable diagnostic tools.

In conclusion, this study has presented basic data on glycan profiles of metacercariae and adult *O. viverrini*. Differences in the amounts of specific glycan structures may reflect their role in development and survival. The detected reduction in antigenicity upon removal of glycans indicates that they contribute to parasite immune responses. Further glycomic investigation of crucial glycoproteins, organ-, cell- or protein-specific glycosylation of the parasite is essential for understanding the glycobiology of host-parasite interactions that may lead to targeted drug discovery.

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Highlights

- *N*- and *O*-linked glycan profiling of metacercaria and adult *Opisthorchis viverrini* by mass spectrometry
- Detection and localization of glycan epitopes of metacercaria and adult *Opisthorchis viverrini* by lectin staining
- Characterization of the immunogenic glycoproteins of adult *Opisthorchis viverrini*

Fig. 1.

MS spectra of permethylated *N*-linked oligosaccharides of metacercariae and adult *Opisthorchis viverrini* by nanospray ionization mass spectrometry (NSI-MS). Glycans released from metacercariae and adult *O. viverrini* glycopeptides were permethylated and analyzed. MS spectra demonstrate the predominance of high mannose and truncated type oligosaccharides in metacercariae (A) and adults (B). Glycans are detected as singly and doubly charged species [2+]. The asterisks indicate fragments of a contaminating hexose ladder that are not derived from parasite material. The total *N*-linked glycan profiles of *O. viverrini* are shown in Tables 1 and 2. Graphical representations of glycan structures are as described in Fig. 2; circles represent hexoses (Man or Glc in this figure) and squares represent N-acetylhexosamines (GlcNAc in this figure).

Fig. 2.

The *N*-linked oligosaccharides of the metacercariae and adult *Opisthorchis viverrini*. The prevalence of each glycan is expressed as a percentage of the total pool of detected glycans (% Total Profile), high mannose and monofucosylated, truncated type oligosaccharides (A), complex and hybrid type glycans (B). Graphical representation of monosaccharide residues are as shown in the legend and are consistent with the suggested nomenclature of the Consortium for Functional Glycomics (<http://glycomics.scripps.edu/CFGnomenclature.pdf>). It is not always possible to assign the specific identity of a hexose (Hex) or *N*-acetylhexosamine (HexNAc) residue by mass spectrometry alone (e.g., Structure 23). In these cases, the circle representing the Hex or the square representing the HexNAc is left unfilled. Numerical designations for glycan structures are summarized in Tables 1 and 2.

The numerical assignments refer to m/z values, not to specific structures. Sets of isobaric glycans (structures with the same molecular weight) are assigned a single number.

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Fig. 3.

Representative MS² and MS³ spectra of permethylated *N*-linked oligosaccharides of *Opisthorchis viverrini*. Fragmentation of parent ion at m/z = 1346 of monofucosylated truncated type structure (A) and parent ion at m/z = 1866 of complex type structure (B) are shown. The parent ion at m/z = 1346 (Structure 6) fragments in MS² to give m/z = 1127 (loss of terminal Hex, $\Delta m/z = 219$), m/z = 894 (loss of reducing end GlcNAc with fucose, $\Delta m/z = 451$), and m/z = 474 (reducing end GlcNAc-fucose with Na⁺). The parent ion at m/z = 1866 (Structure 33) fragments in MS² to give m/z = 1606 (loss of terminal HexNAc, $\Delta m/z = 260$), m/z = 1588 (loss of reducing end GlcNAc, $\Delta m/z = 278$), and m/z = 1402 (loss of terminal HexHexNAc, $\Delta m/z = 464$). Fragmentation at MS³ defines the terminal Hex-HexNAc on extended *N*-linked glycans (C). Fragmentation at m/z = 1026²⁺ (Structure 17) yields the terminal disaccharide at m/z = 486. Further fragmentation at m/z = 486 produces

MS³ ions expected for Hex-HexNAc, as well as a cross-ring ion at $m/z = 329$, which assigns the linkage between the monosaccharides to the 4-position.

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Fig. 4.

A comparison of the *N*-linked oligosaccharides of metacercariae and adult *Opisthorchis viverrini*. The prevalence of each glycan is expressed as a percentage of the total pool of detected glycans (% Total Profile). The metacercariae consisted of high mannose (36.4%) and monofucosylated, truncated-type oligosaccharides (26.3%), complex and hybrid type glycans (37.3%) while the adult consisted of high mannose (67.5%) and monofucosylated, truncated-type oligosaccharides (12.5%), complex and hybrid type (20.0%).

Fig. 5.

Representative MS² and MS³ spectra reveal differential mixtures of the terminal HexNAc-HexNAc permethylated *N*-linked glycans in metacercariae and adult *Opisthorchis viverrini*. Fragmentation of parent ions at m/z = 1046²⁺ (Structures 19) detected in metacercariae and adults exposes the mixture of isobaric constituents that underlie each m/z value. The relative peak intensity of signature fragments indicates higher prevalence of the terminal LacdiNAc of m/z = 1046²⁺ in the adults. Compare fragments at m/z = 527 (A). Fragmentation at MS³ defines the terminal HexNAc-HexNAc on extended *N*-linked glycans. Fragmentation of the adult material at m/z = 1046²⁺ (Structure 19) yields the terminal disaccharide at m/z = 527. Further fragmentation at m/z = 527 produces MS³ ions expected for HexNAc-HexNAc (B).

Fig. 6. MS spectra of permethylated *O*-linked alditols of metacercariae and adult *Opisthorchis viverrini* by nanospray ionization mass spectrometry MS spectra demonstrates the predominance of the core 1 disacchride at *m/z* 534 in metacercariae (A) and adults (B). The total *O*-linked glycan profiles of *O. viverrini* are shown in Table 3.

Fig. 7.

The O-linked oligosaccharides of the metacercariae and adult *Opisthorchis viverrini*. The prevalence of each glycan is expressed as a percentage of the total pool of detected glycans (% Total Profile). The graphical representations and numerical designations of glycan structures are as described in Figs. 2, 6 and Table 3.

Fig. 8. MS² spectra reveal different mixtures of isobaric permethylated *O*-linked glycans in metacercariae and adult *Opisthorchis viverrini*. Fragmentation of parent ions at m/z = 738 (A, Structures 37) and m/z = 779 (B, Structures 38) detected in metacercariae and adults reveals the mixture of isobaric constituents that underlie each m/z value. The relative peak intensity of signature fragments indicates higher prevalence of the linear form of m/z = 738 in the metacercariae and of the linear form of m/z = 779 in the adults. Compare fragments at m/z = 463 and 502 (A), as well as at m/z = 486, 504, and 543 (B).

Fig. 9.

Lectin staining of excysted metacercariae and adult *Opisthorchis viverrini*. The in vitro excysted metacercariae were intensely stained by concanavalin A (ConA), peanut agglutinin (PNA) and wheat germ agglutinin (WGA) FITC-conjugated lectins (A, 400x). In paraffin sections of infected livers (B, 200x), *Pisum sativum* agglutinin (PSA) stained in tegument, tegumental cells, gut, eggs and testes in the adult parasite. PNA intensely stained tegument, tegumental cells and eggs and moderately stained ovaries. *Ulex europaeus* agglutinin (UEA-I) intensely stained gut tissue. WGA intensely stained tegument, tegumental cells, gut, eggs and testes and the biliary epithelium of the hamster. T, tegument; TC, tegumental cell; TS, testes; O, ovary; S, seminal receptacle; E, egg; G, gut; BE, biliary epithelium.

Fig. 10.

Deglycosylation and immunoblotting of *Opisthorchis viverrini* proteins and high performance thin layer chromatography (HPTLC) overlay of neoglycolipids produced from glycans released from *O. viverrini* proteins indicate that infected human serum optimally recognizes a mixed glycan-peptide epitope. Adult *O. viverrini* proteins were deglycosylated by chemical (A) or enzymatic (B) deglycosylation. The resulting proteins were separated by SDS-PAGE and subjected to Coomassie Brilliant Blue staining or immunoblotting using naturally *O. viverrini*-infected or normal human serum. The resulting blots and gels were quantified by densitometry and immunostaining was normalized to total protein (bar graphs; RU, Relative Units). Chemical deglycosylation with trifluoromethanesulfonic acid (TFMS) (A) and enzymatic deglycosylation with PNGaseF (B) both partially reduce immune serum antibody binding. N-linked glycans released from *O. viverrini* proteins by PNGaseF digestion were coupled to dipalmitoylphosphatidylethanolamine (DPPE) to generate neoglycolipids which were spotted onto HPTLC plates, resolved in

chloroform:methanol:water (60:35:8), and probed with immune or control human or rabbit serum (C). While serum from rabbits immunized with parasites recognized *O. viverrini* N-linked glycan neoglycolipids (arrowheads), serum from naturally infected humans did not bind to neoglycolipid glycans. DPPE indicates the position of the non-specific signal associated with residual, uncoupled phospholipid. The entire lane from the indicated TLC plate is shown, from the origin to the solvent front.

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Table 1

The characteristics and prevalence of high mannose and monofucosylated, truncated type *N*-glycans of metacercaria and adult *Opisthorchis viverrini*. The numerical designations assigned to each structure or each set of isobaric structures are used consistently in figures, tables and text. Graphical representations of monosaccharide residues are as described in Fig. 2.

Structure(s)	Observed m/z	Glycan Prevalence (% Total Profile) ^a	
		Metacercaria	Adult
1 MN2	764	2.5	0.7
2 MN2F	938	1.7	0.7
3 M2N2	968	4.0	1.1
4 M2N2F	1142	4.5	3.8
5 M3N2	1172	5.0	1.5
6 M3N2F	1346	3.8	2.6
7 M4N2	1376	3.6	1.6
8 M4N2F	1550	1.2	0.6
9 M5N2	1578	6.5	2.9
10 M6N2	1784	1.9	1.5
11 M7N2	1005 ²⁺ ^b	8.5	9.8
12 M8N2	1108 ²⁺	9.5	15.9
13 M9N2	1210 ²⁺	8.1	34.1
14 GlcM9N2	1312 ²⁺	1.9	3.4

^a The prevalence of each glycan is expressed as a percentage of the total pool of detected glycans (% Total Profile).

^b Doubly charged ions [m+2Na]²⁺ are denoted as ²⁺

Table 2

The characteristics and prevalence of complex and hybrid type *N*-glycans of metacercaria and adult *Opisthorchis viverrini*. The numerical designations assigned to each structure or each set of isobaric structures are used consistently in figures, tables and text. Graphical representations of monosaccharide residues are as described in Fig. 2.

Structure(s)		Observed m/z	Glycan Prevalence (% Total Profile) ^a	
			Metacercaria	Adult
15 GalNM4N2 and NM5N2		924 ²⁺ <i>b</i>	2.1	2.8
16 GalNM4N2F and NM5N2F		1012 ²⁺	1.6	3.5
17 GalNM5N2		1026 ²⁺	4.9	1.8
18 GalN2M3N2F and N2M4N2F		1032 ²⁺	2.4	1.2
19 Hex-GalN2M3N2 and N2M5N2		1046 ²⁺	5.5	0.7
20 N3M3N2F		1052 ²⁺	2.3	2.0
21 GalNM5N2F		1114 ²⁺	1.8	1.6
22 N2M5N2F and Hex-GalN2M3N2F		1134 ²⁺	2.4	1.1
23 Hex-GalN2M4N2 and Hex2N2M4N2		1150 ²⁺	1.6	0.6
24 N4M3N2F		1176 ²⁺	1.6	1.0
25 N3M5N2F and Hex-GalN3M3N2F		1256 ²⁺	1.7	0.8
26 Hex-GalN3M3N2F		1359 ²⁺	1.0	0.5
27 NM3N2		1418	1.8	0.4
28 NM3N2F		1592	1.2	0.7
29 GalNM3N2		1662	1.0	0.4
30 N2M3N2		1662	1.1	0.2
31 GalNM3N2F and NM4N2F		1796	1.1	0.3

Structure(s)	Observed m/z	Glycan Prevalence (% Total Profile) ^a	
		Metacercaria	Adult
32 N2M3N2F	1836	1.1	0.5
33 GalN2M3N2	1867	1.1	0.2

^aThe prevalence of each glycan is expressed as a percentage of the total pool of detected glycans (% Total Profile)

^bDoubly charged ions [m+2Na]²⁺ are denoted as ²⁺

Table 3

The characteristics and prevalence of *O*-linked glycans of metacercaria and adult *Opisthorchis viverrini*. The numerical designations assigned to each structure or to each set of isobaric structures are used consistently in figures, tables and text. Graphical representations of monosaccharide residues are as described in Fig. 2.

Structure(s)	Observed m/z	Glycan Prevalence (% Total Profile) ^a	
		Metacercaria	Adult
34 HexNAc-ol	330	2.1	12.8
35 Hex-HexNAc-ol	534	79.3	51.1
36 HexNAc-HexNAc-ol	576	2.3	5.5
37 Hex-Hex-HexNAc-ol and Hex-[Hex-]HexNAc-ol	and 738	1.4	18.0
38 HexNAc-Hex-HexNAc-ol, Hex-HexNAc-HexNAc-ol, and HexNAc-[Hex-]HexNAc-ol	and and 779	2.8	8.6
39 HexNAc-[HexNAc-]HexNAc-ol	822	0.6	1.5
40 Hex-HexNAc-[Hex-]HexNAc-ol and HexNAc-[Hex-Hex-]HexNAc-ol	and 984	9.7	1.6
41 Hex-HexNAc-[HexNAc-]HexNAc-ol	1024	0.8	0.4
42 Hex-HexNAc-[Hex-HexNAc-]HexNAc-ol and HexNAc-HexNAc-[Hex-Hex-]HexNAc-ol	and 1230	0.6	0.4

^aThe prevalence of each glycan is expressed as a percentage of the total pool of detected glycans (% Total Profile).

Table 4

Carbohydrate recognition preferences of lectins used in this study.

Lectin	Binding preference
Concanavalin A (ConA)	α -Man> α -Glc> GlcNAc
Peanut Agglutinin (PNA)	Gal β 1, 3GalNAc>> α and β Gal
<i>Pisum Sativum</i> Agglutinin (PSA)	α -Man
<i>Ulex europaeus</i> I agglutinin (UEA-I)	α -Fuc
Wheat Germ Agglutinin (WGA)	GlcNAc and Sialic acid

Man, mannose; Gal, galactose; Fuc, fucose; GalNAc, *N*-acetylgalactosamine; GlcNAc, *N*-acetylglucosamine.