

Structural mapping of the glycans from the egg glycoproteins of *Schistosoma mansoni* and *Schistosoma japonicum*: identification of novel core structures and terminal sequences

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The structural diversity of the glycans from *Schistosoma mansoni* and *Schistosoma japonicum* egg glycoproteins was investigated using high sensitivity fast atom bombardment mass spectrometric screening of glycan pools released enzymically or chemically from egg extracts. The egg glycoproteins from the two species carry a comparable range of high mannose and complex type N-glycans with both lacNAc and lacdiNAc constituting the backbones of the antennae in the latter class. Truncated N-glycans similar to those found on nematodes, insects, and plants were also identified. Sequential digestion with peptide N-glycosidase F and peptide N-glycosidase A afforded effective release and separation of N-glycans with nonfucosylated or α 6-monofucosylated trimannosyl N,N'-diacetyl-chitobiose cores from those carrying core α 3-, α 6-difucosylation, all of which were found to be present in both species. Remarkably, a portion of the N-glycans from *S. mansoni* eggs was shown to be based on a xylosylated, α 6-fucosylated trimannosyl core, whereas a portion of those from *S. japonicum* contains a xylosylated α 3-, α 6-difucosylated core which has not been previously described in any organism. O-Glycans were chemically released from the de-N-glycosylated glycopeptides and found to carry terminal sequences similar to those in the N-glycans. This study provides further evidence that multi-fucosylated terminal HexNAc units, previously identified on the cercarial glycocalyx O-glycans and egg glycosphingolipids, and now on the egg N- and O-glycans, are unique features of *S. mansoni* glycans. These multifucosylated terminal structures, which were not detected on the egg glycans of *S. japonicum*, are likely to constitute the cross-reacting epitopes between the eggs and cercariae of *S. mansoni*. Interestingly other HexNAc termini, including an unusual stretch of HexNAc₃, were found to be common to both species. The mapping studies reported in this article provide an important foundation for further structural work in this challenging and important area of glycobiology.

Key words: egg antigens/glycoproteins/mass spectrometry/*Schistosoma mansoni*/*Schistosoma japonicum*

Introduction

Human intestinal schistosomiasis is caused by the two major species of the digenetic trematodes, *Schistosoma mansoni* and *S. japonicum*. The former is endemic in Africa, the Middle East, the Caribbean, and a few countries in South America, whereas the latter is found mainly in China, Philippines and Indonesia. Morbidity is due primarily to deposition of parasite eggs which induce a delayed hypersensitivity granulomatous response, leading to the development of fibrosis, and eventually hepatic destruction, cirrhosis, and liver failure in chronic infections. Although the general pathology is similar, there are notable differences in host specificity and distribution of egg deposition. Thus, *S. mansoni* infects human, primates, and a few rodents, whereas *S. japonicum* infects a broad range of domestic animals, e.g., pigs, water buffalo, rodents, as well as human (Dean, 1985). Adult *S. mansoni* localizes to the portal veins draining the large intestine and deposits eggs in the wall of the colon whereas adult *S. japonicum* lives and deposits eggs in the small intestine. Those eggs which adhere and successfully penetrate the vessels enter the feces and hatch in freshwater into miracidia that infect specific snail hosts, *Biomphalaria glabrata* and *Oncomelania hupensis* for *S. mansoni* and *S. japonicum*, respectively.

Human infection is initiated when the larval worms, called cercariae, penetrate the host skin. The onset of egg-laying elicits strong serological responses that recognize mostly saccharide antigens on the surface of the cercariae in addition to those released by the eggs. Glycoproteins (Boros and Warrens, 1970; Carter and Colley, 1979; Dunne *et al.*, 1981; Tracy and Mahmoud, 1982; Norden and Strand, 1984) and glycolipids (Weiss *et al.*, 1986; Levery *et al.*, 1992) have been identified as egg antigens in both *S. mansoni* and *S. japonicum*. Monoclonal antibodies against the egg antigens of *S. mansoni* have been shown to cross-react with its cercarial glycocalyx and to protect against infection in passive transfer experiments (Grzych *et al.*, 1982; Harn *et al.*, 1984). Conversely, a monoclonal antibody prepared against *S. mansoni* cercarial glycoproteins associated with the glycocalyx was shown to recognize an epitope present on both glycolipid and glycoprotein fractions of the egg antigens (Weiss and Strand, 1985; Weiss *et al.*, 1986; Dalton *et al.*, 1987).

Recent structural studies on the egg glycolipids (Levery *et al.*, 1992; Khoo *et al.*, 1997) and cercarial glycocalyx (Khoo *et al.*, 1995) of *S. mansoni* indicated that both contain similar multifucosylated repeating units and terminal sequences which may constitute the structural basis for their cross-reactivities. No structural data has yet been reported on the *S. mansoni* egg glycoproteins which may be inferred from the immunological data to carry similar fucosylated epitopes (Weiss *et al.*, 1986). In fact, as an understanding begins to emerge of the range of glycan structures present in each developmental stage of

S. mansoni associated with the definitive host (Cummings and Nyame, 1996), the glycan structures of its egg glycoproteins remain the only uncharted territory. On the other hand, virtually nothing is known about the glycan structures of the oriental schistosome *S. japonicum*. Sera of patients infected with *S. japonicum* was shown to immunoprecipitate only a selected few of the major *S. mansoni* egg glycoproteins, indicating that egg antigens from the two species share some common epitopes and yet are sufficiently distinct (Norden and Strand, 1984). Our own recent work demonstrated that the multifucosylated structures which characterize the egg glycosphingolipids of *S. mansoni* are apparently absent in the egg glycolipids of *S. japonicum* (Khoo *et al.*, 1997).

We report here results of a study addressing the structural basis of cross-reacting epitopes in *S. mansoni* egg antigens. In addition we have compared the N- and O-glycans present in the egg glycoproteins of *S. mansoni* with those from *S. japonicum*, as a first step towards identifying species-specific glycans which may influence host specificity and localization to target organs. We show that the structures of the N- and O-glycans are broadly similar in the two species, but, significantly, there are species-specific terminal structures. In addition, both species were found to synthesize unusual N-glycans in which plant-like xylose substitution occurs on the β -mannose of mono- and di-fucosylated trimannosyl cores.

Results

Strategy for probing glycan structures with minimal purification

Delipidated *S. mansoni* and *S. japonicum* egg homogenates were extracted with guanidine hydrochloride to yield a crude preparation of schistosomal egg glycoproteins. Sugar analysis indicated the presence of a large quantity of glucose which was attributable to glycogen-like polymers by subsequent methylation analysis. To remove the majority of these glucan polymers, as well as to facilitate the release of N-glycans without resorting to detergent denaturation, the crude preparation of glycoproteins was first subjected to tryptic/chymotryptic digestion followed by reverse phase C18 Sep-Pak purification. Glycans were then released from the resulting peptides/glycopeptides by sequential digestions with PNGase F and PNGase A, and finally by reductive elimination.

The rationale of using successive PNGase F and PNGase A digestions was based on the established observation that (1) N-glycans which are α 3-fucosylated at the innermost GlcNAc of their *N,N'*-diacetyl-chitobiose core can only be efficiently released by PNGase A and not PNGase F (Tretter *et al.*, 1991); (2) many of the N-glycans from the lower animals including helminths and insects have been reported to carry such core α 3-fucosylation instead of, or in addition to, α 6-fucosylation (März *et al.*, 1995; Haslam *et al.*, 1996). Thus, an initial PNGase F treatment will release N-glycans which are either nonfucosylated or α 6-fucosylated at their *N,N'*-diacetyl-chitobiose cores. Subsequent PNGase A treatment of the recovered glycopeptides will selectively release N-glycans with α 3-fucosylated and α 3-, α 6-difucosylated cores, if present. It should be noted that some residual N-glycans which do not carry core α 3-fucosylation may also be further released during this second PNGase treatment if the first digestion is incom-

plete. Finally the O-glycans are selectively released from the N-deglycosylated glycopeptides by reductive elimination yielding oligosaccharide alditols, i.e., glycans with reduced reducing ends. Each of the *S. mansoni* (Sm) and *S. japonicum* (Sj) egg preparations therefore yielded two distinct populations of N-glycans and a pool of O-glycans, designated as SmNF or SjNF for N-glycans released by PNGase F; SmNA or SjNA for N-glycans released by PNGase A after PNGase F treatment; and SmO or SjO for reduced O-glycans released by reductive elimination.

For a rapid assessment of the range and complexity of these glycan mixtures, samples were permethylated for FAB-MS and linkage analysis. As expected from a direct analysis of such a crude preparation, the FAB-mass spectra of the permethyl derivatives of each of the N- and O-glycan pools comprised a multitude of signals. Although an unambiguous and comprehensive identification of all components was not possible, several important structural features could nevertheless be firmly established based on assignments of the major signals. Thus, the compositions of the molecular ions as deduced from their precise *m/z* values, when considered in conjunction with methylation analysis data and the range of nonreducing end oxonium fragment ions (A-type ions; Dell, 1987) produced, allowed important structural conclusions to be drawn, including the identification of unexpected xylose-containing structures (see below).

Nonreducing terminal sequences

The compilation of terminal A-type fragment ions afforded by each of the glycan pools (Table I) indicates that most of the terminal sequences are common to the N- and O-glycans of a particular species. The differences are largely confined to quantitative variations and the fact that the O-glycans contain a higher proportion of glycans with additional $\pm$ Fuc₁(Hex-HexNAc) units than the N-glycans. Thus, Hex-HexNAc and Fuc₁(Hex-HexNAc) not only constitute the major termini of all glycan pools, but also comprise the inner units of extended antennae. Although stereochemical features remain to be confirmed, the Hex-HexNAc moiety defined by mass spectrometry is likely to be the *N*-acetylglucosamine unit Gal β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ and, when fucosylated, the Lewis x epitope Gal β 1 $\rightarrow$ 4(Fuc α 1 $\rightarrow$ 3) GlcNAc β 1 $\rightarrow$. These sequences may be further galactosylated at the non-reducing end to give the $\pm$ Fuc₁Hex₂HexNAc⁺ type of termini (group II, Table I) which were found on the glycans from *S. japonicum* but not *S. mansoni*.

Nonfucosylated terminal HexNAc was present in all samples but, interestingly, mono- and di-fucosylated HexNAc⁺ were detected as major ions only in the N- and O-glycans of *S. mansoni*. Similarly, although HexNAc₂⁺ and Fuc₁HexNAc₂⁺ were found in all glycan pools, the occurrence of multifucosylated HexNAc₂ units was restricted to *S. mansoni*. It thus appears that both *S. japonicum* and *S. mansoni* are capable of making HexNAc-($\pm$ Fuc)HexNAc- terminal structures, but only *S. mansoni* is able to further fucosylate this structural unit. The presence of Fuc_{1,2}Hex₁HexNAc₃⁺ suggests that HexNAc-($\pm$ Fuc)HexNAc-, like other terminal structures, may be linked to the core via additional [$\pm$ Fuc₁(Hex₁HexNAc₁)]_n units at the reducing end. It is possible that HexNAc-($\pm$ Fuc)HexNAc can also occur as an internal unit linked to a terminal $\pm$ Fuc₁(Hex₁HexNAc₁), i.e., $\pm$ Fuc₁(Hex₁HexNAc₁)-HexNAc-

Table I. Nonreducing terminal structures of the glycans from schistosomal egg glycoproteins as defined by the fragment ions afforded by their permethyl derivatives

Signal assignment		Schistosomal glycans					
<i>m/z</i>	Composition	SmNF	SmNA	SmO	SjNF	SjNA	SjO
I) [$\pm$Fuc₁(Hex-HexNAc)]_n							
464	Hex ₁ HexNAc ₁ ⁺	O	O	O	O	O	O
638	Fuc ₁ Hex ₁ HexNAc ₁ ⁺	✓	O	✓	O	O	O
913	Hex ₂ HexNAc ₂ ⁺	✓	X	✓	✓	✓	✓
1087	Fuc ₁ Hex ₂ HexNAc ₂ ⁺	✓	X	✓	✓	O	O
1261	Fuc ₂ Hex ₂ HexNAc ₂ ⁺	✓	X	✓	✓	✓	O
1536	Fuc ₁ Hex ₃ HexNAc ₃ ⁺	X	X	✓	X	X	✓
1710	Fuc ₂ Hex ₃ HexNAc ₃ ⁺	X	X	✓	X	X	✓
II) $\pm$Fuc₁(Hex₂-HexNAc)							
668	Hex ₂ HexNAc ₁ ⁺	X	X	X	O	✓	✓
842	Fuc ₁ Hex ₂ HexNAc ₁ ⁺	X	X	X	✓	✓	✓
1291	Fuc ₁ [Hex ₂ HexNAc ₁ (Hex ₁ HexNAc ₁)] ⁺	X	X	X	X	X	✓
1465	Fuc ₂ [Hex ₂ HexNAc ₁ (Hex ₁ HexNAc ₁)] ⁺	X	X	X	X	✓	O
III) Fuc_nHexNAc₁₋₃							
260	HexNAc ⁺	O	✓	O	O	O	O
434	Fuc ₁ HexNAc ₁ ⁺	O	✓	✓	X	X	X
608	Fuc ₂ HexNAc ₁ ⁺	O	✓	✓	X	X	X
505	HexNAc ₂ ⁺	✓	✓	✓	✓	✓	✓
679	Fuc ₁ HexNAc ₂ ⁺	✓	✓	✓	O	✓	✓
853	Fuc ₂ HexNAc ₂ ⁺	O	✓	✓	X	X	X
1027	Fuc ₃ HexNAc ₂ ⁺	✓	✓	✓	X	X	X
1201	Fuc ₄ HexNAc ₂ ⁺	✓	✓	✓	X	X	X
1128	Fuc ₁ [HexNAc ₂ (Hex ₁ HexNAc ₁)] ⁺	X	X	✓	X	✓	✓
1302	Fuc ₂ [HexNAc ₂ (Hex ₁ HexNAc ₁)] ⁺	X	X	✓	X	✓	O
750	HexNAc ₃ ⁺	✓	X	✓	✓	✓	✓
924	Fuc ₁ HexNAc ₃ ⁺	✓	✓	✓	✓	✓	✓
1098	Fuc ₂ HexNAc ₃ ⁺	✓	X	✓	X	X	X
1373	Fuc ₁ [HexNAc ₃ (Hex ₁ HexNAc ₁)] ⁺	X	X	✓	X	X	X
1547	Fuc ₁ [HexNAc ₃ (Hex ₁ HexNAc ₁)] ⁺	X	X	✓	X	X	✓
IV) (Hex₁HexNAc₁)-HexNAc							
709	Hex ₁ HexNAc ₂ ⁺	✓	✓	✓	✓	✓	✓
883	Fuc ₁ Hex ₁ HexNAc ₂ ⁺	O	✓	O	O	O	O
1506	Fuc ₂ [Hex ₁ HexNAc ₂ (Hex ₁ HexNAc ₁)] ⁺	X	X	✓	X	✓	O

Data were compiled from at least two separate FAB-MS analyses of each glycan pool. X, not detected; ✓, detected at significant level in at least one preparation; O, consistently present as the major signals.

($\pm$ Fuc)HexNAc, which would yield $\pm$ Fuc₁Hex₁HexNAc₂⁺ (*m/z* 709, 883) as additional terminal fragment ions. Alternatively, $\pm$ Fuc₁Hex₁HexNAc₂⁺ may represent a unique terminal motif (group IV), resulting from further glycosylation of the terminal *N*-acetylglucosamine unit with an additional HexNAc.

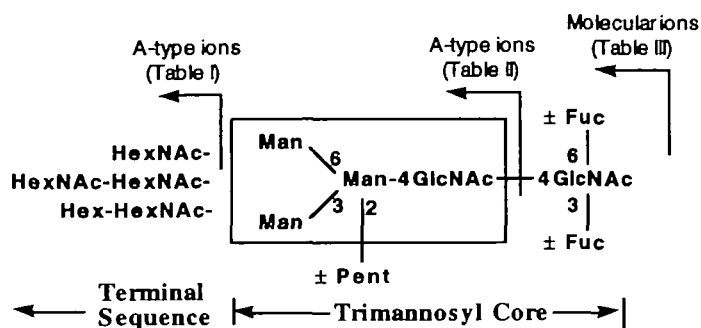
It is unclear how the Fuc₀₋₂HexNAc₃⁺ ions are related to the variably fucosylated HexNAc₁⁺ and HexNAc₂⁺ ions. The absence of Fuc_nHexNAc₄⁺ ions suggests that there is not a stretch of nonreducing terminal HexNAc deriving from two or more consecutive HexNAc₂ units. Instead, the Fuc₀₋₂HexNAc₃ termini are likely to result from addition of a terminal Fuc₀₋₂HexNAc unit to a subterminal HexNAc-($\pm$ Fuc)HexNAc unit. The absence of Fuc₂HexNAc₃⁺ in *S.japonicum* would then be consistent with the absence of fucosylated terminal HexNAc in the glycans of this species.

Mapping the schistosomal egg N-glycans

In addition to fragment ions representing common terminal sequences in N- and O-glycans (Table I), additional sets of

A-type ions unique to the N-glycans are present (Table II). These are derived from cleavage between the two GlcNAc residues of the *N,N'*-diacetyl-chitobiose core (see Scheme 1), the assignments of which were largely corroborated by the identification of corresponding molecular ions in the respective spectra (Table III). Based on the FAB-MS data (Tables I–III) and currently accepted models of eukaryotic N-glycan biosynthesis, these schistosomal egg N-glycans may be conveniently grouped into the following three classes: (1) high mannose structures having from five to nine mannoses and no core fucosylation, (2) truncated structures containing two to four mannoses with no additional Gal or GlcNAc, and (3) complex type structures based on a trimannosyl core and extended with the terminal sequences tabulated in Table I. The high mannose type of structures were present in both species and were completely released by PNGase F as expected. In contrast, the latter two classes were distributed between the PNGase F and PNGase A released materials depending on the kind of core fucosylation present. The salient and unique structural features of the complex and truncated types of N-glycans from the eggs of the two schistosome species are described in scheme 1.

Scheme 1



N-glycans from the eggs of *S. japonicum*

The permethyl derivatives of SjNF (Figure 1) afforded four major molecular ions which correspond to the truncated class, namely $\pm$ Fuc₁Hex₂HexNAc₂ (m/z 945, 1119) and $\pm$ Fuc₁Hex₃HexNAc₂ (m/z 1149, 1323). Of the complex type, the most prominent ones were Fuc₁Hex₃HexNAc₃ (m/z 1568) and Fuc₁Hex₅HexNAc₄ (m/z 2221), corresponding to a trimannosylated core with an additional HexNAc and two additional Hex₁HexNAc₁ units, respectively (see Table III). Most of the fucosylated components were efficiently defucosylated by α -fucosidase from bovine kidney as demonstrated by the disappearance of the molecular ion signals attributed to fucosylated components in the FAB-spectrum of the resulting permethyl derivatives (data not shown).

Methylation analysis of SjNF showed the presence of terminal Fuc, terminal Man/Glc, terminal Gal, 2-linked Man, 3,6-linked Man, 4-linked GlcNAc, and 3,4-linked GlcNAc as major peaks. At a lower level, 3-linked Gal, 2,4-linked Man, 2,6-linked Man, terminal GalNAc, terminal GlcNAc, 3-linked GalNAc, and 4,6-linked GlcNAc were also detected. Thus, the major peaks observed are consistent with normal biantennary N-glycans terminating with Gal-4($\pm$ Fuc-3)GlcNAc, as well as truncated trimannosyl core structures. The low level of 3-linked Gal suggested that the *N*-acetylglucosamine unit occurred mainly as a terminal sequence although a small proportion also constituted the internal unit -3Gal-4($\pm$ Fuc-3)GlcNAc-. The presence of terminal GlcNAc, terminal GalNAc, and 3-linked GalNAc indicated that the Fuc₁HexNAc₁₋₃ terminal sequence may be a mixture of different structures based on -4($\pm$ Fuc-3)GlcNAc and -3GalNAc-, terminating in either GalNAc or GlcNAc whereas 4,6-GlcNAc supported the presence of core α 6-fucosylation which was susceptible to α -fucosidase digestion.

Despite similarity in A-type ions (Table I), the FAB-spectrum of SjNA (Figure 2) differed significantly from that of SjNF (Figure 1) in the molecular ion region (Tables II, III). Notably, all the molecular ion signals afforded by SjNA contain at least two Fuc residues whereas the major molecular ions in SjNF correspond to nonfucosylated or monofucosylated components. Treatment with bovine kidney α -fucosidase effected complete defucosylation of SjNF glycans but only efficiently removed a single fucose from the difucosylated components of SjNA suggesting that the second fucose in SjNA glycans is resistant to this enzyme. A second striking difference was the presence in SjNA spectra, but not in SjNF spectra, of an ion series corresponding to glycans containing a pentose residue (see Table III).

Assignments of molecular ions for most of the smaller di-

Table II. Core-related fragment ions afforded by the permethyl derivatives of schistosomal egg N-glycans

Signal assignment		Schistosomal egg N-glycans			
m/z	Composition	SmNF	SmNA	SjNF	SjNA
872	Hex ₃ HexNAc ₁ ⁺	X	X	✓	X
1032	Pent ₁ Hex ₃ HexNAc ₁ ⁺	O	X	X	✓
1117	(HexNAc ₁)Hex ₃ HexNAc ₁ ⁺	X	X	✓	X
1277	(HexNAc ₁)Pent ₁ Hex ₃ HexNAc ₁ ⁺	✓	X	X	✓
1321	(Hex ₁ HexNAc ₁)Hex ₃ HexNAc ₁ ⁺	X	✓	✓	X
1495	(Fuc ₁ Hex ₁ HexNAc ₁)Hex ₃ HexNAc ₁ ⁺	X	X	X	✓
1770	(Hex ₂ HexNAc ₂)Hex ₃ HexNAc ₁ ⁺	X	O	X	✓
1944	(Fuc ₁ Hex ₂ HexNAc ₂)Hex ₃ HexNAc ₁ ⁺	X	O	X	X
2118	(Fuc ₂ Hex ₂ HexNAc ₂)Hex ₃ HexNAc ₁ ⁺	X	✓	X	X
1522	(HexNAc ₂)Pent ₁ Hex ₃ HexNAc ₁ ⁺	X	X	X	✓
1726	(Hex ₁ HexNAc ₂)Pent ₁ Hex ₃ HexNAc ₁ ⁺	X	X	X	✓
1811	(Hex ₁ HexNAc ₃)Hex ₃ HexNAc ₁ ⁺	X	X	X	✓
1852	(HexNAc ₄)Hex ₃ HexNAc ₁ ⁺	X	X	X	✓
1076	Hex ₄ HexNAc ₁ ⁺	X	X	✓	X
1280	Hex ₃ HexNAc ₁ ⁺	✓	X	✓	X
1484	Hex ₆ HexNAc ₁ ⁺	✓	X	✓	X
1688	Hex ₇ HexNAc ₁ ⁺	✓	X	✓	X
1892	Hex ₈ HexNAc ₁ ⁺	✓	X	✓	X
2096	Hex ₉ HexNAc ₁ ⁺	✓	X	✓	X

The absence of a particular A-type ion may be due to high chemical noise in the relevant region of the spectrum and/or weak molecular ions and does not necessarily negate the assignment of a particular molecular ion signal in Table III. X, Not detected; ✓, detected at significant level in at least one preparation; O, consistently present as the major signals.

fucosylated components in SjNA were corroborated by their respective fragment ions resulting from cleavage of the chitobiose core (Tables II, III; Scheme 1). Thus, for example, the Pent-containing molecular ions Fuc₂Pent₁Hex₃HexNAc₂ (m/z 1657) and Fuc₂Pent₁Hex₃HexNAc₃ (m/z 1902) gave the A-type fragment ions Pent₁Hex₃HexNAc₁⁺ (m/z 1032) and Pent₁Hex₃HexNAc₂⁺ (m/z 1277), respectively. The Pent residue appeared to be associated with the trimannosyl core since (1) it could be detected in molecular ions as small as Fuc₂Pent₁Hex₃HexNAc₂ and (2) Pent-containing terminal sequence ions (Table I, Scheme 1) were not found. In addition, the nonfucosylated fragment ions produced indicated that both Fuc residues were attached to the reducing end GlcNAc, consistent with an α 3-, α 6-difucosylated core structure which would only be released by PNGase A and not PNGase F. This conclusion is also supported by our fucosidase experiments coupled with previous reports (Takahashi *et al.*, 1990; Kubelka *et al.*, 1993) which demonstrated that α -fucosidase from bovine kidney could only remove the α 6-Fuc and not the α 3-Fuc when used at low dose (0.1 mU).

The linkage analysis profile of SjNA was similar to SjNF with a few notable differences. Firstly, 3,4,6-linked GlcNAc was detected and not 4,6-linked GlcNAc, consistent with the assignment of an α 3-, α 6-difucosylated core structure. Secondly, 2,3,6-linked Man and a terminal Pent were found to be present only in SjNA. While the small amount of 2,4- and 2,6-linked Man that were detected in both SjNA and SjNF was an indication of the presence of tri- and tetraantennary types of complex N-glycans, 2,3,6-linked Man is not a residue com-

Table III. Assignment and tentative interpretation of the molecular ions afforded by the permethyl derivatives of schistosomal egg N-glycans

Signal assignment		Schistosomal egg N-glycans			
<i>m/z</i>	Composition, [M + H] ⁺	SmNF	SmNA	SjNF	SjNA
High mannose type structures					
1557	Hex ₅ HexNAc ₂	✓	X	✓	X
1761	Hex ₆ HexNAc ₂	✓	X	✓	X
1965	Hex ₇ HexNAc ₂	✓	X	✓	X
2169	Hex ₈ HexNAc ₂	✓	X	✓	X
2373	Hex ₉ HexNAc ₂	✓	X	✓	X
Truncated core structures					
945	Hex ₂ HexNAc ₂	✓	X	✓	X
1119	Fuc ₁ Hex ₂ HexNAc ₂	✓	X	O	X
1279	Fuc ₁ Pent ₁ Hex ₂ HexNAc ₂	O	X	X	X
1149	Hex ₃ HexNAc ₂	✓	X	O	X
1323	Fuc ₁ Hex ₃ HexNAc ₂	✓	X	✓	X
1483	Fuc ₁ Pent ₁ Hex ₃ HexNAc ₂	O	X	X	X
1497	Fuc ₂ Hex ₃ HexNAc ₂	X	X	X	✓
1657	Fuc ₂ Pent ₁ Hex ₃ HexNAc ₂	X	X	X	✓
1353	Hex ₄ HexNAc ₂	✓	X	✓	X
1527	Fuc ₁ Hex ₄ HexNAc ₂	✓	X	✓	X
Complex type structures^a					
HexNAc ₁ -Man ₃ GlcNAc ₂					
1394	Hex ₃ HexNAc ₃	X	X	✓	X
1568	Fuc ₁ Hex ₃ HexNAc ₃	✓	X	O	X
1728	Fuc ₁ Pent ₁ Hex ₃ HexNAc ₃	✓	X	X	X
1902	Fuc ₂ Pent ₁ Hex ₃ HexNAc ₃	X	X	X	✓
Hex ₁ HexNAc ₁ -Man ₃ GlcNAc ₂					
1772	Fuc ₁ Hex ₄ HexNAc ₃	X	X	✓	X
1946	Fuc ₂ Hex ₄ HexNAc ₃	X	✓	X	X
Hex ₂ HexNAc ₁ -Man ₃ GlcNAc ₂ ^b					
1802	Hex ₄ HexNAc ₃	✓	X	✓	X
1976	Fuc ₁ Hex ₅ HexNAc ₃	✓	X	✓	X
2150	Fuc ₂ Hex ₅ HexNAc ₃	✓	X	✓	X
HexNAc ₂ -Man ₃ GlcNAc ₂					
1813	Fuc ₁ Hex ₃ HexNAc ₄	✓	X	✓	X
1973	Fuc ₁ Pent ₁ Hex ₃ HexNAc ₄	✓	X	X	X
2147	Fuc ₂ Pent ₁ Hex ₃ HexNAc ₄	X	X	X	✓
2321	Fuc ₃ Pent ₁ Hex ₃ HexNAc ₄	✓	X	X	X
Hex ₁ HexNAc ₂ -Man ₃ GlcNAc ₂					
1843	Hex ₄ HexNAc ₄	✓	X	✓	X
2017	Fuc ₁ Hex ₄ HexNAc ₄	O	X	✓	X
2191	Fuc ₂ Hex ₄ HexNAc ₄	✓	X	✓	X
HexNAc ₃ -Man ₃ GlcNAc ₂					
1884	Hex ₃ HexNAc ₅	X	X	✓	X
2058	Fuc ₁ Hex ₃ HexNAc ₅	✓	X	✓	X
2566	Fuc ₃ Pent ₁ Hex ₃ HexNAc ₅	✓	X	X	X
(Hex ₁ HexNAc ₁) ₂ -Man ₃ GlcNAc ₂					
2221	Fuc ₁ Hex ₅ HexNAc ₄	O	X	O	X
2395	Fuc ₂ Hex ₅ HexNAc ₄	✓	O	✓	✓
2569	Fuc ₃ Hex ₅ HexNAc ₄	✓	O	X	✓
2743	Fuc ₄ Hex ₅ HexNAc ₄	X	✓	X	✓
(Hex ₁ HexNAc ₁)(HexNAc ₂)-Man ₃ GlcNAc ₂					
2262	Fuc ₁ Hex ₄ HexNAc ₅	✓	X	✓	X
2436	Fuc ₂ Hex ₄ HexNAc ₅	X	X	✓	✓
2610	Fuc ₃ Hex ₄ HexNAc ₅	X	X	X	✓
2784	Fuc ₄ Hex ₄ HexNAc ₅	X	X	X	✓
(HexNAc ₂) ₂ -Man ₃ GlcNAc ₂					
2303	Fuc ₁ Hex ₃ HexNAc ₆	X	X	✓	X
2477	Fuc ₂ Hex ₃ HexNAc ₆	X	X	X	✓
2651	Fuc ₃ Hex ₃ HexNAc ₆	X	X	X	✓
(Hex ₁ HexNAc ₁)(Hex ₁ HexNAc ₂)-Man ₃ GlcNAc ₂					
2466	Fuc ₁ Hex ₅ HexNAc ₅	✓	X	✓	X
2640	Fuc ₂ Hex ₅ HexNAc ₅	X	X	✓	X
2814	Fuc ₃ Hex ₅ HexNAc ₅	X	X	✓	X
(Hex ₁ HexNAc ₁) ₃ -Man ₃ GlcNAc ₂					
2496	Hex ₆ HexNAc ₅	✓	X	X	X
2670	Fuc ₁ Hex ₆ HexNAc ₅	O	X	✓	X
2844	Fuc ₂ Hex ₆ HexNAc ₅	X	X	✓	X

X, Not detected; ✓, detected at significant level in at least one preparation; O, consistently present as the major signals.

^aTentative assignment based on the assumption that each N-glycan contains a trimannosyl *N,N'*-diacetyl-chitobiose core. A further truncated dimannosyl *N,N'*-diacetyl-chitobiose core which is extended with additional terminal glycosylation at either the Man α 1 $\rightarrow$ 6 or Man α 1 $\rightarrow$ 3 arm only is also possible. Likewise, alternative assignment as hybrid type structures is also consistent with some of the compositions. The position of fucosylation is not differentiated in this table but may be partly inferred from their composition and sensitivity towards PNGase F and PNGase A. Each of the saccharide modules in parentheses may be linked to one another in one linear sequence or distributed among the antenna.

^bSince the Hex₂HexNAc₁⁺ fragment ion was not detected in SmNF, Hex₃HexNAc₃ in SmNF is likely to be of hybrid type, possibly (Hex₁HexNAc₁)-Man₄GlcNAc₂.

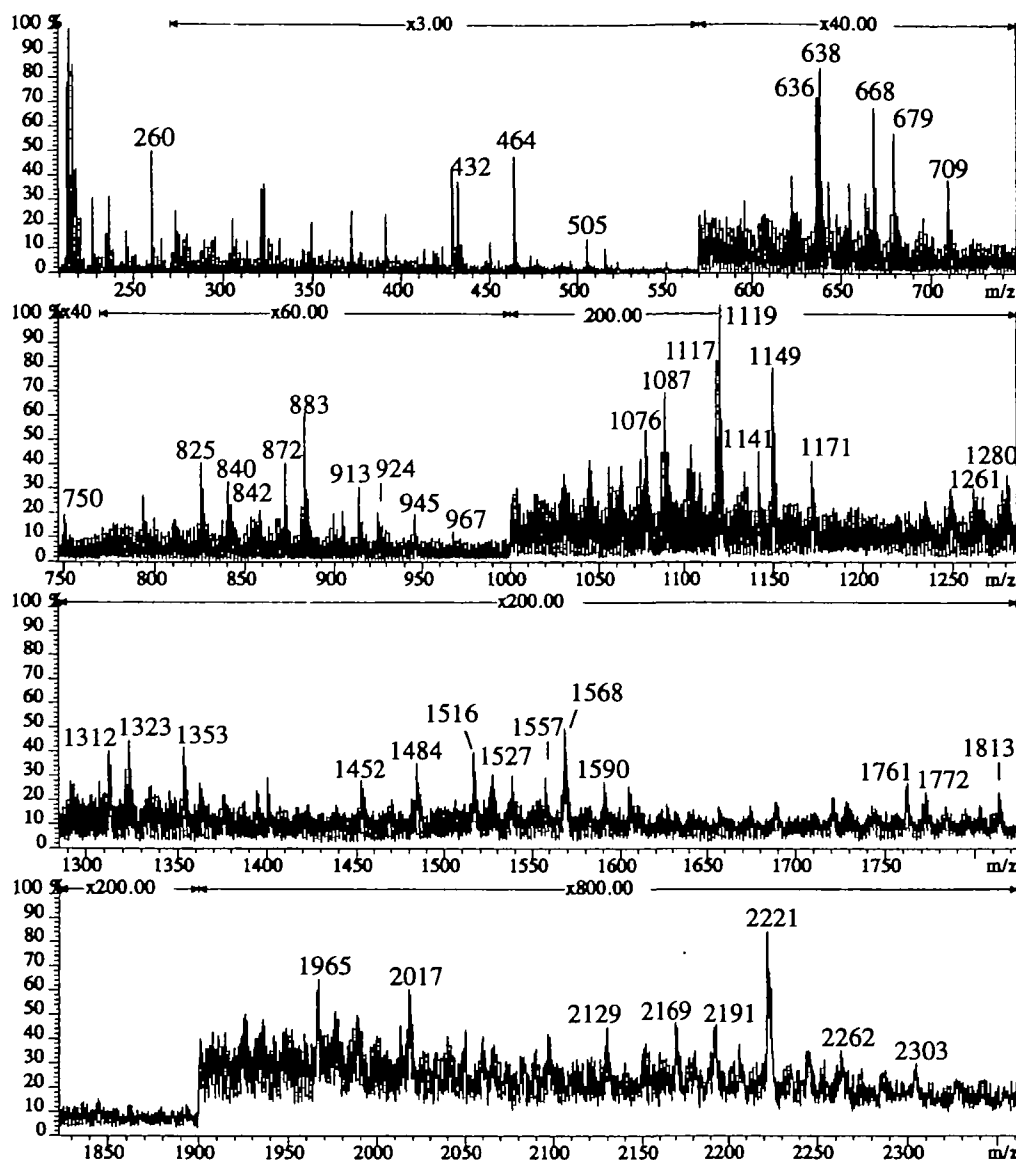


Fig. 1. FAB-mass spectrum of the permethyl derivatives of SjNF. In addition to those listed in Tables I–III, signals at 22 u higher correspond to sodiated in place of protonated molecular ions; signals at 32 u lower correspond to elimination of a methanol moiety; signals at m/z 1312 and 1516 correspond to molecular ions of $\text{Hex}_4\text{HexNAc}_1$ and $\text{Hex}_3\text{HexNAc}_1$, probably as a result of contaminating endo H glycosidase activity in peptide N-glycosidase F.

monly associated with N-glycans. It is, however, consistent with the xylosylated core structures present on the N-glycans from snail hemocyanins and also many plant glycoproteins where a xylose residue is attached to position 2 of the 3,6-linked β -Man (Scheme 1; Kamerling, 1991). Since the only Pent detected in the sugar analysis of SjNA coincided with the retention time of a xylose residue, we conclude that SjNA contains a novel core structure which carries difucosylation as well as xylosylation.

N-glycans from the eggs of *S. mansoni*

In experiments on *S. mansoni*, sugar analysis indicated that most of the N-glycans were released by PNGase F and that, unlike *S. japonicum*, xylose was present in the PNGase F sensitive glycans. The linkage analysis profile of SmNF was found to be similar to that of SjNF except for (1) the presence of a 2,3,6-linked Man and a terminal Pent (as in SjNA); and (2) a higher abundance of terminal Fuc and the presence of a 2-linked Fuc not detected in SjNF or SjNA. The FAB-MS and

linkage data suggest that the xylosylated core structures are mono-fucosylated in *S. mansoni*, in contrast to *S. japonicum* where they are difucosylated. In the former species the fucose must be 6-linked because of the PNGase F sensitivity. A higher abundance of terminal Fuc and the presence of a 2-linked Fuc in *S. mansoni* are consistent with the FAB data described earlier, i.e., the multifucosylated HexNAc termini such as $\text{Fuc}_{1,2}\text{HexNAc}$ and $\text{Fuc}_{2,4}\text{HexNAc}_2$ are synthesized by *S. mansoni* and not *S. japonicum*. Thus, the egg glycoproteins of *S. mansoni* probably contain the $\pm\text{Fuc}\rightarrow 2\text{Fuc}\rightarrow 3\text{GalNAc}\rightarrow 4(\pm\text{Fuc}\rightarrow 2\text{Fuc}\rightarrow 3)\text{GlcNAc}$ epitope previously identified in the egg glycolipids and cercarial glycocalyx (Khoo *et al.*, 1995, 1997).

SmNA afforded two major molecular ions (Figure 3) which were assigned as $[\text{M}+\text{Na}]^+$ of di- and tri-fucosylated $\text{Hex}_5\text{HexNAc}_4$. The corresponding prominent A-type ions at m/z 1770 and 1944 are consistent with both these components having a difucosylated core and thus producing A-type ions of compositions $\text{Hex}_5\text{HexNAc}_3^+$ and $\text{Fuc}_1\text{Hex}_5\text{HexNAc}_3^+$ via

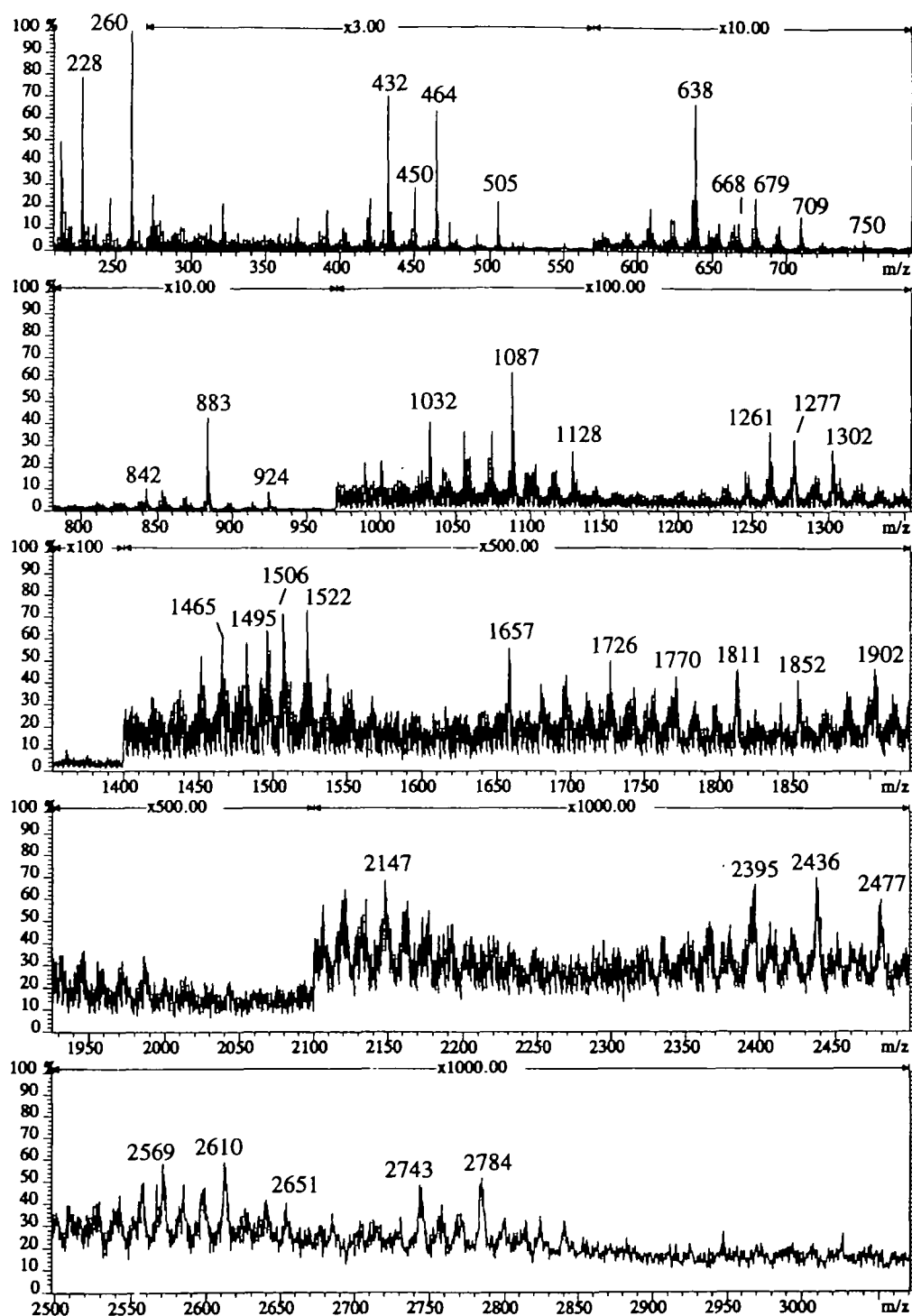


Fig. 2. FAB-mass spectrum of the permethyl derivative of SjNA. Signals at 32 u lower correspond to elimination of a methanol moiety.

cleavage of the *N,N'*-diacetyl-chitobiose (see Scheme 1). Amongst the A-type ions derived from the antennae (Table I) *m/z* 464 is the most prominent indicating that Hex-HexNAc is a major nonreducing structure. The molecular and fragment ion data are consistent with the major glycans released from *S.mansoni* by PNGase A being biantennary structures difucosylated on the proximal GlcNAc of the core and carrying either two lacNAc antennae or one lacNAc and one Lewis x antenna. Unlike *S.japonicum* no xylose-containing glycans were detected in this PNGase F-resistant fraction.

Mapping the schistosomal egg O-glycans

As mentioned above, the O-glycans share the same range of terminal structures as the N-glycans (see Table I) but with a higher proportion containing additional $\pm\text{Fuc}_1(\text{Hex}_1\text{HexNAc}_1)$ unit(s). Interestingly, despite the presence of the terminal $\text{Fuc}_{0-4}\text{HexNAc}_2$ unit in SmO, the larger fragment ions and the more abundant molecular ions did not carry more than two Fuc residues. It thus appears that fucosylation is largely restricted to nonreducing end moieties and that multifucosylated structures constitute only a minor proportion of the nonreducing

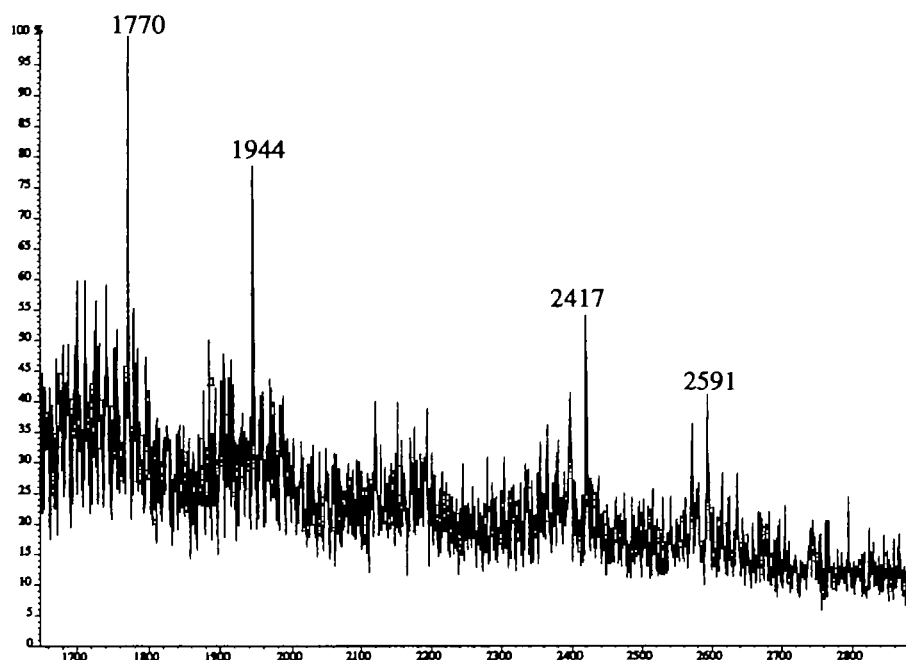
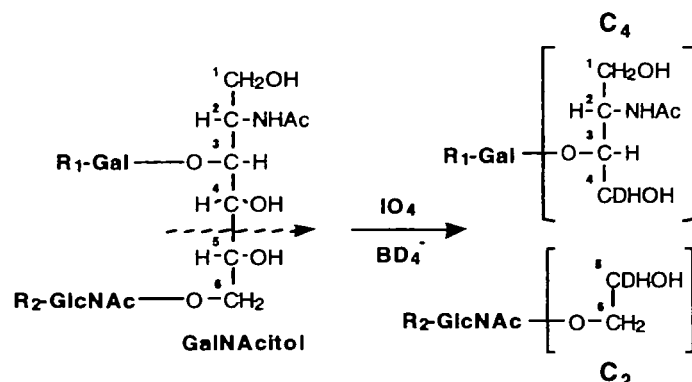


Fig. 3. The molecular ion region of the FAB-mass spectrum of the permethyl derivative of SmNA, showing the two major molecular ions detected together with their corresponding A-type fragment ions. Other weak molecular ion signals may be present amongst the high background noise.

termini of SmO. The major molecular ions afforded by the permethyl derivatives of SmO (Figure 4) and SJO are listed in Table IV together with tentative assignments of sequences. In addition to lacking multifucosylated terminal HexNAc structures (Table I), SJO differed from SmO in having a terminal Hex₂HexNAc unit (Table I) which was further corroborated by the presence of (Hex₂HexNAc₁)-Hex-HexNAcitol in SJO, not found in SmO.

As found in our previous work on the cercarial O-glycans (Khoo *et al.*, 1995), periodate oxidation afforded an efficient means of defucosylation, as well as providing information on core structures. The most common O-glycans are based on either core 1, R₁→Galβ1→3GalNAcα1→Ser/Thr, or core 2, R₁→Galβ1→3(R₂→GlcNAcβ1→6)GalNAcα1→Ser/Thr, where R represents further elongation or termination with a variety of structures (Brockhausen, 1995). Thus, periodate oxidative cleavage between carbons 4 and 5 of the reducing end GalNAcitol yields two distinct products defined by the reducing end fragments carried, as shown in Scheme 2.

Scheme 2



Accordingly, O-glycans based on core 2 afford both R₁-Hex-C₄ and R₂-HexNAc-C₂ types of ions whereas O-glycans based on core 1 and other O-glycans with no or a single substituent on C6 would not yield R₂-HexNAc-C₂ upon oxidation. In cases where complex mixtures of O-glycans such as SmO and SJO are examined, the relative amount of R₁-Hex-C₄ versus R₂-HexNAc-C₂ ions is an indication of the extent of branching on the reducing GalNAc.

The permethyl derivatives of periodate oxidized, borodeuteride reduced SmO yielded both -C₂ and -C₄ series of ions (Figure 5), consistent with the presence of substituents on carbons 3 and 6 of a reducing end GalNAcitol. R₁ for the major R₁-Hex-C₄ molecular ions detected was assigned as HexNAc (*m/z* 692), Hex₁HexNAc₁ (*m/z* 896), HexNAc₂ (*m/z* 937), HexNAc₃ (*m/z* 1182), HexNAc₄ (*m/z* 1427), together with their relatives containing additional Hex₁HexNAc₁ unit(s) (449 u) such as HexNAc+Hex₁HexNAc₁ (*m/z* 1141) and HexNAc+Hex₂HexNAc₂ (*m/z* 1590). Likewise, the major R₂-HexNAc-C₂ molecular ions may be assigned as HexNAc-C₂ (*m/z* 359), Hex₁HexNAc₁-C₂ (*m/z* 563), HexNAc₂-C₂ (*m/z* 604), HexNAc₃-C₂ (*m/z* 849), HexNAc₄-C₂ (*m/z* 1094), and their analogs [Hex₁HexNAc₁]_n higher, including HexNAc-[Hex₁HexNAc₁]₁₋₃-C₂ (*m/z* 808, 1257, 1706). The spectrum of periodate oxidized SJO was similar to that of SmO except for the absence of most of the -C₂ series of ions. Thus only HexNAc-Hex₁HexNAc₁-C₂ (*m/z* 808) was detected as a minor component alongside the major -C₄ series of ions (data not shown) indicating that SJO contains a smaller proportion of core type 2 compared with SmO.

Taking into consideration that nonsubstituted terminal Fuc, Hex, or HexNAc residues will be oxidized and subsequently lost in the periodate procedure, the compositions of the periodate oxidized products are consistent with the assignments given in Table IV which are derived from all the data obtained including the FAB data from intact glycans discussed earlier. Thus, HexNAc-[Hex₁HexNAc₁]_n-Hex-C₄ and HexNAc-

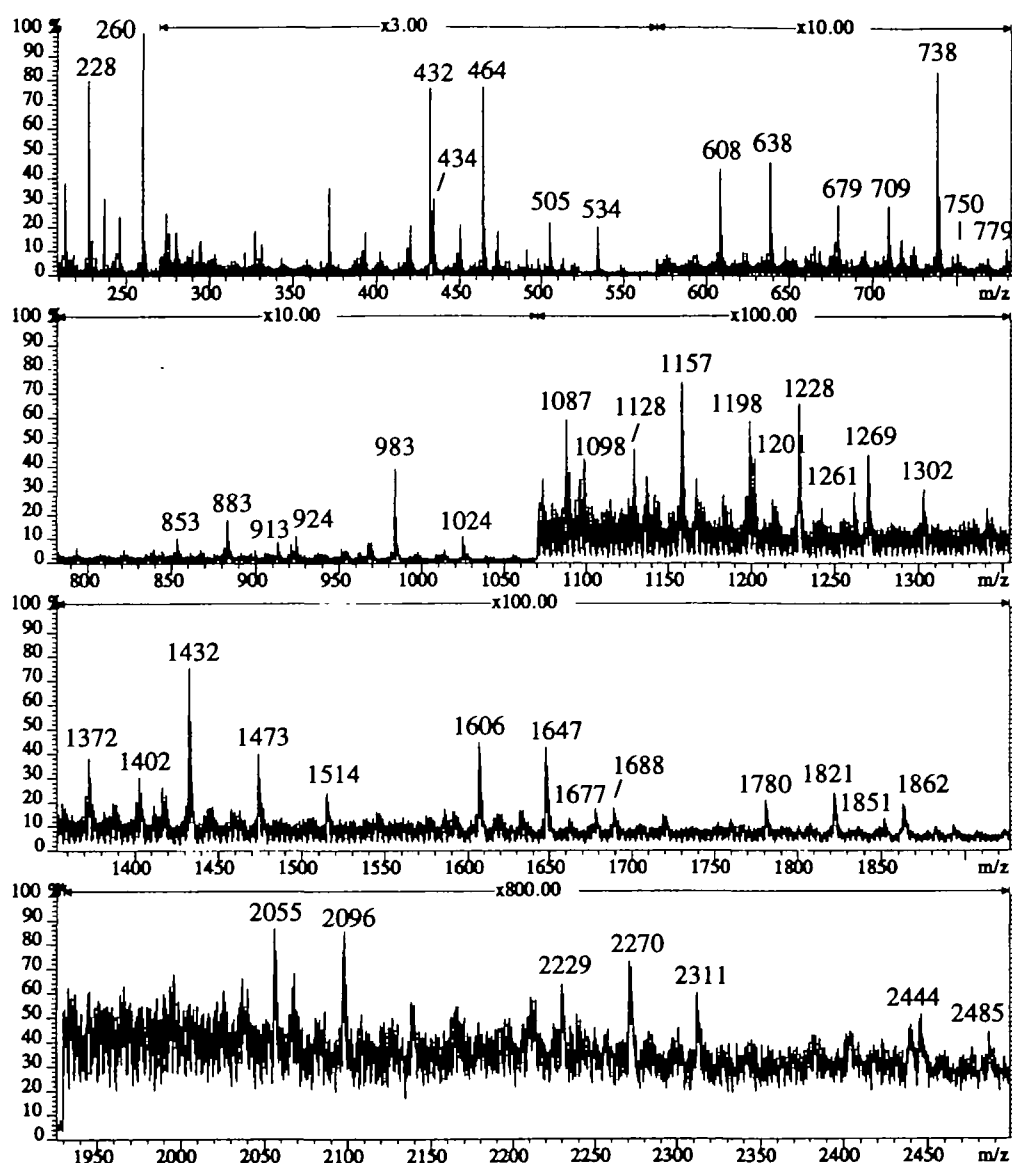


Fig. 4. FAB-mass spectrum of the permethyl derivatives of SmO, optimized for the molecular ion signals. Signals at 32 u lower correspond to elimination of a methanol moiety.

[Hex₁HexNAc₁]_n-C₂, are the expected products of periodate cleavage of fucosylated structures having poly-*N*-acetyl-lactosamine backbones linked to cores 1 and/or 2. In addition, the presence of ions such as HexNAc₂-C₂, HexNAc₃-C₂ and HexNAc₄-C₂ in oxidized SmO firmly established that a stretch of up to at least four HexNAcs can be directly linked to the reducing end core residue. These and the corresponding -C₄ ions support the assignment of molecular ions terminating with fucosylated HexNAc residues (Table IV). Finally, the relatively low abundance but significant presence of Hex₁HexNAc₁-C₂ and Hex₁HexNAc₁-Hex-C₄ is consistent with a $\pm$ Fuc(HexNAc-3Hex-HexNAc)- type of terminal structure (A-type ions at *m/z* 709, 883, Table I) in which only the nonfucosylated, nonreducing terminal HexNAc was oxidized and cleaved during the periodate reaction. The Hex-Hex-HexNAc terminal sequence uniquely present in *S.japonicum* (see earlier) might also yield these oxidative cleavage products depending on the as yet undefined linkage of the subterminal Hex.

Discussion

We have effected a structural mapping of the glycans carried on the egg glycoproteins of both *S.mansoni* and *S.japonicum* based on highly sensitive and definitive mass spectrometric analysis in conjunction with selective use of enzymatic and chemical modifications. The precise mass data afforded constitutes the unique strength of mass spectrometry in the analysis of such crude complex mixtures since the composition of each component can be defined. The preponderance of A-type ions resulting from facile cleavage at each HexNAc residue in the FAB-MS analysis of permethyl derivatives (Dell, 1987) afforded key information on the range of terminal sequences present. The very high sensitivity associated with the detection of A-type ions in these experiments means that, although the corresponding molecular ions may not be detected either because of low abundance and/or high molecular weight, a comprehensive mapping of terminal epitopes, which often represent the most important immunogenic structural elements in-

Table IV. Assignment and preliminary interpretation of the molecular ions afforded by the permethyl derivatives of schistosomal egg O-glycans

<i>m/z</i>	Composition, [M + Na] ⁺	Probable structural configuration ^a
534	Hex ₁ HexNAcitol	Core 1
738	Hex ₂ HexNAcitol	Novel core
779	Hex ₁ HexNAc ₂ -itol	(HexNAc)-Hex-HexNAcitol
1269	Hex ₁ HexNAc ₄ -itol	(HexNAc)(HexNAc ₂)-Hex-HexNAcitol
(Hex₁HexNAc₁)_n-Hex-HexNAcitol		
983	Hex ₂ HexNAc ₂ -itol	(Hex ₁ HexNAc ₁)-Hex-HexNAcitol
1157	Fuc ₁ Hex ₂ HexNAc ₂ -itol	
1432	Hex ₃ HexNAc ₃ -itol	(Hex ₁ HexNAc ₁) ₂ -Hex-HexNAcitol
1606, 1780	Fuc _{1,2} Hex ₃ HexNAc ₃ -itol	
1881	Hex ₄ HexNAc ₄ -itol	(Hex ₁ HexNAc ₁) ₃ -Hex-HexNAcitol
2055, 2229	Fuc _{1,2} Hex ₄ HexNAc ₄ -itol	
(HexNAc₂)_{1,2}(Hex₁HexNAc₁)_n-Hex-HexNAcitol		
1024	Hex ₁ HexNAc ₃ -itol	(HexNAc ₂)-Hex-HexNAcitol
1198, 1372	Fuc _{1,2} Hex ₁ HexNAc ₃ -itol	
1473	Hex ₂ HexNAc ₄ -itol	(HexNAc ₂)(Hex ₁ HexNAc ₁)-Hex-HexNAcitol
1647, 1821,	Fuc _{1,2} Hex ₂ HexNAc ₄ -itol	
1514	Hex ₁ HexNAc ₅ -itol	(HexNAc ₂) ₂ -Hex-HexNAcitol
1688, 1862	Fuc _{1,2} Hex ₁ HexNAc ₅ -itol	
2096, 2270, 2444	Fuc _{1,3} Hex ₃ HexNAc ₅ -itol	(HexNAc ₂)(Hex ₁ HexNAc ₁) ₂ -Hex-HexNAcitol
2311, 2485	Fuc _{2,3} Hex ₂ HexNAc ₆ -itol	(HexNAc ₂) ₂ (Hex ₁ HexNAc ₁)-Hex-HexNAcitol
(Hex₁HexNAc₂)(Hex₁HexNAc₁)_n-Hex-HexNAcitol		
1228	Hex ₂ HexNAc ₃ -itol	(Hex ₁ HexNAc ₂)-Hex-HexNAcitol
1402	Fuc ₁ Hex ₂ HexNAc ₃ -itol	
1677	Hex ₃ HexNAc ₄ -itol	(Hex ₁ HexNAc ₂)(Hex ₁ HexNAc ₁)-Hex-HexNAcitol
1851	Fuc ₁ Hex ₃ HexNAc ₄ -itol	
(Hex₂HexNAc₁)-Hex-HexNAcitol^b		
1187	Hex ₃ HexNAc ₂ -itol	(Hex ₂ HexNAc ₁)-Hex-HexNAcitol
1361	Fuc ₁ Hex ₃ HexNAc ₂ -itol	

^aThe exact sequence was not defined. Each of the saccharide modules in parenthesis may be linked to one another in one linear sequence (core 1, R₁ → 3Galβ1 → 3GalNAcitol) or distributed between the two arms of a core-2 type structure, i.e., R₁ → 3Galβ1 → 3(R₂ → GlcNAcβ1 → 6)GalNAcitol. Fucosylation is expected to be mainly on the HexNAc residues. Other arrangements are possible and further work is needed to substantiate the proposed configurations.

^bDetected only in SJO and not in SmO.

involved in host-parasite interactions, is still possible. In the context of limited supplies of source materials and the absence of earlier structural data on egg glycoproteins, our current mass spectrometric studies have achieved an important objective, namely a broad survey of the structural diversity present and the partial characterization of new or rare structures, a knowledge of which will greatly facilitate their eventual detailed characterization if merited.

The detection of fucosylated terminal HexNAc epitopes in the egg glycoproteins of *S. mansoni* furnishes the crucial link to previous immunological and structural studies on schistosomal egg antigens and the cercarial glycocalyx. Both the egg glycosphingolipids and the cercarial O-glycans of *S. mansoni* were previously shown to contain multifucosylated terminal HexNAc epitopes which were characterized as $\pm$ Fuc→2Fuc→3GalNAc→4(Fuc₂ or ₃→3)GlcNAc. In the cercarial glycocalyx, this type of terminal unit is carried on repeating units of →[3Galα1→3GalNAcβ1→4(Fuc₂ or ₃→3)GlcNAcβ1→] of core 1 and core 2 O-glycans (Khoo *et al.*, 1995), whereas in egg glycosphingolipids, it is linked to a stretch of →4GlcNAc each of which may carry up to two Fuc residues at carbon 3 (Leverly *et al.*, 1992; Khoo *et al.*, 1996). In contrast, multifucosylated terminal HexNAc moieties were not detected in the egg glycosphingolipids of *S. japonicum* (Khoo *et al.*, 1996). Likewise in our studies of egg glycoproteins reported in this article, although glycan chains terminating with

HexNAc₁₋₃ and Fuc₁HexNAc_{2,3} were found on both species, terminal Fuc_{1,2}HexNAc, Fuc_{2,4}HexNAc₂ and Fuc₂HexNAc₃ could only be detected in the glycans of *S. mansoni* and not *S. japonicum*. It thus appears that there are two distinct types of HexNAc termini as a consequence of differential fucosylation: (1) HexNAc-($\pm$ Fuc)HexNAc type which is common to both species; and (2) Fuc_{1,2}HexNAc-(Fuc_{1,2})HexNAc which is specific to *S. mansoni* egg antigens. Both types of terminal structure can be linked to the cores of N- and O-glycans either directly or via additional saccharide units and may be further glycosylated with a third HexNAc at the nonreducing end.

The presence of a range of very similar and yet distinct terminal glycosylation sequences in the egg antigens of the two schistosome species supports the findings of both species-cross reactive and species-specific saccharide epitopes on the egg glycoproteins and glycolipids (Norden and Strand, 1984; Weiss and Strand, 1985; Weiss *et al.*, 1986). Notably, most of the immunogenic *S. mansoni* egg glycolipids were found to contain epitopes reactive with two of the available monoclonal antibodies (mAb), 128C3/3 and 485D2/7, which were originally raised against *S. mansoni* cercarial and egg glycoproteins, respectively. While mAb 485D2/7 was shown to be *S. mansoni*-specific, mAb 128C3/3 has demonstrable reactivity against a number of *S. japonicum* egg glycoproteins as well as the glycolipid fractions. It is likely that both mAbs recognized the variably fucosylated HexNAc units in subtly different epitopes

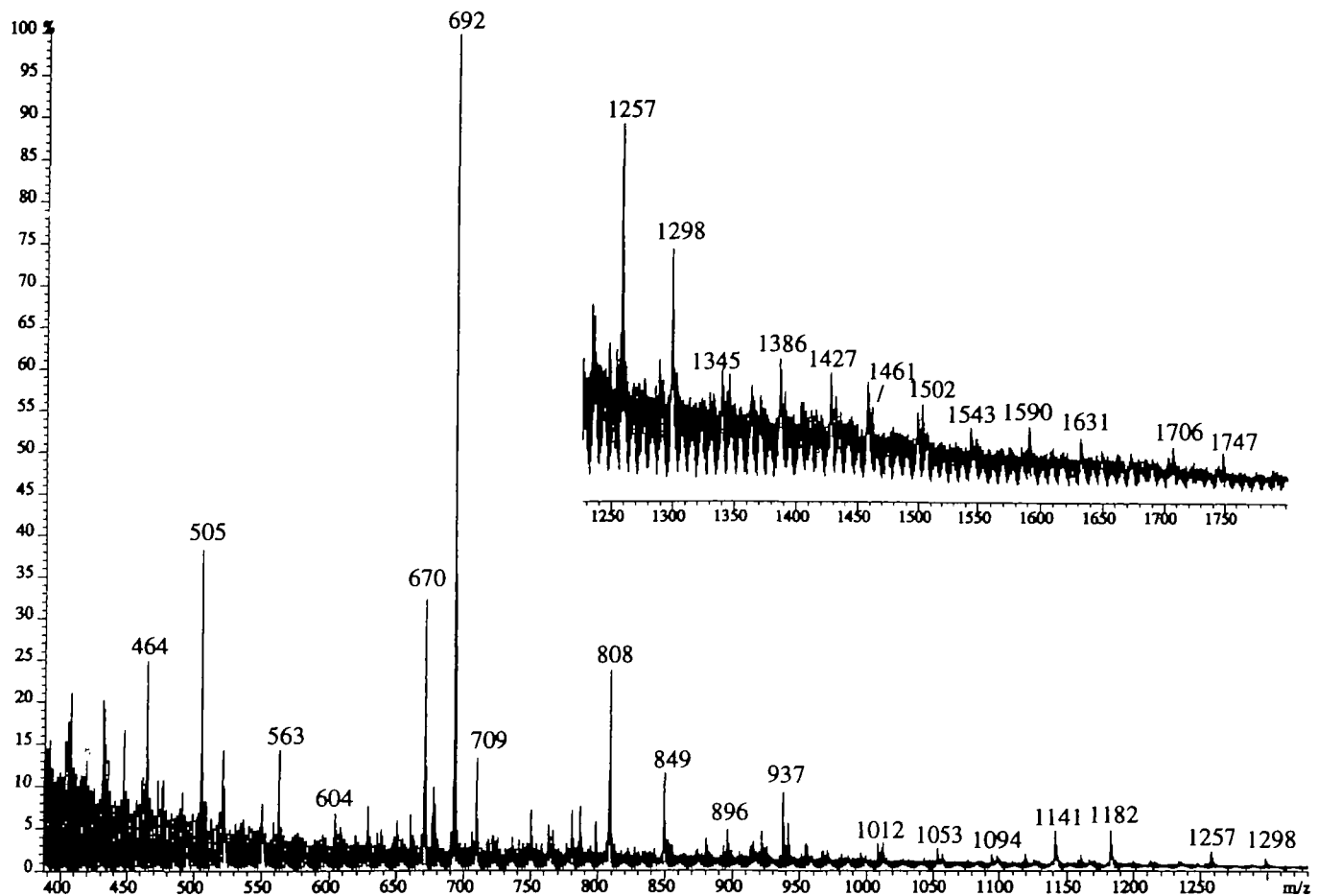


Fig. 5. FAB-mass spectrum of the permethyl derivative of periodate oxidized SmO. Inset: the mass region from m/z 1320 to 1800 shown on a different scale for a better presentation of the weaker ions detected above the high background noise.

as indicated by the structural data now available. In particular, mAb 128C3/3 which was shown to recognize immobilized L-Fuc but not the normal range of blood group antigens (Levery *et al.*, 1992) seems unlikely to recognize exclusively a Fuc α 1 $\rightarrow$ 2Fuc epitope since this is only found on *S.mansoni* cercarial glycocalyx and egg antigens but not on *S.japonicum* eggs.

In recent years, GalNAc β 1 $\rightarrow$ 4GlcNAc (*N,N'*-diacetyl-lactosamine, lacdiNAc) has increasingly been identified as an antenna building block replacing the more common Gal β 1 $\rightarrow$ 4GlcNAc (*N*-acetyl-lactosamine, lacNAc), particularly in lower animals (for references see van den Eijnden *et al.*, 1995). To date, a whole spectrum of further substituted lacdiNAc structures have been identified which closely resemble their lacNAc analogs in complex type glycan structures. Thus, lacdiNAc units may be capped by sialylation or sulfation. They may contain a Fuc residue in α 1 $\rightarrow$ 3-linkage to GlcNAc to form the lacdiNAc analog of the Lewis x (Gal β 1 $\rightarrow$ 4(Fuc α 1 $\rightarrow$ 3)GlcNAc) structure. In *S.mansoni*, both lacdiNAc and α 3-fucosylated lacdiNAc have been identified on the complex N-glycans synthesized by adult worms (Nyame *et al.*, 1989; Srivatsan *et al.*, 1992a) which also contain (poly)lacNAc, and Lewis x structures (Srivatsan *et al.*, 1992b). In addition, Lewis x was identified as terminal and internal repeating units of the O-linked polysaccharide chains derived from circulating cathodic antigen secreted by the adult worm (van Dam *et al.*, 1994). Our current data are in support of both lacNAc and

lacdiNAc types of terminal structures, including Lewis x, being carried on the N- and O-glycans of the egg glycoproteins of both schistosomal species. It should be noted that, although the presence of the Lewis x epitope on the egg antigens of *S.mansoni* has been indirectly implicated through several immunological studies (Ko *et al.*, 1990; Köster and Strand, 1994), it has not actually been identified and characterized as a constituent of egg glycans.

Although the lacdiNAc analogs of the rather common poly-lacNAc and α 3-galactosylated lacNAc units have not been described, the backbone of the cercarial O-glycans of *S.mansoni* which comprises repeating units of Gal α 1 $\rightarrow$ 3GalNAc β 1 $\rightarrow$ 4GlcNAc (Khoo *et al.*, 1995) may be viewed as a hybrid of these structures. It is not known whether a true poly-lacdiNAc chain exists in nature. Our data on *S.mansoni* glycosphingolipids indicated that HexNAc-rich backbones are more likely to have the sequence GalNAc β 1 $\rightarrow$ [4GlcNAc β 1 $\rightarrow$] $_n$ although the possibility that such sequences might be interspersed by 3-linked GalNAc has not been ruled out. Our present work on the egg glycoproteins detected the presence of nonreducing terminal HexNAc $_3$, as well as a stretch of up to HexNAc $_4$ directly linked to the O-glycan cores. To our knowledge, a minor GalNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ 2Man antenna in the N-glycans of batroxobin from *Bothrops moojeni* venom as reported by Lochnit and Geyer (1995), and our own observation of a HexNAc $_3$ terminal epitope as a minor constituent of a glycoprotein from *Acanthocheilonema viteae*

(Haslam *et al.*, 1997) are the only examples of terminal HexNAc₃ that are known to be present on glycoproteins. Baker *et al.* (1994) have identified a novel UDP-GlcNAc:GlcNAc β -R β 1 $\rightarrow$ 4GlcNAc transferase (β 4-GlcNAc-T) in the pond snail *Lymnaea stagnalis* which may be able to catalyze the synthesis of the [$\rightarrow$ 4GlcNAc β 1]_n type of glycan chain although the occurrence of such structures in *L. stagnalis* has not been investigated. Further work is required to establish the precise structures of putative oligomeric HexNAc moieties in schistosomes and their hosts.

The detection of various lacNAc- and lacdiNAc-based terminal sequences (Table I) clearly indicates the ability of both schistosome species to synthesize complex type N-glycans in addition to the high mannose type (Table III). In this respect, the N-glycans from schistosomal eggs resemble those from *S. mansoni* adult worms and schistosomula stages (Nyame *et al.*, 1988a,b, 1989; Srivatsan *et al.*, 1992a,b). However, the truncated structures, xylosylation and core difucosylation are novel features not previously identified in schistosomal glycoconjugates (Cummings and Nyame, 1996). In fact, although each of these structural entities has a precedent in other lower animals, a trimannosyl core with both xylosylation and difucosylation has never been described before.

Core α 3-fucosylation and xylose β 1 $\rightarrow$ 2 linked to the core β -Man were first identified on plant N-glycans which typically comprise (1) high mannose type of compositions Man₅GlcNAc₂, and (2) complex type with additional non-reducing terminal glycosylation on a β 2-xylosylated α 3-fucosylated mannotriosyl *N,N'*-diacetyl-chitobiose core (Xyl₁Fuc₁Man₃GlcNAc₂). Nonextended Xyl₁Fuc₁Man₃GlcNAc₂, further truncated Xyl₁Fuc₁Man₂GlcNAc₂, as well as nonfucosylated Xyl₁Man₃GlcNAc₂ and Xyl₁Man₄GlcNAc₂ have also been characterized (reviewed in Sturm, 1995). The only two examples of xylose-containing N-glycans in animals prior to this work are the hemocyanins of the snails *Helix pomatia* and *Lymnaea stagnalis*. In addition to both nonfucosylated and α 6-fucosylated truncated β 2-xylosylated trimannosyl *N,N'*-diacetyl-chitobiose structures, complex type N-glycans with a nonfucosylated, β 2-xylosylated trimannosyl *N,N'*-diacetyl-chitobiose core were shown to be extended at either or both Man α 1 $\rightarrow$ 3 and Man α 1 $\rightarrow$ 6 arms to give mono- and biantennary structures (reviewed in Kamerling, 1991). Interestingly, the N-glycans from hemocyanin of *L. stagnalis* were found to carry a terminal sequence X-Gal β 1 $\rightarrow$ 3GalNAc β 1 $\rightarrow$ 4GlcNAc β 1 $\rightarrow$ (X = 3-O-Me or Fuc α 1 $\rightarrow$ 2) (van Kuik *et al.*, 1987) which may now be viewed as a further substituted lacdiNAc.

Apart from a few noted exception where sialylated complex type structures have been identified, insect N-glycans characterized thus far (reviewed in Kubelka *et al.*, 1995; März *et al.*, 1995) in general fall into either the high mannose type or truncated type categories. Complex type N-glycans, when present, are restricted to addition of a single GlcNAc β 1 $\rightarrow$ 2 or a GalNAc β 1 $\rightarrow$ 4(Fuc α 1 $\rightarrow$ 3)GlcNAc β 1 (fucosylated lacdiNAc) to the trimannosyl core which may be nonfucosylated, α 6- or α 3-monofucosylated, or α 6- and α 3-difucosylated. Xylosylation has not been observed in insect glycoproteins and their N-glycans may be truncated as far as the β -Man. To date, no other core α 3-fucosylated N-glycans from animals has been reported by other groups although our own data has revealed the presence of such structural features in the nematodes (Haslam *et al.*, 1996; Dell and Khoo, unpublished observa-

tions) which typically also synthesize a high proportion of high mannose and truncated N-glycans.

It thus appears that a portion of the schistosomal egg N-glycans are similar to the commonly found mammalian complex type and high mannose type structures which also characterize the N-glycans of the adult worms and schistosomula. In addition, a subset of the egg N-glycans may be identified as truncated structures, very similar to those identified on plants, insects, and nematodes. In accordance with the currently accepted or proposed biosynthetic pathways for the N-glycans of mammals (Kornfeld and Kornfeld, 1985; Verbert, 1995; Schachter, 1995), plants (Sturm, 1995), insects (Altmann and März, 1995; Altmann *et al.*, 1995; März, 1995; Wagner *et al.*, 1996), and snails (Mulder *et al.*, 1995), these "truncated" structures would require the trimming action of α -mannosidase II on a GlcNAc-Man₃GlcNAc₂ substrate, followed by nonobligatory, overlapping, or sequential actions of α 6-fucosyltransferase, α 3-fucosyltransferase, and β 2-xylosyltransferase on the core (Figure 6). Addition of further GlcNAc residues by GlcNAc transferase II and other transferases will initiate the synthesis of complex type structures via lacNAc, lacdiNAc and/or chitobio pathways (van den Eijnden *et al.*, 1995). On the other hand, a β -N-acetylglucosaminidase activity such as that identified in insect cells (Altmann *et al.*, 1995; Wagner *et al.*, 1996) is required to account for the removal of the GlcNAc residue on the Man α 1 $\rightarrow$ 3 arm to yield the Man₃GlcNAc₂-based truncated core structures. The shorter core structures would require further trimming by one or more mannosidases.

It is of interest to note that difucosylated core structures in *S. mansoni* are much less abundant than nonfucosylated or α 6-fucosylated core structures and only the latter also carry xylosylation. In contrast, xylosylated core structures in *S. japonicum* are mainly difucosylated. Since α 3-Fuc on the core GlcNAc has been identified as an antigenic determinant (Prenner *et al.*, 1992), it is conceivable that the variously substituted N-glycan cores identified in this study contribute to cross-reactive and species-specific epitopes in the egg antigens of *S. mansoni* and *S. japonicum*.

Materials and methods

Preparation of eggs

Biomphalaria glabrata infected with a Puerto Rican strain of *S. mansoni*, were obtained from Dr. Fred Lewis of the Bethesda Biomedical Research Institute. Eggs were obtained from mice infected 6 weeks previously with cercariae by the method of Boros and Warren (1970) and stored frozen in saline. *S. japonicum* eggs were obtained from Dr. Yuan Hong Chang of Shanghai Medical University. Infected *Onchomelania hupenensis* snails were obtained in Anhui province. Cercariae were allowed to infect rabbits and eggs were harvested by a slight modification of the method used for *S. mansoni*.

Extraction of glycoproteins and preparation of glycans

Schistosomal egg glycoproteins were obtained from the same batches of eggs used to prepare the glycolipids (Khoo *et al.*, 1997). Delipidated egg homogenates after lipid extraction with chloroform/methanol/water were sonicated in 4 M guanidine hydrochloride and the supernatants were dialyzed against running water. The crude glycoprotein extracts were first digested with non-TPCK treated trypsin (Sigma) (1:50 enzyme:protein ratio, w/w; 37°C for 5 h in 50 mM ammonium bicarbonate buffer, pH 8.4) followed by C18 Sep-pak (Waters) purification. Glucan polymer and other hydrophilic contaminants were washed off with 5% aq. acetic acid and the bound peptides/glycopeptides were eluted with a step gradient of 20, 40, and 60% propan-1-ol in 5% acetic acid. All the eluted fractions were pooled, dried down, and then incubated with N-glycosidase F (1 unit, Boehringer Mannheim) overnight at 37°C in 50 mM ammonium bicarbonate buffer, pH 8.4. Released N-glycans were separated

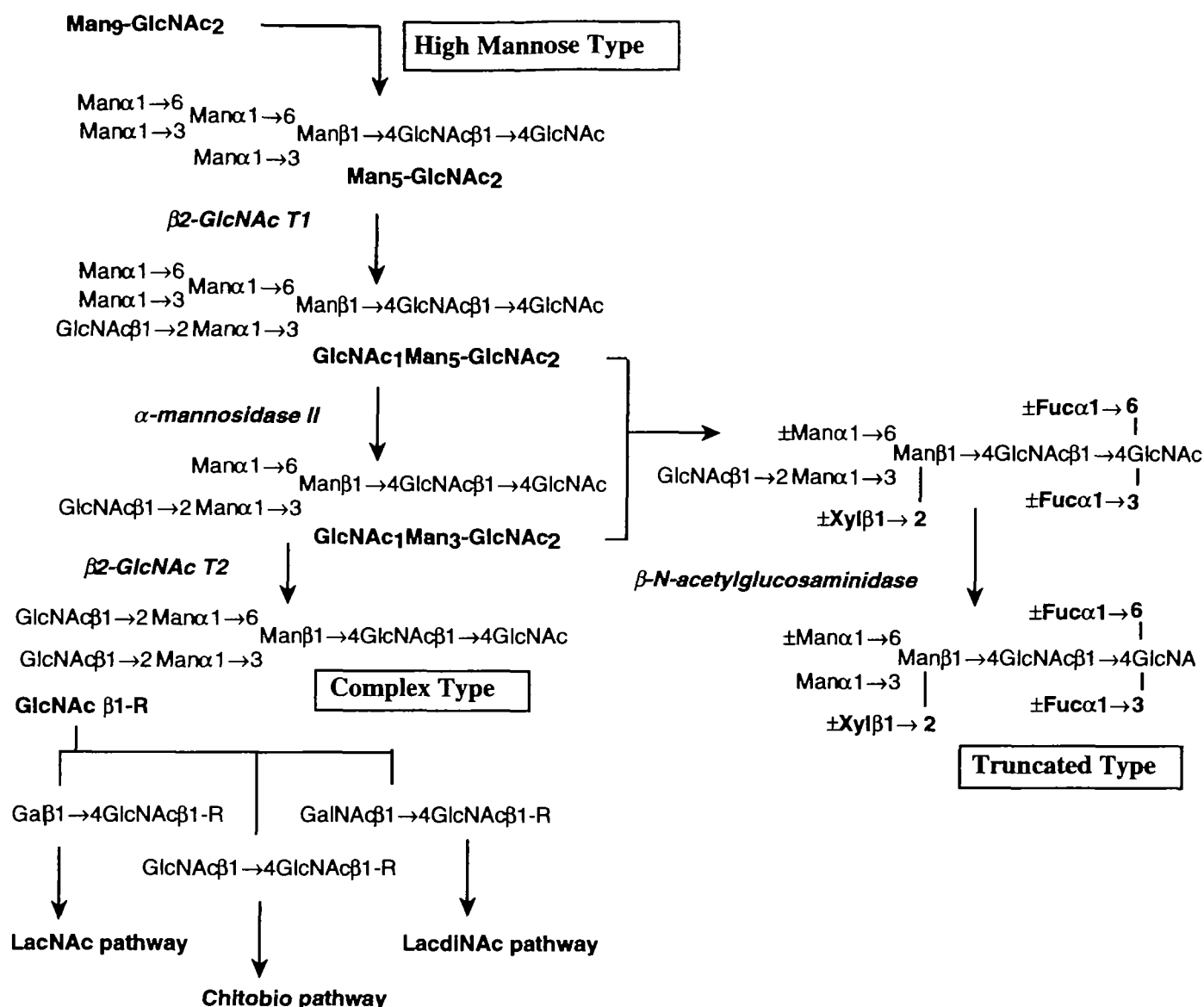


Fig. 6. A proposed biosynthetic scheme from which the different classes of N-glycans may be derived, based on that established for mammals, plants, insects, and snails. The schistosomal N-glycans are likely to be similarly derived although none of the critical enzymes shown in this scheme has been identified in a schistosomal system. Core fucosylation and xylosylation are believed to take place after the action of β 2-GlcNAc T1 since the β 2-Xyl and α 6-Fuc transferases identified in plants and *L. stagnalis* were found to be inactive against mannosyl-only substrates, i.e., those without the GlcNAc added by β 2-GlcNAc T1. This also implies that a β -N-acetylglucosaminidase is required to produce truncated structures such as Man₂3GlcNAc₂. The nomenclature of LacNAc, LacdiNAc, and Chitobio pathways was coined by van den Eijnden (van den Eijnden *et al.*, 1995); see the text for other relevant references.

from peptides/glycopeptides using the same C18 Sep-pak procedure. The pooled peptides/glycopeptides fractions were then digested with glycopeptidase A from almond (0.2 mU, Seikagaku) in 50 mM ammonium acetate buffer, pH 5, at 37 °C overnight. N-glycans released by glycopeptidase A were likewise separated from the de-N-glycosylated peptides/glycopeptides by the same C18 Sep-pak procedures. Finally, O-glycans were reductively eliminated from the pooled 20–60% propan-1-ol fractions with 1 M sodium borohydride solution in 0.05 M aqueous sodium hydroxide (45 °C overnight) and desalted by passing through a Dowex (50W-X8, 50–100 mesh, protonated form) column in 5% acetic acid. Borates were removed by repeated coevaporation with 10% aq. acetic acid in methanol. Partial defucosylation was carried out with α -fucosidase from bovine kidney (0.1 U at 1 U/ml, Boehringer Mannheim) in 50 mM ammonium acetate buffer, pH 4.5, at 37 °C for 24 h.

Chemical derivatization and FAB-MS analysis

Samples were permethylated using the NaOH/dimethyl sulfoxide slurry method as described by Dell *et al.* (1994). Periodate oxidation and subsequent work-up steps was carried out as described previously (Khoo *et al.*, 1995).

FAB-mass spectra were obtained using a VG Analytical ZAB-2SE 2FPD or Autospec mass spectrometer fitted with a cesium ion gun operated at 25–30 kV. Data acquisition and processing were performed using the Opus software. Monothiolglycerol was used as matrix.

Monosaccharide composition and linkage analysis

Partially methylated alditol acetates for GC-MS analysis were prepared from permethyl derivatives after FAB-MS analysis according to the procedures described by Albersheim (Albersheim *et al.*, 1967). GC-MS was carried out using a HP Gas Chromatograph 5890 connected to a HP 5790 Mass Selective Detector. Each sample was dissolved in acetone prior to injection on a DB-5 fused silica capillary column (30 m, 0.25 μ m film thickness, 0.25 mm I.D., J&W Scientific) at 80 °C. The temperature was increased to 180 °C over 20 min and then increased to 280 °C in 8 min. For monosaccharide composition analysis, N- and O-glycan pools were methanolysed with 0.5 M methanolic-HCl (Supelco) at 80 °C for 16 h, re-N-acetylated with 500 μ l of methanol, 10 μ l of pyridine, and 50 μ l of acetic anhydride, and then treated with the Tri-Sil TMS-derivatizing reagent (Pierce) for 15 min at room temperature. GC-MS

analysis of the TMS derivatives was performed on the same HP system using a temperature gradient of 140°C to 200°C at 5°C/min, increased to 300°C at 10°C/min.

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Abbreviations

FAB-MS; fast atom bombardment-mass spectrometry; GC-MS, gas chromatography-mass spectrometry; Hex, hexose; HexNAc, *N*-acetylhexosamine; MeOH, methanol; mAb, monoclonal antibody; lacdiNAc, *N,N'*-diacetylactosamine; lacNAc, *N*-acetylactosamine; Pent, pentose; PNGase A, peptide *N*-glycosidase A; PNGase F, peptide *N*-glycosidase F; SjNA, *N*-glycans of *S. japonicum* released by PNGase A after PNGase F treatment; SjNF, *N*-glycans of *S. japonicum* released by PNGase F; SjO, *O*-glycans from *S. japonicum* released by reductive elimination on de-*N*-glycosylated sample; SmNA, *N*-glycans of *S. mansoni* released by PNGase A after PNGase F treatment; SmNF, *N*-glycans of *S. mansoni* released by PNGase F; SmO, *O*-glycans from *S. mansoni* released by reductive elimination on de-*N*-glycosylated sample.

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