Targeted identification of Schistosoma mansoni egg glycans

Robijn, M.L.M.

Targeted identification of *Schistosoma mansoni* egg glycans

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Cover illustration: *Schistosoma mansoni* egg, visualised with immunofluorescence microscopy. The egg and excreted antigens are stained with monoclonal antibody (mAb) 114-4D12. MAb 114-4D12 recognises a series of specifically fucosylated glycoprotein and glycolipid antigens. In addition, mAb 114-4D12 recognises unconjugated glycans that are excreted by the *S. mansoni* egg. A specific MALDI-TOF MS peak pattern is shown indicating fucosylated free glycans that were obtained from *S. mansoni* infection urine (front cover) and egg incubation medium (back cover), after affinity purification with mAb 114-4D12.

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PROEFSCHRIFT

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Co-promotor:	Dr. C.H. Hokke
Referent:	Prof. Dr. P.M. Rudd *University College Dublin, Ireland*
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Aan Martijn

Verlies is een ervaring naar een nieuwe weg. Een nieuwe gelegenheid om op een andere manier te denken. Verliezen is niet het einde van alles, maar het einde van een bepaalde manier van denken. Wie ergens valt, staat ergens anders weer op. Dat is de wet van het leven.

Uit Spijkerschrift van Kader Abdolah –van de Perzische dichter Mohammad Mokhtari.

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General introduction

SCHISTOSOMIASIS: DISTRIBUTION AND LIFE CYCLE

Schistosomiasis is caused by digenetic blood flukes (trematodes) of the genus *Schistosoma*. The adult schistosome worms live in a mammalian host (humans) while the intermediate hosts, as for all digenetic trematodes, are snails. Schistosomiasis is also known as bilharzia since in 1851 Dr. Theodore Maximiliar Bilharz discovered these adult worms during an autopsy (Bilharz, T: Fernere Beobachtungen Über das die Pfortader des Menschen bewohnende *Distomum haematobium* und sein Verhältnis zu gewissen pathologischen Bildungen. Zeitschrift Wissenschaft Zool. 4: 72, 1852, Leipzig ; http://www.schisto.org/Schistosomiasis/).

There are five species of schistosomes infecting man. The first major schistosome species is *Schistosoma mansoni*, which is prevalent in sub-Saharan Africa, Egypt, Libia and the Middle East as well as in South America, in particular in Brazil and the Caribbean. *S. mansoni* is transmitted by snails of the genus *Biomphalaria*, aquatic snails that thrive in irrigation canals and along lake shores. The second major species *S. haematobium* occurs in Africa, Asia Minor and Arabia and is transmitted by *Bulinus* snails, which inhabit less permanent water bodies, because they hibernate in mud during the dry season. Amphibious snails of the genus *Oncomelania* transmit the third major schistosome species *S. japonicum*, which not only affects man but also (domestic) animals (Wang *et al.*, 2005). *S. japonicum* is today only endemic in some parts of China, Indonesia and the Philippines, but used to be more widespread (Ebisawa, 1998; Zhou *et al.*, 2005). The two ‘minor’ species of schistosomiasis are *S. intercalatum*, prevalent in West Africa and *S. mekongi* predominantly found in Southeast Asia.

Schistosomiasis is after malaria the second most prevalent parasitic disease. In sub-Saharan Africa an estimated 170 million people are infected and an additional 30 million people in North-Africa, Asia and South America (Chitsulo *et al.*, 2000). The disease is a huge public health problem strongly correlated with poverty and is of great socio-economic importance in the developing world. By the improvement of health education, training in hygiene, water supply and sanitation prevalence rates can be reduced (Utzinger *et al.*, 2003). Strong socio-economic development can even result in the local eradication of schistosomiasis which occurred in, for example, Japan, and in parts of China, Brazil and the Caribbean (Engels *et al.*, 2002).

The schistosome life cycle is intriguing and complex (Figure 1). In the vertebrate (human) host sexual reproduction takes place, whereas in the intermediate molluscan (snail) host

asexual reproduction occurs. People become infected when they come into contact with fresh water infested with cercariae. Upon penetration of the skin the parasite undergoes different developmental changes: the cercariae shed their bifurcated tails and transform into schistosomula that enter the capillaries. Via the blood they migrate through the lungs to the portal veins of the liver (*S. mansoni* and *S. japonicum*) or the bladder plexus (*S. haematobium*). There the worms mature and pair. The adult male worm is 1 to 2 cm long and embraces the longer and thinner female worm in a groove (the gynaecophoric canal). The adult female worms may produce, depending on the species, up to several hundreds (*S. mansoni*) or thousands (*S. japonicum*) of eggs daily. It is assumed that approximately half of the eggs migrate through the wall of the intestine or bladder to be excreted by faeces or urine, but the other half is taken by the blood flow and becomes lodged in liver, intestines or urinary tract, where a plethora of excreted egg antigens induce strong granulomatous inflammatory responses. The excreted eggs hatch in fresh water to become ciliated miracidia, which in turn infect the intermediate snail host. In the snail massive asexual multiplication occurs. After two generations of sporocysts (mother and daughter sporocysts) cercariae are released into the fresh water and thus the life cycle is completed.

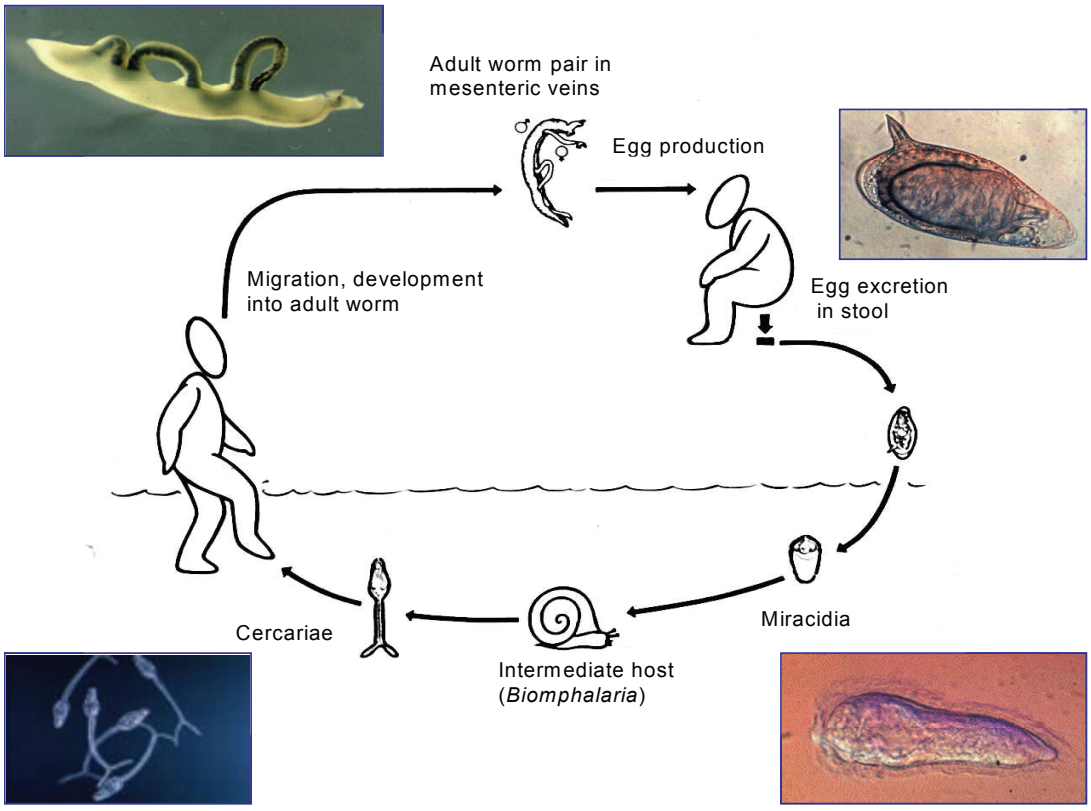


Figure 1. Life cycle of *Schistosoma mansoni*. Life cycle with courtesy of Dr. A.M. Polderman. Pictures with courtesy of Dr. D.W. Dunne.

PATHOLOGY AND MORBIDITY

The global burden of the disease is high; approximately 20 million people suffer from severe consequences of infection, and annually in an estimated 280.000 cases the disease progresses to a lethal outcome (van der Werf *et al.*, 2003). However, the majority of the globally 200 million infected individuals show relatively mild or no symptoms. Still, the effect of schistosomiasis on the health status of infected individuals is clearly not negligible. The evidence-based reassessment of schistosomiasis-related disability as published in The Lancet in 2005, significantly associated diarrhoea, pain, and fatigue, and other objective findings such as haemoglobin deficit, undernutrition and reduced exercise tolerance with human schistosome infection (King, Dickman & Tisch, 2005). This study on the global prevalence of schistosome infection suggests that the true public health burden of schistosomiasis is probably substantially greater than previously thought. However, the unspecific indirect morbidity such as pain and fatigue is difficult to measure and to dissociate from other poverty-related health problems and is therefore subject of a continuous debate (Gryseels *et al.*, 2006; King *et al.*, 2006). This debate has been revived in the light of the Disease Control Priorities Project (DCPP), a joint project of the Fogarty International Center of the US National Institutes of Health, the WHO, and The World Bank, was launched in 2001 to identify policy changes and intervention strategies for the health problems of low-income countries (Laxminarayan *et al.*, 2006).

The term schistosomiasis generally refers to the chronic stage of the disease, which has the biggest impact on the infected individual. However, three distinct syndromes are caused by schistosomes: cercarial dermatitis, acute schistosomiasis and chronic schistosomiasis.

Cercarial dermatitis

The so-called cercarial dermatitis or swimmer's itch is caused by an acute inflammatory reaction that occurs upon penetration of cercariae into the human skin, which may provoke a rash. This dermatological response in the human host is variable and dependent on the degree of hypersensitivity induced by previous exposure. Initial exposure to cercariae produces only mild, transient reactions that often pas unnoticed. Swimmer's itch may also result from invasion of non-human (e.g. avian) schistosome cercariae (genus Trichobilharzia) that do not mature in man (Blazova & Horak, 2005). Cercarial dermatitis is therefore also seen in non-tropical countries, non-endemic to human schistosomes (Verbrugge *et al.*, 2004; Ferte *et al.*, 2005).

Acute schistosomiasis

Acute schistosomiasis or Katayama syndrome (as reviewed by (Ross *et al.*, 2007)) appears between 14-84 days after non-immune individuals are exposed to first schistosome infection or heavy reinfection. To others this early phase of infection is often asymptomatic. Disease onset is associated with migrating schistosomula and egg deposition. The symptoms are non-specific and include: fever, headache, cough, loss of appetite, abdominal tenderness, diarrhoea and often eosinophilia. Clinical diagnosis is very difficult at this stage, mainly because it is still too early to reliably demonstrate eggs in the excreta (Polderman *et al.*, 1989; Visser, Polderman & Stuiver, 1995). Based on positive serology for schistosomal antibodies and high eosinophil counts (Bierman, Wetsteyn & van Gool, 2005) and on a history of fresh water exposure in an endemic area 14-84 days before presentation of clinical symptoms, more and more travellers and tourists returning home from an endemic country are diagnosed with acute schistosomiasis (Whitty *et al.*, 2000).

Chronic schistosomiasis

The chronic stage of infection may occur even without recognisable symptoms and can last for decades. The manifestations of the disease (diarrhoea, dysentery, abdominal pain, fatigue, weight loss, bloody stool or haematuria), are due to chronic inflammatory reactions induced by the eggs. *S. haematobium* infections are characterised by blood in the urine (haematuria) caused by the inflammatory reactions around the eggs trapped in the bladder wall, which may ultimately lead to bladder cancer (Norden & Gelfand, 1972). The eggs of the other schistosome species that do not leave the body with the excreta may be deposited in the intestines (intestinal schistosomiasis) or are swept into the periportal circulation and get trapped in the perisinusoidal capillary venules of the liver (hepatosplenic schistosomiasis). The trapped eggs excrete antigens through microscopic pores in the egg shell, which induce a cellular, granulomatous response leading to fibrosis. Severe infections can result in portal hypertension, hepatomegaly, splenomegaly, ascites and bleeding varices, which may lead to death.

The egg induced granuloma formation (Figure 2) is a delayed type hypersensitivity (Th2 type) reaction, and, although eventually resulting in severe pathology appears to be a necessary protective host response against the excreted egg antigens (Stadecker *et al.*, 2004). The granulomata formed around the egg consist of a number of different immune cells, including T and B lymphocytes, macrophages, giant cells, epitheloid cells, mast cells, plasma cells, fibroblasts and eosinophils. The inflammatory response is most enhanced at the acute stage (8-10 weeks) and is down-modulated at the chronic stage. The severity of the disease depends

on the intensity and duration of infection, but immunological and genetic factors of the host also play a role.

DIAGNOSIS AND DIAGNOSTIC METHODS

A simple, cheap, sensitive and highly specific assay for routine diagnosis is not yet available. A commercially available easy to perform assay with potential, but which is not yet widely used, is the reagent strip test for detection of the adult worm antigen circulating cathodic antigen (CCA) in urine. The lateral flow test based on the capture of the immune complex of CCA and a carbon-labeled anti-CCA antibody shows high sensitivity, association with the intensity of infection and a high specificity (van Dam *et al.*, 2004), but the added value of the reagent strip test for diagnosis in low endemic areas has not been proven yet (Legesse & Erko, 2007). Methods that allow infections to be correctly diagnosed are a prerequisite for effective disease control. This applies not only to those living in endemic areas, but also to tourists and other travellers to an endemic region who may return home infected (Whitty *et al.*, 2000).

Schistosomiasis can be diagnosed by direct or indirect methods.

Direct parasitological methods

There are two main variants of direct methods. The first is microscopic detection of parasite eggs in the excreta or in tissues of infected individuals. The second direct method is the detection of parasite-derived material in the circulation or excreta.

The detection of schistosome eggs by microscopy is still most widely used. The eggs of the different schistosome species are identifiable by egg morphology. Eggs of *S. mansoni* are approximately 140 x 60 µm in size and have a characteristic lateral spine. The eggs of *S. haematobium* have the same size as those of *S. mansoni*, but have a terminal spine and the eggs of *S. japonicum* are smaller (80 x 63 µm) and have a strongly reduced terminal spine. The Kato-Katz thick smear (Katz, Chaves & Pellegrino, 1972) is the most extensively used method for examining stool for *S. mansoni* and *S. japonicum* eggs while sedimentation or filtration of urine is used for *S. haematobium* eggs. These microscopy-based methods are very specific, simple and cheap and require, in areas of high endemicity, personnel with only basic training. However, an important disadvantage of the Kato-Katz thick smear is the lack of sensitivity (De Vlas & Gryseels, 1992; Engels *et al.*, 1997). Due to the uneven distribution of *S. mansoni* eggs in solid excreta and the considerable day-to-day fluctuation, infections (especially in those individuals with low infection intensity) are easily missed.

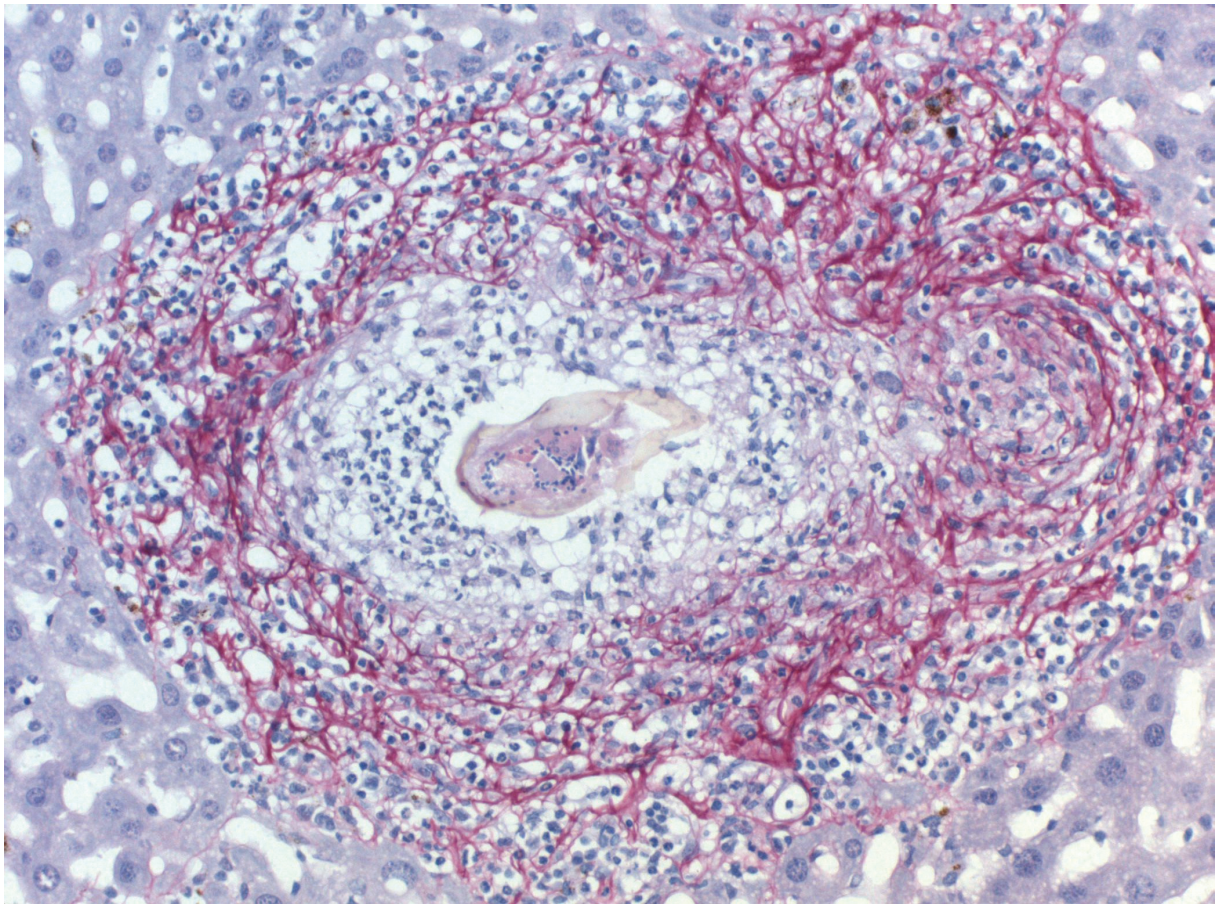


Figure 2. Granuloma around a *Schistosoma mansoni* egg in the liver of an 8 weeks infected Swiss outbred (OF1) mouse, stained with Sirius Red.

Several schistosome-specific antigens excreted by cercariae, schistosomula, adult worms or eggs have been found in blood or urine of the host. Parasite-derived circulating antigens are generally detected in an antibody sandwich enzyme-linked immunosorbent assay (ELISA); a (96-well) ELISA plate is coated with monoclonal or polyclonal antibodies directed against schistosome specific antigens. The schistosome antigens in the blood or urine of infected patients bind to the antibody coating and are 'sandwiched' by conjugated schistosome specific mono- or polyclonal antibodies that are applied in the next step. When the same monoclonal antibody is used for the coating and for the conjugation step, only antigens with a repetitive (glycan-)epitope can be captured in the sandwich ELISA.

The two schistosome circulating antigens that have been studied most extensively are the adult worm gut-associated circulating anodic antigen (CAA) and circulating cathodic antigen (CCA), named according to their migratory behaviour in immuno-electrophoresis (Deelder *et al.*, 1976). Circulating antigen levels are relatively sensitive, specific and quantitative markers of active schistosome infections. The CAA/CCA assays have therefore proven valuable diagnostic tools for the follow-up of chemotherapy and reinfection, as a screening test in

control programmes and as an epidemiology research tool (Polman, 2000). Although it was shown that the concentration of CAA/CCA correlates with the number of living worms (De Jonge *et al.*, 1990) and with egg excretion (De Jonge *et al.*, 1989), the levels of these worm-antigens do not merely reflect the tissue egg load.

It is generally accepted that the detection of egg antigens - more than the detection of adult worm antigens - would reflect the tissue egg load and correlated morbidity (Nibbeling *et al.*, 1998b; Hassan *et al.*, 1999). Various studies have demonstrated circulating soluble egg antigens (CSEA) in serum and urine of infected individuals (Fu & Carter, 1990; Hassan, Badawi & Strand, 1992; Nourel Din *et al.*, 1994b; Nibbeling *et al.*, 1997; Nibbeling *et al.*, 1998a). The sandwich ELISA, which was developed in our laboratory to detect circulating egg antigens in blood or urine of *S. mansoni* infected individuals plays a central role in this thesis. In this diagnostic ELISA two IgG monoclonal antibodies (mAbs) 114-4D12 and 114-5B1 (Nourel Din *et al.*, 1994a) are used that are reactive with two different repetitive sugar epitopes on soluble egg antigens (SEA). MAbs 114-4D12 and 114-5B1 are cross-reactive with other life cycle stages (Bogers *et al.*, 1994; Nourel Din *et al.*, 1994a; Robijn *et al.*, 2005; Robijn *et al.*, 2007a). However, the recognised epitopes are at least 10,000 times more concentrated in eggs than in adult worms (Nourel Din *et al.*, 1994a), which makes this ELISA highly egg antigen-specific. In mice the CSEA become detectable from eight weeks post-infection onwards, when egg laying has started (Nourel Din *et al.*, 1994a). The CSEA levels are strongly correlated to egg output (Nourel Din *et al.*, 1994b) (Figure 3). Similar correlations have been found for egg counts and CSEA levels using a comparable *S. haematobium* CSEA detection assay in which another mAb is used (290-2E6) (Nibbeling *et*

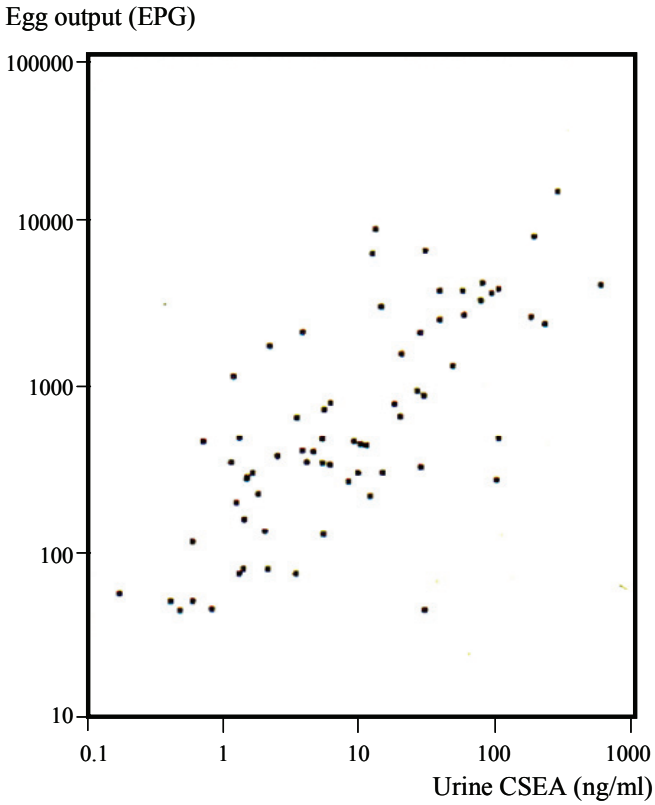


Figure 3. Correlation between the concentration of circulating soluble antigen (CSEA) in urine determined by the combined enzyme-linked immunosorbent assay and egg output in *Schistosoma mansoni*-infected individuals. EPG = eggs per gram of faeces. Figure reproduced from Nourel Din *et al.* (1994) Am. J. Trop. Med. Hyg. 50:585-594

al., 1998a). Moreover, *S. haematobium* CSEA levels are independent of the day-to-day fluctuation in egg excretion and are correlated to ultrasound detectable pathology (Kahama *et al.*, 1998). Nevertheless, the additional value of the current CSEA detection assays appears to be limited, since sensitivity of the assays remains too low to compete with the generally used egg counts or the assays to detect the adult worm antigens CAA/CCA.

More recently the potential for detecting schistosome DNA in faeces by polymerase chain reaction (PCR) is being investigated (Pontes *et al.*, 2003). Another new tool that is currently heavily investigated and may lead to a novel approach for diagnosis, based on the detection of parasite-proteins is 'parasitoproteomics' or proteomic fingerprinting (Biron *et al.*, 2005; Agranoff *et al.*, 2005).

Indirect parasitological methods

The indirect methods are based on the detection of schistosomiasis related factors, such as the examination of morbidity by questionnaires, observation of intestinal pathology by imaging techniques (X-ray or ultrasound) or the detection of immunological measures. Methods that involve detection of schistosome-specific antibodies constitute the bulk of indirect methods (Doenhoff, Chiodini & Hamilton, 2004), but also the more "conceptual" approach of detecting biomarkers based on the metabolic changes that occur in a schistosome-infected animal (Wang *et al.*, 2004), belongs to this category.

Indirect diagnostic methods are generally less specific than direct methods. Specific antibody detection does not differentiate between present and past infections, but is generally sensitive, which makes it more suitable in specific situations, e.g. for diagnosis of incidentally exposed individuals (travellers) or for diagnosis in areas where the prevalence of schistosomiasis is so low that direct methods fail (Deelder, Duchenne & Polderman, 1989; Polderman *et al.*, 1989).

CONTROL, TREATMENT AND VACCINATION

Currently, the cheap drug Praziquantel is the common platyhelminthicide used to treat schistosomiasis. Recently it was shown that Praziquantel predominantly binds schistosome actin on the surface membrane of adult worms (Tallima & El Ridi, 2007). Praziquantel has few and mild side effects and a high activity against all five species of schistosomiasis. A single oral dose of 40mg/kg body weight of Praziquantel is generally sufficient to give cure rates of between 60-90% and reductions of 90-95% in the average number of excreted eggs. In individuals from non-endemic situations (immigrants and travellers, in which re-infection does not occur) the cure-rate is said to be nearly 100% (Whitty *et al.*, 2000). However, therapeutic failures to Praziquantel at a single dose have been reported (Alonso *et al.*, 2006).

There is a fear for development of praziquantel resistance among schistosomes, but fortunately no resistance to praziquantel has yet developed (Fenwick & Webster, 2006). A higher dose of praziquantel or drug combinations (praziquantel combined with artemether or oxamnique) may help to improve treatment success rates (Utzinger *et al.*, 2001). Artemisinin-based combination therapy can be used alternatively to Praziquantel and may have special value for young children co-infected with Plasmodium sp. Preclinical studies showed the promising activity of artemisinins against intestinal and liver flukes in vivo, as well as against cancer cells (Utzinger *et al.*, 2007).

The WHO member states currently implement a strategy of repetitive treatment of clinical cases and groups at high risk of morbidity. Based on the WHO strategy (WHA 54.19, May 2001) the Schistosomiasis Control Initiative (SCI; http://www.schisto.org) aims for the regular administration of chemotherapy to at least 75% and up to 100% of all school-age children at risk of morbidity by 2010 (Fenwick *et al.*, 2006). This policy is focussed on the control of morbidity rather than on the prevention of transmission. The improvement of water and sanitation facilities, health education and integration of the control measures into existing public health services are a prerequisite for long-term control (Engels *et al.*, 2002).

Treatment does not prevent reinfection; however, recent studies have suggested that treatment not only results in reduced morbidity but additionally would lead to increased resistance to reinfection as a result of altered immune responses to antigens that become available after the adult worms die (Colley & Evan, 2004; Mutapi *et al.*, 2005). Although it is evident that humans can acquire immunity to schistosomes (Woolhouse *et al.*, 1991), so far the development of an effective vaccine has failed.

The first vaccination experiments were performed in the early eighties in mice and baboons with irradiation attenuated cercariae (Sturrock *et al.*, 1980; Hsu, Hsu & Burmeister, 1981; Hsu *et al.*, 1983; Damian *et al.*, 1984). These studies were partly successful and supported the idea that a vaccine for schistosomiasis would be feasible. It would be impossible, however, to use irradiated cercariae for vaccination of man, since the possibility exists that (less) irradiated cercariae cause pathology e.g. attenuated larvae may reach sites such as the brain. Therefore many more initiatives were undertaken for vaccine development in the past decade. The approaches ranged from using native or recombinant proteins and peptide constructs to nucleic acid vaccines (as reviewed by (Kalinna, 1997; Bergquist *et al.*, 2002; Capron *et al.*, 2002; Lebens *et al.*, 2004; Wu, Lu & Yu, 2005)). However, so far only one anti-schistosome vaccine candidate, a 28kD glutathione-S-transferase, has been tested in clinical trials (Capron

et al., 2005). This recombinant protein vaccine (rSh 28GST) that showed significant inhibitory effect on the female worm fecundity and egg viability was found safe for use in humans. The next phases of clinical trials are being prepared for efficacy tests in infected children (Capron *et al.*, 2005). The other current vaccine candidates have so far shown limited efficacy in trials with mice. Two-dimensional (2D) electrophoresis, liquid chromatography, mass spectrometry and the advances of the schistosome genomic and postgenomic investigations result in the identification of novel vaccine and drug candidates (Wu, Lu & Yu, 2005; Wilson & Coulson, 2006b; Hokke, Fitzpatrick & Hoffmann, 2007). The completion of the genomes of *S. mansoni* and *S. japonicum*, which is anticipated for 2008, and the ongoing development of tools for gene manipulation and transgenesis of schistosomes will also be of significant assistance to the field of vaccine development (Brindley & Pearce, 2007).

Relatively new is the glycan-based approach to vaccine development to fight parasitic infections (Dennis, 2003). The first conjugate vaccine candidates containing synthetic oligosaccharide antigens are reaching preclinical and clinical trials against parasitic infections (Seeberger & Werz, 2007). Many of the glycans (oligosaccharides/carbohydrate chains/sugar chains) that schistosomes carry are clearly distinctive from those of their host, which is a basic requirement for any type of vaccine molecule. They are widely displayed on many different proteins and lipids that are often exposed on the outer surface of the parasite or secreted in the case of proteins, which makes glycans good targets by their availability for immune interactions. Moreover they are less subjective to antigenic variation than proteins and seem to be central to the parasites' ability to conquer the host defences in infections (Dennis, 2003). Encouraging results have been achieved by the immunisation of animals with partially defined glycoprotein antigens, as reviewed by Nyame, Kawar & Cummings. (2004). Keyhole limpet hemocyanin (KLH), a glycoprotein from the mollusc *Megathura crenulata* has been shown to induce protection against cercarial challenge in rats through a *S. mansoni*-cross-reactive glycan epitope (Grzych *et al.*, 1987). Alternative vaccination strategies may make use of peptide mimotopes that mimic the glycan components (Nyame, Kawar & Cummings, 2004). In addition, schistosomal glycoconjugates are of interest for use as adjuvants. KLH is often used in vaccine studies as a carrier because it generates impressive immune responses (Gathuru *et al.*, 2005; Slovin, Keding & Ragupathi, 2005) that may arise through its unusual glycans (Kantelhardt *et al.*, 2002; Wuhrer *et al.*, 2004b). Okano *et al.*, (Okano *et al.*, 1999) showed that the carbohydrates on SEA are not themselves the targets of the induced IgE response but that they act as Th2-activating adjuvants. It would appear that an anti-schistosome vaccine may ultimately become available, but there is still a long way to go.

Biological roles of schistosome glycoconjugates

As indicated above, the schistosome glycans linked to proteins or lipids (glycoconjugates) play an important role in the interaction between the schistosome and its host. Glycans or glycoconjugates are important targets for the host innate and adaptive immune responses. On the other hand it has become increasingly clear that the parasite benefits from the expression of these glycoconjugates e.g. in evading the immunological measures of the host. About the fundamental roles of glycans in the parasite's development little is yet known. Especially very few specific and authentic glycans or glycoconjugates that induce a particular effect have been identified at molecular level (Hokke *et al.*, 2007b)

In this thesis we focus on the identification and characterisation of some exceptional glycans found in schistosomes. It is of great importance to study the structures of the parasite's glycans in order to gain insight into their functions, immunomodulatory effects and to find new diagnostic or intervention tools. The possible functions of schistosome glycans and their interaction with the host's immune system will be briefly discussed, followed by a more detailed description of the synthesis, structural components and techniques for the analysis of glycans and glycoconjugates.

Generally, it is very difficult to predict the function of a given oligosaccharide on a given conjugate (protein or lipid), or the relative importance to the organism (Varki, 1993). But the observations that recombinant (non-glycosylated) schistosome proteins induce different immune responses than their natural (glycosylated) counterparts (Asahi & Stadecker, 2003), together with the observations that periodate-treated antigens have different Th-stimulatory effects (Okano *et al.*, 1999) strongly suggest that these glycans do specifically mediate the protein's function. Common features of oligosaccharides are that they mediate specific recognition events or that they modulate biological processes.

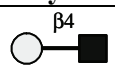
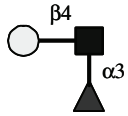
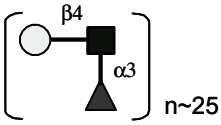
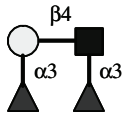
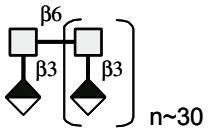
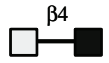
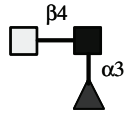
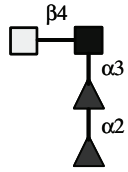
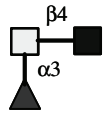
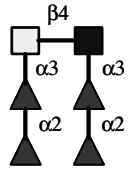
All schistosome life cycle stages abundantly express glycoconjugates and their expression is developmentally regulated. In addition, in adult schistosomes gender-specific glycosylation occurs as became evident from combined dioecious transcriptomic and proteomic data (Hokke *et al.*, 2007a) as well as from comparison of released glycans from males and females by mass spectrometry (Wuhrer *et al.*, 2006d). The sex- and stage-specific expression of glycans may reflect the specific demands of the different schistosome life cycle stages for their interaction with their host and environment. For example the unique complex oligosaccharide structures isolated from the glycocalyx, a dense layer of glycans and glycoconjugates that covers the surface of the free living stages of the parasite, cercariae and

miracidia, may play a role in the mechanical stabilization of the surface of the schistosome larvae in fresh water (Chiang & Caulfield, 1988; Nanduri *et al.*, 1991). In the snail, and arguable also in man, specific carbohydrate structures seem to be involved in the process of self-nonself discrimination. By expressing carbohydrate structures that are similar to those of their intermediate host, schistosomes can create molecular mimicry at their surface (Dissous, Grzych & Capron, 1986; Damian, 1987; Neeleman, van der Knaap & van den Eijnden, 1994).

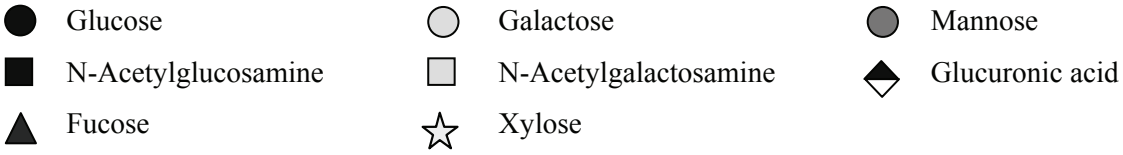
Interesting research has been done on parasite-host interactions involving the (developing) worm stages. To survive, the adult worms have to evade or resist the activity of the defence system of the human host. Schistosomes are very successful in doing so, as adult worms are able to survive inside the hostile environment of the blood vessels for up to 35 years (Fulford *et al.*, 1995). Many immune evasion mechanisms that involve glycans have been proposed to explain the survival of the parasite including: molecular mimicry (Damian, 1987; Damian, 1989), masking of the parasite surface by acquisition of host molecules (McLaren, 1984), physicomechanical resistance of the multilayered tegumental membrane (McLaren & Hockley, 1977), inhibition of complement activation (Horta & Ramalho-Pinto, 1991; van Dam *et al.*, 1993b) and shedding of antigen-antibody complexes (Pearce *et al.*, 1991).

Nevertheless, schistosome glycans do induce different immune responses. In infected individuals antibody responses are found against different glycan-structures (Lewis X (LNFPIII), LDN, F-LDN, LDN-F, LDN-DF, F-LDN-F, FFGn, FFFGn see Table I) (Eberl *et al.*, 2001; Nyame *et al.*, 2003; van Roon *et al.*, 2005) with the strongest responses predominantly of the IgG1 isotype against those oligosaccharides that are unique to the parasite (van Remoortere *et al.*, 2001; Kantelhardt *et al.*, 2002; Naus *et al.*, 2003; van Remoortere *et al.*, 2003). Antibodies of the IgG2 and IgM isotypes are predictive for susceptibility to reinfection (Butterworth *et al.*, 1988; Demeure *et al.*, 1993). The IgG2 and IgM responses, that are higher in young children than in (more resistant) adults, may block effector antibodies like IgG1 and IgE, and fail to mediate antibody-dependent cytotoxicity (Dunne *et al.*, 1987b; Butterworth, 1992). It has been suggested that the anti-glycan antibodies, which are mainly induced by the eggs, may function as a smokescreen to prevent anti-schistosome immunity to build up (Eberl *et al.*, 2001) as only during the chronic stage of infection (protective) antibodies to peptide epitopes become prominent.

Table I. Terminal glycan structures found on schistosome glycoconjugates.

Abbreviation	Structure	Structure in symbols *
LN, LacNAc	Galβ1- 4GlcNAcβ1-	
Lewis^x	Galβ1- 4GlcNAcβ1- Fucα1-3	
CCA	[Galβ1- 4GlcNAcβ1- Fucα1-3]$_{n\sim25}$	
Pseudo-Lewis^y	Galβ1- 4GlcNAcβ1- Fucα1-3 Fucα1-3	
CAA	-6GalNAcβ1 -[6GalNAcβ1- GlcAβ1-3 GlcAβ1-3]$_{n\sim30}$	
LDN, LacdiNAc	GalNAcβ1 - 4GlcNAcβ1-	
LDN-F	GalNAcβ1 - 4GlcNAcβ1- Fucα1-3	
LDN-DF	GalNAcβ1- 4GlcNAcβ1- Fucα1-3 Fucα1-2	
F-LDN	GalNAcβ1 - 4GlcNAcβ1- Fucα1-3	
DF-LDN-DF	GalNAcβ1→4GlcNAcβ1- Fucα1-3 Fucα1-3 Fucα1-2 Fucα1-2	

**Symbolic representation according to nomenclature adopted by the Consortium for Functional Glycomics (CFG) (http://www.functionalglycomics.org/static/consortium/).*



In contrast to the adult worms around which, in the vasculature of infected mice and chimpanzees no overt cellular inflammation has been detected (Keating, Wilson & Skelly, 2006), around eggs robust cellular inflammation occurs. The schistosome eggs cause the major stimuli for the Th2 type response that is so characteristic for helminth infections (Pearce, 2005) next to being active inducers of the anti-glycan humoral immune response. It seems that egg excretion from the human body - and therewith survival of the schistosome species - is dependent upon the host's immune response, as egg excretion does not occur in immunocompromised animals (Pearce, 2005). Upon egg deposition, the initial Th1 type immune response is skewed towards Th2 (Grzych *et al.*, 1991; Pearce & MacDonald, 2002). In the strictest sense, the egg-directed Th2 response is host-protective: mice lacking the ability to develop a normal Th2 type response die soon after egg-laying has started (Doenhoff *et al.*, 1986; Brunet *et al.*, 1997).

Okano *et al.*, were the first who showed that glycans are the important components for the induction of Th2 responses: egg antigens lost their ability to induce Th2 responses after all glycans were destroyed by periodate treatment (Okano *et al.*, 1999). In a second paper they also showed that one specific glycan LNFPIII, which contains Lewis X, when conjugated to human serum albumin (HSA) could induce a strong Th2 response (Okano *et al.*, 2001). Moreover LNFPIII acted as an adjuvant inducing anti-HSA antibody production for which conjugation to a protein and the fucose residue were essential. More Th2-driving helminth glycans have been defined (Van der Kleij *et al.*, 2002b; Faveeuw *et al.*, 2003) but about the mechanisms by which (schistosome) glycans bias the immune response (reviewed by (Thomas & Harn, Jr., 2004)) little is yet known. Thomas, Harn and co-workers (Thomas *et al.*, 2005) found that DC2 maturation is promoted by LNFPIII/Lewis X via a toll-like receptor 4 (TLR4)-dependent pathway. More recently it was demonstrated that the internalization of *S. mansoni* SEA by multiple C-type lectins may be important to regulate the response of immature DCs to TLR-induced signals (van Liempt *et al.*, 2007). Molecular modelling studies increase our knowledge on the recognition patterns and preferred binding modes of C-type lectins (L-SIGN) to schistosome oligosaccharides (Meyer *et al.*, 2007). However, to acquire a good understanding of how DC's or other antigen presenting cells (APC) are activated to drive Th2-type and anti-inflammatory responses more research is needed, which may have importance for the use of glycans (as adjuvants) for vaccines, as well as for the development of novel anti-inflammatory therapeutics that could be used to treat Th1-mediated autoimmune diseases.

Much longer it has been established that glycans play an important role in the formation of granuloma around trapped eggs in the tissues (Weiss, Aronstein & Strand, 1987; Jacobs, Deelder & Van Marck, 1999; Van de Vijver *et al.*, 2004), which is associated with Th2 type immune responses. Research to determine exactly which glycans or glycoconjugates are responsible for each of these processes is often hampered by a lack of sufficient amounts of purified material and lack of availability of synthetic structures that correspond to the exceptional parasite glycans (van Roon *et al.*, 2005). Only recently Van de Vijver *et al.* (2006) showed that terminal Galβ1-4GlcNAc (LacNAc, LN) or GalNAcβ1-4GlcNAc (LacdiNAc, LDN) (see Table I) glycan elements specifically harbour granuloma-inducing activities, whereas other synthetic glycan elements (Lewis X, FFGn and F-LDN-F) did not. At the molecular level, various other interactions between schistosome glycans and host immune cells via receptors such as C-type lectins and toll-like receptors have been identified (as reviewed by (Hokke & Yazdanbakhsh, 2005)), but it remains to be seen how exactly, these interactions have implications for the immunomodulatory and/or stimulatory effects of schistosome glycans observed *in vitro* and *in vivo*.

Schistosome glycans and glycoconjugates

Direct structural and indirect histochemical studies have shown that schistosomes produce an enormous variety of glycans. Evidently, schistosomes express many different glycosyltransferases, on which the glycan biosynthesis is based. Relatively few of the schistosome glycosyltransferases have yet been identified. Some of the described enzymes are very distinct from their mammalian counterparts and therewith form a basis for glycans that seem to be unique for schistomes or helminths. Of particular interest are the fucosyltransferases since schistosome glycoconjugates differ from typical mammalian glycoconjugates by their relatively high amount of fucose. Fucose (Fuc) has been found in schistosome glycoconjugates linked to *N*-acetylglucosamine (GlcNAc), *N*-acetylgalactosamine (GalNAc) or galactose (Gal) in the form of Fucα1-3GlcNAc, Fucα1-3GalNAc, Fucα1-3Gal, Fucα1-2Fuc and Fucα1-6GlcNAc linkages. Different α3-fucosyltransferases of *S. mansoni* have been described (DeBose-Boyd, Nyame & Cummings, 1996; Marques, Jr., Weiss & Strand, 1998; Trottein *et al.*, 2000), but it is expected that more schistosome α3-fucosyltransferases exist. In humans already six different α3-fucosyltransferases have been identified that all catalyse the formation of the same Fucα1-3-linkage to GlcNAc, but with different expression patterns or substrate specificity (reviewed by (de Vries *et al.*, 2001)).

The (±Fucα1-2)Fucα1-2Fuc moiety so far seems unique for schistosomes. The α2-fucosyltransferase that accounts for this specific Fucα1-2Fuc linkage, has been identified in cercariae of the avian schistosome-species *Trichobilharzia ocellata* (Hokke *et al.*, 1998). It is anticipated that one or more similar α2-fucosyltransferases exist in the human schistosome species. Differences in the levels of enzymatic activities as well as the occurrence of their products indicate that fucosyltransferases are differentially regulated during development. Much higher (up to 50-fold) fucosyltransferase activity is found in egg extracts compared to cercarial or worm extracts (Marques Jr *et al.*, 2001). The presence of higher fucosyltransferase activity in eggs may reflect the importance of fucosylated structures to the egg biology, and possible roles in mediating adhesion and trafficking, induction of Th2 and granulomatous responses have been suggested (Marques Jr *et al.*, 2001).

Schistosomes, like other eukaryotes, produce different classes of glycoconjugates which are defined according to the nature of their glycan core-region and the nature of the aglycone (protein or lipid): proteoglycans, glycosphingolipids and glycoproteins. Glycans are fundamentally different from their protein-or lipid backbones in that the possible combination of different monosaccharides in a linear or branched orientation with different possible linkages results in a large variety of possible structures. In addition, numerous other modifications, such as phosphorylation, sulphation or methylation may occur, but so far none of these modifications have been demonstrated to occur in schistosomes.

In the next paragraphs, previously to the introduction on the different schistosome glycoconjugates, some general information on the synthesis and structural components of each category of glycoconjugates (proteoglycans, glycosphingolipids and glycoproteins) will be given.

Proteoglycans

A proteoglycan is a protein with one or more covalently attached glycosaminoglycan (GAG) chains (Molecular weight (MW): roughly 10,000 to 2 million Da). The glycosaminoglycan chains consist of repeating disaccharide units that are normally build on the tetrasaccharide GlcAβ1-3Galβ1-3Galβ1-4Xylβ- linked to Serine (Ser). The GAG are roughly divided into different groups (hyaluronic acid (HA), chondroitin sulphate (ChS), dermatan sulphate (DS), heparan sulphate (HS) heparin (Hep), and keratan sulphate (KS)) according to the nature of the repeating disaccharide that form the inside of the GAG. These disaccharide repeating-units are not uniform but show diversity in number and position of the sulphate groups and hence have various physiological active domains.

The first schistosome antigen identified as a schistosome-derived 'proteoglycan-like antigen' was called the gut-associated proteoglycan (GASP) (Nash, Lunde & Cheever, 1981). However, later studies indicated that the initial name was incorrect as this antigen was no proteoglycan but a glycoprotein identical to the adult worm gut-derived antigen CAA (Nash & Deelder, 1985), which consists of a repeating disaccharide unit -6)-[β-D-Glc*p*A-(1-3)]-β-D-Gal*p*NAc-(1- arranged as a polymeric GalNAc-chain substituted with GlcA and is linked through an unknown core via a GlcNAc to a threonine (Thr) (Bergwerff *et al.*, 1994). Since then, only a few studies have characterised proteoglycans/glycosaminoglycans in schistosomes. In *S. mansoni* heparin and/or heparin sulphate, chondroitin sulphate and hyaluronic acid were found in the carcass and tegument of the adult worm (Robertson & Cain, 1985) and dermatan sulphate and chondroitin sulphate have been identified in adult worms of *S. haematobium* (Hamed, Maharem & El Guindy, 1997). No information is available about proteoglycans from *S. japonicum*.

Glycosphingolipids

Glycosphingolipids contain glycan chains based on a basic unit, which generally consist of -3Manβ1-4Glc-, -3Galβ1-4Glc-, -4Galβ1-4Glc-, -Galα1-4Gal- linked to the ceramide (Cer). Schistososomes produce a unique glycosphingolipid core: GalNAcβ1-4Glcβ1-Cer therefore called the 'schisto-core' (Makaaru *et al.*, 1992). Lipid extracts of eggs, worms and cercariae of *S. mansoni* have shown to contain a large number of highly immunogenic glycolipids (Weiss, Magnani & Strand, 1986). The simple core is extended by complex, neutral glycans of which (terminal) elements are also commonly expressed on schistosome glycoproteins (Robijn *et al.*, 2005). In both *S. mansoni* and *S. japonicum* the glycolipids may be extended with a heterogeneously (mono-, di-, tri-) fucosylated linear GlcNAc-chain, containing up to 5 GlcNAc, terminating with GalNAc (Wuhrer *et al.*, 2002). The terminal GalNAc is often difucosylated in *S. mansoni*, but not in *S. japonicum* (Khoo *et al.*, 1997a). *S. mansoni* glycosphingolipids from cercariae are dominated by terminal Lewis X or pseudo-Lewis Y (Wuhrer *et al.*, 2000b) (see Table I).

Three different classes of glycans occur on glycoproteins: glycans can form a glycosylphosphatidylinositol (GPI) anchor or are linked to the polypeptide backbone via N- (N-glycans) or O- glycosidic linkages (O-glycans) (Figure 4).

GPI anchors

The biosynthesis of GPI anchors occurs in two major steps. First the GPI is preassembled in the endoplasmatic reticulum (ER), oligosaccharides and phosphoethanolamine are

sequentially added to the phosphatidylinositol, resulting in a complete GPI precursor. Then the GPI precursor is transferred to carboxy-terminal of certain membrane-associated proteins (Hart, 1999).

Several proteins containing a GPI anchor have been identified in schistosomula (Pearce & Sher, 1989) and adult worms (Sauma & Strand, 1990; Jones *et al.*, 2002), but not in 7-day-old lung stage parasites (Pearce *et al.*, 1989), which suggests developmental regulation of expression. The GPI anchored surface membrane proteins have been shown to be immunogenic and are spontaneously released from the surface of the parasite, which may contribute to immune evasion. A 200kD GPI anchored glycoprotein is exposed on the surface of *S. mansoni* adult worms following praziquantel treatment (Sauma & Strand, 1990). An acetylcholinesterase found on the surface as well as in muscle from *S. haematobium* adult worms exists as a GPI anchored dimer, which is highly unusual for acetylcholinesterases (Jones *et al.*, 2002). Furthermore the sequence of the C-terminus of the schistosome-derived enzyme was not conform the current consensus for GPI modification, which indicates that schistosome GPI anchors may differ from those of the mammalian host.

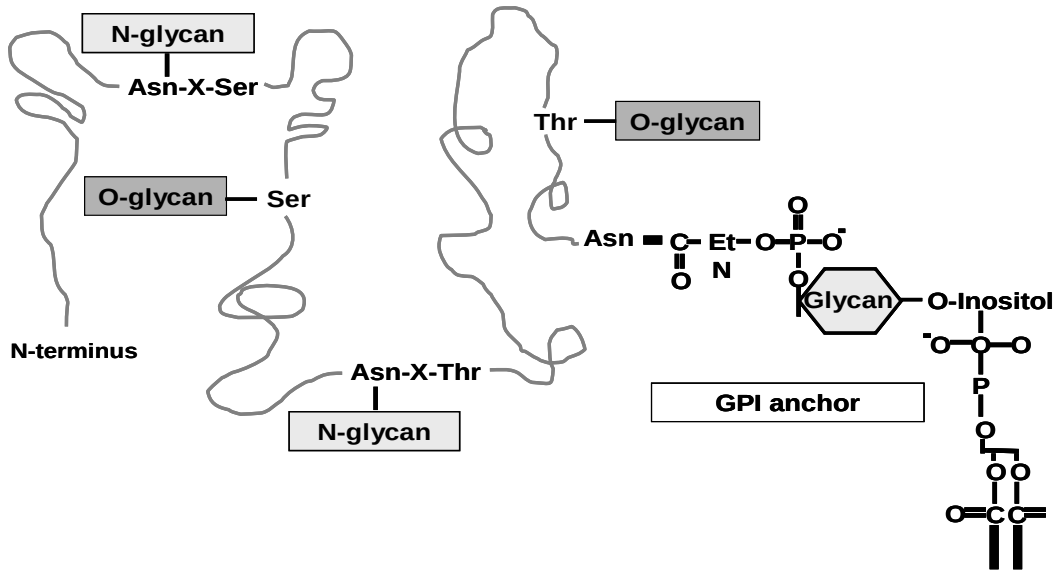


Figure 4. Carbohydrate-peptide linkages in glycoproteins. Adapted with courtesy from Dr. P. Rudd.

In this thesis the emphasis lies on the structural characterisation of glycans on a subset of soluble egg glycoproteins and therefore characteristics and biosynthesis of N- and O-glycoprotein-linked glycans will be described most extensively.

N-glycans

N-glycans are covalently linked to an asparagine (Asn) residue when the Asn lies within the consensus sequence Asn-Xxx-serine/threonine/cysteine (Ser/Thr/Cys) (Figure 4) in a polypeptide chain (where Xxx is any amino acid except proline (Pro) or aspartate (Asp)). N-glycans share a common core-structure consisting of two N-acetylglucosamine (GlcNAc) residues and three mannose (Man) residues and can generally be divided into three main classes: oligomannosidic-type, complex-type and hybrid-type (Figure 5). The biosynthesis of all types N-glycans starts in the rough ER by the stepwise formation of the precursor-structure (A) Glc3Man9GlcNAc2 linked to the membrane-bound lipid dolichol (Kornfeld & Kornfeld, 1985; Schachter, 1991; van den Eijnden, 2000). This precursor structure is transferred en bloc to the Asn in the consensus sequence of a newly synthesized polypeptide at the ribosome. Then the oligosaccharide is trimmed by different subsequently acting glycosidases (α-glucosidases I, II and α-mannosidase I) resulting in (B) an oligo-mannosidic type N-glycan.

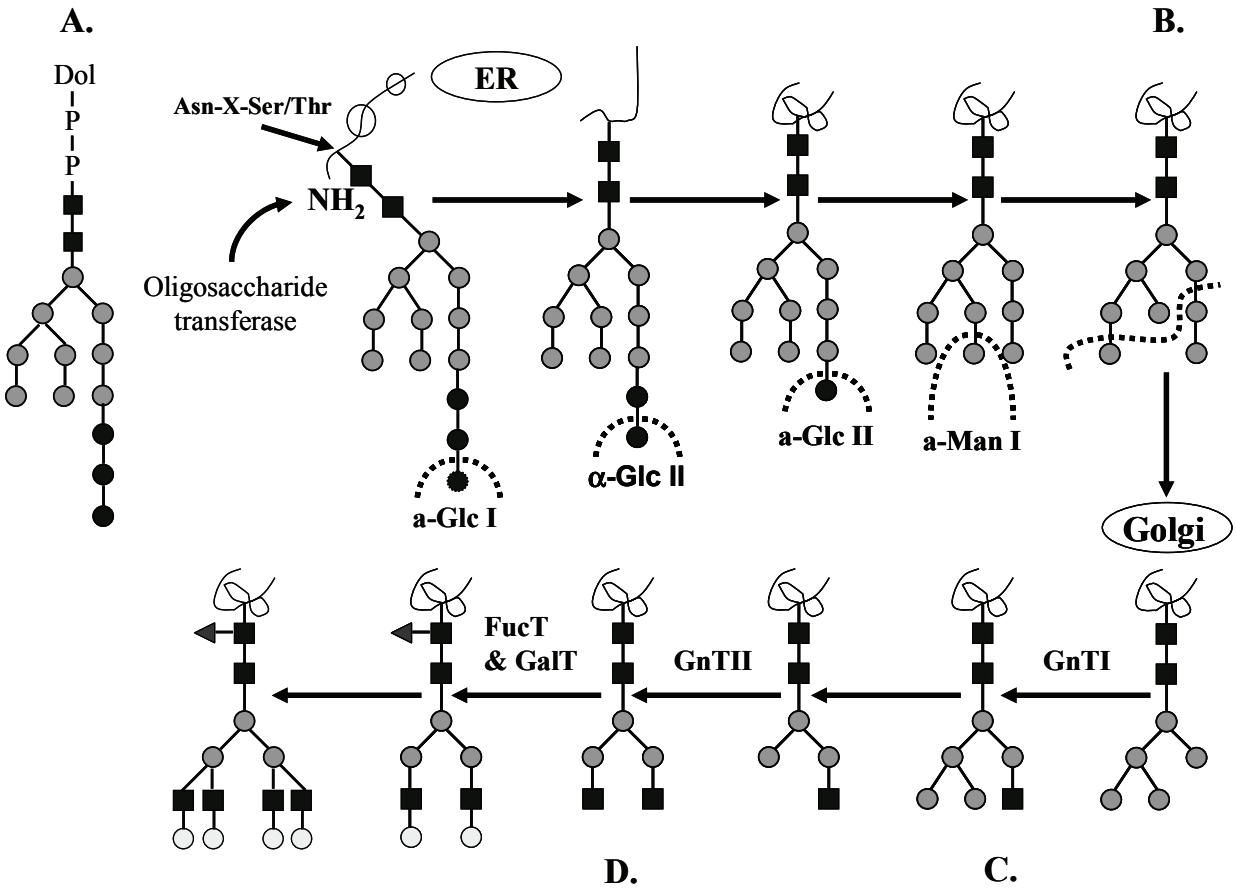


Figure 5. Biosynthesis of N-glycans. For further explanation see text.
Symbols are shown according to nomenclature adopted by the CFG .

The oligomannose structure may be transferred to the medial- or trans- Golgi cisternae where it can be converted into (C) hybrid or (D) complex (di-, tri- or tetra- antennary and/or core-fucosylated) structures by subsequently acting specific glycosidases and glycosyltransferases.

Schistosome N-glycans partly resemble those found in mammals: high mannose-type structures ($Man_{5-9}GlcNAc_2$-Asn) (Nyame, Cummings & Damian, 1988a) or complex-type di-, tri- or tetra- antennary structures (Nyame, Cummings & Damian, 1988b; Nyame *et al.*, 1989) were found in different stages of the parasite. Most schistosome N-glycans, however, are very different from their mammalian counterparts.

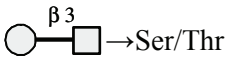
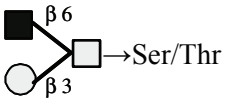
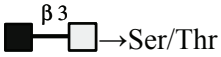
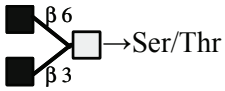
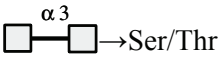
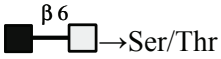
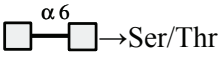
Schistosome N-glycans contain many different terminal motifs, such as mono- or poly-Galβ1-4GlcNAc (LN) or GalNAcβ1-4GlcNAc (LDN) (Nyame *et al.*, 1989; Srivatsan, Smith & Cummings, 1992a; Wuhrer *et al.*, 2006c), or fucosylated variants Galβ1-4(Fucα1-3)GlcNAc (Lewis X) (Srivatsan, Smith & Cummings, 1992b), GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) and Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (DF-LDN-DF) (Khoo *et al.*, 1997b) (Table I). The DF-LDN-DF terminal motif is found on N-glycans of *S. mansoni* eggs (and cercarial and egg O-glycans), but not in *S. japonicum* glycans, which do not contain the difucosylated terminal GalNAc (Khoo *et al.*, 1997b). Di-antennary N-glycans containing a terminal LDN(-F) or Lewis X motif have been found in glycoproteins of both invertebrate and vertebrate origin (Gooi *et al.*, 1981; Fox *et al.*, 1983; van den Eijnden *et al.*, 1997). In humans these structures are usually further modified, the GalNAc residue of LDN is 4-O-sulfated in human pituitary hormones (Baenziger, 1996) and Lewis X is often α2-3 sialylated.

Nonfucosylated, α6-monofucosylated as well as α3- and α6-difucosylated trimannosyl N -chitobiose core structures are found amongst schistosome N-glycans. A portion of these N-glyans from *S. mansoni* eggs is based on a β2-xylosylated, α6-fucosylated core, and *S. japonicum* egg glycoproteins may contain a remarkable β2-xylosylated, α3-, α6-fucosylated core (see Table I), which so far has not been described in any other species (Khoo *et al.*, 1997b). Core- α3 fucosylated and β2-xylosylated N-glycans are also found on other helminth-, plant-, insect-, and mollusc glycoproteins, but not on mammalian glycoproteins. No β2-xylosylation nor α3-fucosylation occurred in *S. japonicum* cercariae and adult worms (Khoo, Huang & Lee, 2001).

O-glycans

O-glycans are covalently linked to a Ser or Thr aminoacid residue via an *N*-acetylgalactosamine. No general core-structure is shared between different classes of O-glycans as is the case with the N-glycans. Thus, no *en bloc* transfer of a lipid linked precursor but the sole transfer of a GalNAc to Ser or Thr initiates O-glycosylation. The GalNAc is extended in at least 7 different linear or branched ways (see Table II) leading to a great variety of O-glycans.

Table II. Core structures of the GalNAcα1→Ser/Thr type O-glycans.

Core	Symbolic representation*	Structure
Core 1		Galβ1→3GalNAcα1→Ser/Thr
Core 2		GlcNAcβ1↘ 6 3 GalNAcα1→Ser/Thr ↗ Galβ1
Core 3		GlcNAcβ1→3GalNAcα1→Ser/Thr
Core 4		GlcNAcβ1↘ 6 3 GalNAcα1→Ser/Thr ↗ GlcNAcβ1
Core 5		GalNAcα→3GalNAcα1→Ser/Thr
Core 6		GlcNAcβ1→6GalNAcα1→Ser/Thr
Core 7		GalNAcα→6GalNAcα1→Ser/Thr

** Symbolic representation according to nomenclature adopted by the CFG.*

■ N-Acetylglucosamine

○ Galactose

□ N-Acetylgalactosamine

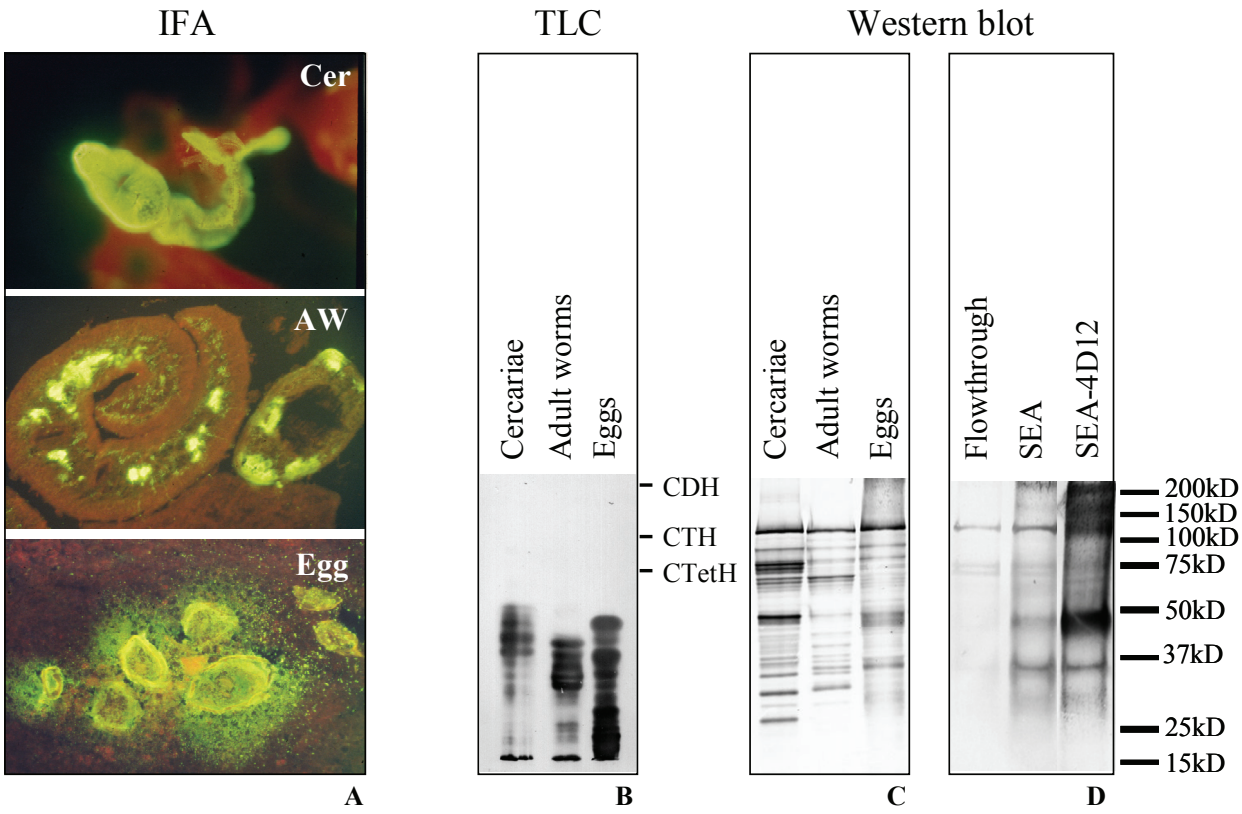


Figure 6. Immunostainings with monoclonal antibody 114-4D12. (*A*) Immunofluorescence assays (IFA) of *Schistosoma mansoni* cercaria (*Cer*), a male (left) and a female (right) adult worm (*AW*) and eggs (*Egg*). (*B*) High performance thin-layer chromatography (TLC)-resolved cercarial, adult worm and egg complex glycolipids (50ng of carbohydrate per lane). The migration positions of orcinol/H2SO4-stained globoside standards (CDH, CTH and CtetH) are indicated. (*C*) Western blot analysis of cercarial, adult worm and egg glycoproteins. (*D*) Western blot analysis of the affinity purified 114-4D12 binding subfraction of SEA (SEA-4D12), the flow-through and the starting material SEA. Masses of a precision protein standard are indicated.

Schistosome O-glycans range from single O-linked GlcNAc or GalNAc residues or short Galβ1-3GalNAcα1-Ser/Thr mucin-type disacharides on glycoproteins from *S. mansoni* schistosomula and adult worms (Nyame, Cummings & Damian, 1987; Nyame Cummings & Damian, 1988b) to very large and complex oligosaccharides. The adult worm gut antigens CAA and CCA contain very high molecular mass O-glycans and differ dramatically in structure. CAA is a unique threonine-linked polysaccharide consisting of a repeating motif of polymeric GalNAc residues substituted with GlcA, probably connected to the protein via a, yet unknown, core saccharide with GlcNAc at the reducing end (Bergwerff *et al.*, 1994). CAA is so far the only glycan found in schistosomes that contains an acidic glycan group.

CCA consists of an O-linked poly-Lewis X carbohydrate chain with approximately 25 repeating units, containing GalNAc as the reducing terminal monosaccharide (van Dam *et al.*, 1994). The poly-Lewis X chains are attached to the protein backbone via core 1 and/or core 2 O-glycan structures.

Glycoproteins from the cercarial glycocalyx are also high molecular mass O-glycans. These O-glycans consist of an unusual backbone of a trisaccharide-repeating unit, -3GalNAcβ1-4GlcNAcβ1-3Galα1- substituted with di- and tri-fucosyl residues (Khoo *et al.*, 1995). These structures were in line with previous findings that the cercarial glycocalyx predominantly consists of fucose, galactose, galactosamine and glucosamine (Xu *et al.*, 1994) and are attached via a core 2 or (the smaller glycans) via core 1 (see Table II) (Khoo *et al.*, 1995). These multifucosylated terminal DF-LDN-DF epitope-containing glycans were later also found to be the main structures in the O-glycan pool from egg glycoproteins (Khoo *et al.*, 1997b). Like for *S. japonicum* glycosphingolipids, *S. japonicum* O-glycans only contained terminal LDN and LDN-F and no DF-LDN-DF.

Another type of cercarial O-glycans terminating with Lewis X or LacNAc, which could be separated from the multifucosylated glycans by an AA lectin column are carried by a novel bi-antennary like O-glycan core structure; –3(±Galβ1-6)Galβ1-3(Galβ1-6)GalNAc (Huang, Tsai & Khoo, 2001). In *S. mansoni* such novel core structures could be detected among the O-glycans synthesized by adult worms, eggs and miracidia, in addition to the cercariae. In *S. japonicum* and *S. haematobium* the Hex_2-HexNAc-ol entity could also be found in cercarial and egg extracts (Huang *et al.*, 2001).

The cercarial and egg O-glycans are associated with extreme heterogeneity, which is due to: the variable degree of fucosylation, incomplete extensions, the presence or absence of the 6-arm on the core GalNAc and the variable number of repeating units on each arm of the often branched O-glycan structure. All schistosome N- or O- glycosylated amino acids may have a wide variety of different glycan structures attached leading to pronounced ‘microheterogeneity’, albeit less extreme. In addition ‘macroheterogeneity’ occurs, which means that different glycosylation sites of a certain protein may be only partly and differentially glycosylated resulting in different isoforms of that one protein. The extreme heterogeneity, together with the specific and unusual characteristics of schistosome glycans makes their analysis a real challenge.

GLYCAN ANALYSIS: TECHNIQUES

Immunolocalisation of carbohydrate epitopes

With the use of synthetic glycoconjugates many anti-carbohydrate monoclonal antibodies that were raised in schistosome infected or immunized mice have been successfully characterised (van Remoortere *et al.*, 2000; van Roon *et al.*, 2005). These defined mAbs are useful to map expression profiles of specific carbohydrate epitopes. The differential expression of certain terminal glycan motifs can be efficiently screened by (Figure 6A) immunofluorescence assays (IFA), by (B) high performance thin-layer chromatography (TLC) (glycolipids) or by (C) Western blot analysis (glycoproteins) to localise the epitope on sections of worms and eggs (van Dam *et al.*, 1993a; Deelder *et al.*, 1996; van Remoortere *et al.*, 2000; Robijn *et al.*, 2005). These carbohydrate motifs that form the epitopes of the antibodies are generally located at the non-reducing termini of the larger glycan structure.

One of the main objectives of this thesis was to fully sequence the glycoprotein-derived glycans that carried the epitope recognised by one specific mAb 114-4D12. From the immunoreactivity pattern of mAb 114-4D12 with *S. mansoni* egg glycoproteins obtained from Western blot analysis (Figure 6 C, D) we learned that our target glycoproteins of unknown origin were of different size, mostly large (> 50kD) and heavily glycosylated (smear). For the relative little amount of mAb 114-4D12 affinity purified schistosome egg-material the preferred method of choice was to release the glycans, fluorescent label them for easy purification and increased sensitivity and analyse these oligosaccharides by mass spectrometry. Although with the current “standard” mass spectrometry equipment recording spectra of intact glycoproteins is possible, in practice individual glycoforms are only resolved from small proteins (up to ~20-40kD) containing a limited number of glycans, preferably attached to a single site (Harvey, 2005).

Mass spectrometry (MS) is becoming widely accepted as method for obtaining extensive structural information from small amounts of glycans (reviewed by (Harvey, 2005)). Although some information such as the nature of individual monosaccharides (e.g. galactose, glucose) is not immediately clarified by MS it is increasingly accepted as oligosaccharide sequencing technique for studies on small amounts of material (such as in proteomic work). The classical structural determination by gas chromatography mass spectrometry (GC-MS) and nuclear magnetic resonance (NMR) do simply not provide the required sensitivity. The oligosaccharide sequencing techniques that were employed in this study will be discussed.

Enzymatic release of N-glycans

Due to their conserved core structure N-glycans can be released by several enzymes. The most widely used enzyme is *N*-glycosidase F or PNGase-F. PNGase-F releases most N-glycans except those that contain fucose α1-3 linked to the Asn-linked GlcNAc (Tretter, Altmann & Marz, 1991), which occurs in schistosomes. In those situations *N*-glycosidase A or PNGase-A is used. PNGase-A has the disadvantage, however, that it is often less effective and only releases glycans from smaller peptides (Kolarich & Altmann, 2000). Both PNGase-F and –A are amidases that cleave the intact glycan as glycosylamine, which leaves the aspartic acid in place of the asparagines at the *N*-linked site of the protein. The reducing termini of the glycans are left intact, which allows the glycans to be derivatized with fluorescent or other reagents to aid detection.

Chemical release of N- and O-linked glycans

Compared to enzymatic release, chemical release has the advantage of being non-selective, however, it may introduce artefacts. Both N- and O- linked glycans can be released by hydrazinolysis. Using anhydrous hydrazine a cleavage is introduced between peptide bonds, including that between the N-linked glycan and asparagines. O-glycans are specifically released at 60°C, whereas 95°C is required to release N-glycans (Merry *et al.*, 2002). This method preserves the reducing terminus of the glycan, which has the advantage that the glycans remain reactive for fluorescent labelling, which allows easy purification and improves sensitivity. As hydrazine cleaves all peptide bonds all information on the protein is lost.

As O-glycans consist of many different core-structures (more different core structures than the seven illustrated in table II are currently known) no universal enzyme is available for their release. O-glycans can be released chemically from the serine or threonine residues by reductive β-elimination. This reaction also converts the amino-acids to unsaturated hydroxyamino acids. The oligosaccharides are released by addition of sodium hydroxide. As a result of the high pH 3-sybstituted reducing HexNAc residues are cleaved, giving rise to the so-called 'peeling reaction'. Reduction of released carbohydrate chains using reducing agents such as sodium borohydride or sodium borodeuteride stabilizes them. Unfortunately, this technique reduces the carbonyl moiety from the reducing terminus to alcohol, thus preventing the attachment of a fluorescent tag. Reductive β-elimination also releases N-glycans (Karlsson & Packer, 2002). Recently a method was introduced to directly release O-glycans from in-gel glycoproteins using reductive β-elimination (Taylor, Holst & Thomas-Oates, 2006).

Analysis of released glycans

Released glycans can be profiled by high performance liquid chromatography (HPLC) or by mass spectrometry (MS). Different types of HPLC columns can be used for glycan analysis such as normal phase (NP), graphitized carbon or reverse phase (RP), of which the latter is only applicable to most fluorescent labelled but not to underivatized glycans. NP-HPLC gives some structural information as retention time is directly proportional to size (Royle *et al.*, 2002). Fluorescent labelling of the glycans allows direct quantitation from the HPLC profiles, if the peaks represent single glycan structures.

Mass measurements yield more detailed composition information in terms of the non-isobaric monosaccharide composition (Table III). Structural sequences may be obtained by MS fragmentation (for more information on MS see below) or by sequential exoglycosidase digestions combined with HPLC-profiling (Rudd *et al.*, 1997a) or MS. In this approach aliquots of a total pool of fluorescent labelled glycans are simultaneously digested with a series of multiple enzymes. From the combined data of incremental values of the oligosaccharides from MS composition and fragmentation analysis and the linkage-information based on the exoglycosidase digestions for both N- (Rudd *et al.*, 1997a) and O-glycans (Royle *et al.*, 2002) a structural database has been constructed, which enables identification of glycans at subpicomolar level. The database contains the structure, molecular weight, and both NP and RP HPLC elution positions determined in glucose (NP) or arabinose (RP) units for each glycan.

This technology has been used for comparison of glycosylation profiles of IgG glycans from normal and rheumatoid IgG (Wormald *et al.*, 1997), for the analysis of the 36 major N-glycans attached to human erythrocyte CD59 (Rudd *et al.*, 1997b), or for the identification of O-glycans on human neutrophil gelatinase B and secretory IgA (Royle *et al.*, 2002). However, for non-mammalian oligosaccharides this method is often only partly functional as no exoglycosidases are available for the specific oligosaccharide features that schistosomes produce, for example the unique (±Fucα1-2)Fucα1-2Fuc moiety. In the cases of unusual glycan structures a powerful technique is 2- or 3- dimensional HPLC analysis in which glycan pools are resolved into individual sugars by successive passages through different HPLC columns (Tomiya *et al.*, 1991; Takahashi *et al.*, 1995). The isolated glycans from the individual HPLC peaks can then be used for further analysis, preferably by MS. Depending on the types of glycans and the heterogeneity of the glycan pool this technique is much less sensitive (at least 100x) than the above described oligosaccharide sequencing technology.

Table III. Residue masses of monosaccharides commonly found in schistosome oligosaccharides.

Monosaccharide class	Specific Monosaccharides	Symbol	Residue formula	Monoisotopic mass *
Pentose	Xylose, Xyl	☆	$C_5H_8O_4$	132.0423
Deoxyhexose	Fucose, Fuc	▲	$C_6H_{10}O_4$	146.0579
Hexose	Galactose, Gal Glucose, Glc	○ ●	$C_6H_{10}O_5$	162.0528
Hexuronic acid	Glucuronic acid	◆	$C_6H_8O_6$	176.0321
N-Acetylaminohexose	*N*-Acetylgalactosamine, GalNAc *N*-Acetylglucosamine, GlcNAc	□ ■	$C_8H_{13}NO_5$	203.0794

* The oligosaccharide masses are obtained by addition of the residue masses together with the mass of one molecule of water (18.0106 monoisotopic mass). Only charged ions can be measured in the mass spectrometer. For the mass of the $[M+H]^+$ ion, an additional 1.0078 mass units should be added and for the mass of the [M+Na]+ ion, an additional of 22.9899 (monoisotopic) mass units should be added.

Moreover, MS does often not provide the linkage positions of the monosaccharide and also the nature of the individual monosaccharides (e.g. galactose or mannose) remain unknown. When specific knowledge of the glycosylation enzymology is not available for the system under investigation additional techniques are required for full characterisation of the oligosaccharide. Classically, structural determination of glycans have been performed by gass chromatography (GC)-MS using specific derivatization techniques such as methylation analysis or nuclear magnetic resonance (NMR) spectroscopy, but for characterization of limited (parasitic) glycan material these methods often do not provide the necessary sensitivity.

Anyway, mass spectrometry is an important and advancing technology in structural glycobiology. In an MS experiment samples are ionized by one of several techniques, of which the most important are electrospray-ionisation (ESI) and matrix-assisted laser desorption ionisation (MALDI) (Wada *et al.*, 2007). Fast atom bombardment-mass spectrometry (FAB-MS) has also been useful for compositional and sequential analysis of complex glycan mixtures (Haslam, Morris & Dell, 2001; Morelle & Michalski, 2005), however FAB-MS is much less sensitive and has now been largely replaced by matrix-

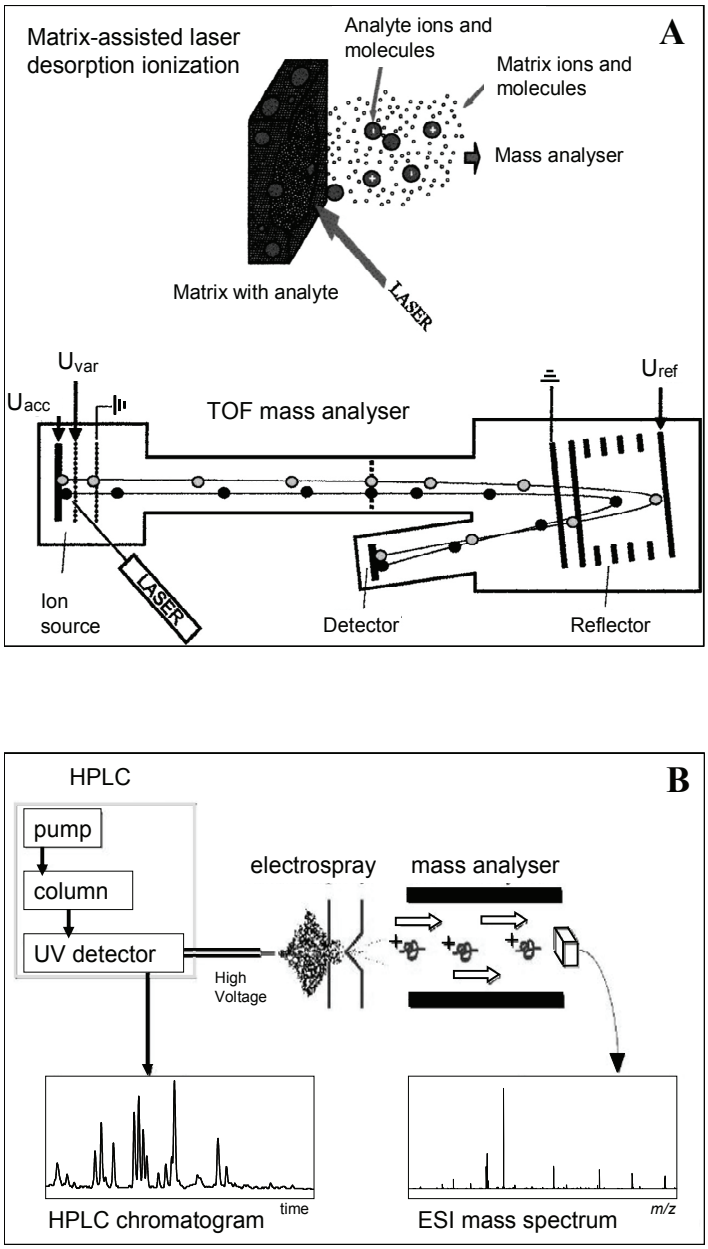


Figure 7. Schematic representation of two commonly used mass spectrometry techniques. *A.* Matrix-assisted laser desorption ionization (MALDI) time-of-flight (TOF). The analyte (molecules of interest) is dissolved with a matrix and applied on a target plate, which is allowed to dry prior to insertion into the high vacuum of the mass spectrometer. When the laser is fired the matrix transforms the laser energy into excitation energy for the sample, which leads to sputtering of analyte and matrix ions from the surface of the mixture. The time-of-flight analyser separates ions according to their mass-to-charge (m/z) ratios by measuring the time it takes for ions to travel from the ion source via the reflector to the detector. *B.* Liquid chromatography electrospray ionisation MS (LC-ESI MS) has the advantage of online purification or separation of the sample immediately prior to MS. The by HPLC purified analytes are ionised at the tip of a capillary to which a high voltage of 3 or 4 kV is applied. As a consequence of this strong electric field the sample is dispersed into an aerosol of highly charged droplets. The droplets diminish in size by solvent evaporation assisted by a drying gas (warm nitrogen) and the dried charged sample ions pass the sampling cone into the mass analyser.

assisted laser desorption ionization time-of-flight (MALDI-TOF) MS and ESI-MS. FAB-MS was not used in any of the experiments performed for this thesis. Each of these technologies has its own individual strengths and weaknesses and for determining a glycan structure often more than one technique has to be used.

Principles of MALDI-TOF MS

In MALDI-TOF MS (see Figure 7A) the sample is embedded in a low-molecular weight, ultraviolet-absorbing matrix, and ionization is effected by a pulsar laser. The energy of the laser is absorbed by the matrix and enough energy is transferred to the sample (by mechanisms not completely understood) to enable formation of molecular ions. These

molecular ions are accelerated through an electric field and subsequently drift through a vacuum flight tube until detected. The time each molecule needs to traverse this region, the "time of flight" or TOF, depends on their mass/charge (m/z) ratio. MALDI is a highly sensitive technique (femtomoles to picomoles) and therefore very suitable to measure the small available amounts of parasite glycans. In addition, MALDI-TOF can be used to analyse underivatised molecules and complex mixtures, producing spectra uncomplicated by multiple charging or significant fragmentation.

Although sample preparation is relatively easy and salts and buffers are to a certain extent tolerated by MALDI-MS, the presence of too much salts or other impurities such as plasticizers in the sample have an adverse effect on ion yield and crystal formation in the matrix. When the impurities are in the molecular weight range of the sample these will surely disturb the analysis of the sample. Therefore it is essential to purify and desalt the glycans or glycoproteins of interest by chromatography or dialysis techniques before applying them to the MALDI target plate.

Principles of ESI-MS

In electrospray-ionisation MS (ESI-MS) (see Figure 7B), a stream of volatile liquid containing the (non-volatile) molecules of interest is introduced into the atmospheric pressure ion source of a mass spectrometer via a metal-tipped glass capillary. An aerosol of highly charged microdroplets is generated in the source. Accelerated by a high voltage and encountering a drying gas, the net effect is the creation of dried gaseous ions. The ions charge depends on the amount of ionisable groups in the molecule. The beam of ions resulting from this still not fully understood ionization mechanism are directed to the mass analyzer through a series of lenses (Morelle & Michalski, 2005). The ESI-MS experiments described in this thesis were performed by a nanospray MS, which due to the reduced internal diameter of tubing and low flow rate (nanoliters/min.) is more sensitive than the more conventional microspray MS. ESI is a very gentle process compared to other ionization techniques, which has made this technique an indispensable tool for analysis of many different biological molecules including glycans, glycoproteins and glycolipids. The compatibility of electrospray with liquid separation techniques has resulted in LC-MS, which has become a powerful technique for glycan analysis like for many other biomolucules. Normal phase nano-scale liquid chromatography combined with ESI-MS can produce fragmentation data of small underivatized N-linked glycans at the low-femtomole level (Wuhrer *et al.*, 2004a). NP separations provide correlation between structure of the underivatised glycan and retention time. On-line chromatography by NP, RP or other separation techniques prior to MS results in

the introduction of isomeric compounds into MS at different timepoints. This enables the production of fragmentation spectra of isomeric compounds which would otherwise produce mixed spectra. Porous graphitized carbon columns have also been used to separate glycans in an on-line HPLC-MS system (Kawasaki *et al.*, 1999; Barroso, Didraga & Bischoff, 2005) and for 2-aminobenzamide labelled glycans reverse phase separation can be applied (Wuhrer *et al.*, 2004b).

Fragmentation of Glycans by MS/MS

Through fragmentation of glycans by MS-MS, usually by collision or laser-induced dissociation, their monosaccharide sequences, branching patterns and sometimes glycosydic linkages can be determined. The sequence of a glycan is most easily obtained; glycosidic cleavages implying rupture of the bond between two monosaccharides give information on the sequence and composition of the glycan. The oligosaccharide mass is the sum of the glycosidically-linked monosaccharide masses with the addition of 18.01 Da (H_2O) at the reducing end for underivatised glycans, or the mass of the label for derivatised glycans (Table III). From the order in which monosaccharides are lost from the parent structure the sequence and also the branching pattern can be determined.

Linkages can be determined from cross-ring cleavages that involve the rupture of two bonds in the same sugar residue ring structure. The type of the parent ion that is used for MS/MS has a significant effect on the fragmentation (Ngoka, Gal & Lebrilla, 1994). Decay rates of the protonated (H^+) ions are faster than that of any alkali metal adducts, such as sodiated (Na^+) ions. Fragmentation spectra of $[M+H]^+$ ions from glycans are often relatively simple as primarily glycosidic cleavages occur. However, rearrangement ions can appear, examples of which are the migration of fucose towards the derivatised reducing terminus or between antennae of fucosylated N-glycans (Harvey *et al.*, 2002; Harvey, 2005). Rearrangements of sodiated ions as observed for protonated ions have not been reported (Brull *et al.*, 1998) which is in line with our observations (Robijn *et al.*, 2007a; Robijn *et al.*, 2007b).

Determination of the glycosylation site and glycopeptide analysis

Sometimes it is possible to obtain the glycosylation site(s) of a glycoprotein after cleavage of the glycoprotein with a protease (e.g., trypsin or pronase) and analysis of the resulting glycopeptides. Although improving this approach has several limitations. It is not always possible to produce peptides containing only one glycosylation site, for example O-glycans often exist in groups on closely positioned amino acids, and many tryptic peptides are not amenable for optimum mass spectral analysis (Harvey, 2005). The N-glycosylation site of

glycopeptides is more easily obtained than the O-glycosylation site based on the conversion of Asn to Asp upon treatment with the enzyme *N*-glycosidase F (PNGaseF), which is easily identified by conventional MS techniques.

Mass spectrometric detection of glycopeptides in the presence of peptides is often difficult because of suppression effects (Annesley, 2003). However, in the last years methods to determine the glycosylation as well as the glycosylation site have been studied with increased attention and incorporated into the conventional proteomics techniques. To analyse the complex mixtures of glycoprotein digests containing peptides and glycopeptides of one or more (co-migrating) glycoproteins isolated from SDS-PAGE gels several approaches have been developed to enrich the glycopeptides from the mixture and for the proceeding glycopeptide analysis multiple tandem MS techniques can be used (as reviewed by (Wuhrer *et al.*, 2006b)). Glycopeptide ionisation by MALDI eg. by metastable dissociation in a MALDI-TOF/TOF-MS results in fragmentation of glycosidic bonds and additionally provides peptide sequence tags. These glycopeptides generally retain the attached glycan moiety, thereby corroborating the attachment site of the glycan moiety (Wuhrer, Hokke & Deelder, 2004). It has recently been shown that with the use of ion trap mass spectrometers very detailed characterization of glycopeptides can be performed using two different approaches: In the so far most used approach (glyco)peptide fragment ions obtained by collision induced dissociation (CID) are isolated in repetitive cycles of MS/MS. In the first cycle predominantly glycosidic bonds are cleaved, whereas in the second cycle the peptide is fragmented providing information on the peptide sequence and glycan attachment site. In the newer approach, electron transfer dissociation (ETD) resulting in peptide sequence information and the glycan attachment site is used in combination with CID fragmentation which provides information on the glycan structure. Similarly, such complementary datasets can be obtained by a Fourier transform ion cyclotron resonance (FT-ICR) MS employing infrared multi photon dissociation (IRMPD) for glycan fragmentation and electron capture dissociation (ECD) for peptide fragmentation. These very recent techniques have not been applied in this thesis.

Despite the significant progress realised in the analysis of specific schistosoma glycoconjugates so far (as outlined in this thesis) it is evident that the rapid progress in MS instrumentation, both at the level of more sensitive instrumentation and of novel ionization techniques, like ETD, will in the near future allow much more detailed analysis at sensitivity levels needed for the often very small amounts of parasite material present.

2

Mapping fucosylated epitopes on glycoproteins and glycolipids of *Schistosoma mansoni* cercariae, adult worms and eggs.

MARJOLEIN L.M. ROBIJN[1], MANFRED WUHRER[1,2], DIEUWKE KORNELIS[1], ANDRÉ M. DEELDER[1], RUDOLF GEYER[2], and CORNELIS H. HOKKE[1]

1 Department of Parasitology, Centre of Infectious Diseases, Leiden University Medical Centre, P.O. Box 9600, 2300 RC Leiden, The Netherlands; 2 Institute of Biochemistry, Faculty of Medicine, University of Giessen, Friedrichstrasse 24, D-35392 Giessen, Germany

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SUMMARY

The developmental expression of the antigenic fucosylated glycan motifs Fucα1-3GalNAcβ1-4GlcNAc (F-LDN), Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F), GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F), Galβ1-4(Fucα1-3)GlcNAc (Lewis X), and GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF) in *Schistosoma mansoni* cercariae, adult worms and eggs, was surveyed using previously defined anti-carbohydrate monoclonal antibodies (mAbs). Lewis X was found both on glycolipids and glycoproteins, yet with completely different expression patterns during the life cycle: on glycolipids, Lewis X was mainly found in the cercarial stage, while protein-conjugated Lewis X was mainly present in the egg stage. Also protein-conjugated LDN-F and LDN-DF were most highly expressed in the egg-stage. On glycolipids LDN-DF was found in all three examined stages, whereas LDN-F containing glycolipids were restricted to adult worms and eggs. The motifs F-LDN and F-LDN-F were found both on glycoproteins and glycolipids of the cercarial and egg stage, while in the adult stage, they appeared to occur predominantly on glycolipids. Immunofluorescence assays (IFA) showed that these F-LDN and F-LDN-F containing glycolipids were localised in a yet undefined duct or excretory system of adult worms. Murine infection serum showed major reactivity with this adult worm duct-system, which could be fully inhibited by preincubation with keyhole limpet hemocyanin (KLH). Altogether, the use of defined mAbs provides a quick and convenient way to map expression profiles of carbohydrate epitopes.

INTRODUCTION

Schistosomiasis is the second most important human parasitic disease after malaria. In tropical and sub-tropical countries, over 200 million people are infected with schistosomes. One of the major human schistosome species is *Schistosoma mansoni*, of which the adult worms live as pairs in the mesenteric vessels where they produce hundreds of eggs per day. It has been estimated that about half of the eggs of *S. mansoni* leave the body with the faeces, whilst the rest of the eggs are deposited in the liver and other organs of the host where they cause granulomatous inflammation. Miracidia that hatch from the eggs in fresh water are able to infect the intermediate host: snails of the genus *Biomphalaria*. Cercariae are formed by asexual replication and are released in the water to infect the definitive human host (Jordan, Webbe & Sturrock, 1993; Ross *et al.*, 2002).

Throughout their intriguing and complex life cycle schistosomes express a complex set of glycoproteins and glycolipids that play a major role in the immunology of schistosomiasis ((Hokke & Deelder, 2001), and references cited therein). These glycoconjugates display exceptional glycan motifs, including carbohydrate chains of the gut-associated antigens CAA and CCA (Bergwerff *et al.*, 1994; van Dam *et al.*, 1994), (Fucα1-2)$_{1-2}$Fucα1-3- elements (Khoo *et al.*, 1995; Khoo *et al.*, 1997a; Wuhrer *et al.*, 2002) and the Fuc(α1-3)GalNAc- motif (Kantelhardt *et al.*, 2002), which induce high antibody responses in humans and primates (van Remoortere *et al.*, 2001; Kantelhardt *et al.*, 2002; Naus *et al.*, 2003; van Remoortere *et al.*, 2003). The more widely expressed glycans GalNAcβ1-4GlcNAc (LDN), Galβ1-4(Fucα1-3)GlcNAc (Lewis X) and GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F), which are shared between schistosomes and their mammalian hosts (Fox *et al.*, 1983; Bergwerff *et al.*, 1992; Yan, Chao & van Halbeek, 1993; De Graaf *et al.*, 1993) generally induce weaker antibody responses (van Remoortere *et al.*, 2000; van Remoortere *et al.*, 2003), but may have immunomodulatory effects. In addition, schistosome infections induce antibodies to keyhole limpet hemocyanin (KLH) (Eberl *et al.*, 2001; Kantelhardt *et al.*, 2002). It has been shown that Fucα1-3GalNAc- moieties in the N-linked glycans of the highly immunogenic KLH glycoprotein are the main schistosome cross-reactive epitopes (Kantelhardt *et al.*, 2002; Geyer *et al.*, 2004) and these enable the use of KLH for serodiagnostic purposes (Grzych *et al.*, 1987; Hamilton *et al.*, 1999).

Fucosylated glycans have been shown to be able to induce the Th2 type immune responses that are characteristic for infections with schistosomes and other helminths (Okano *et al.*, 1999; Faveeuw *et al.*, 2002; Gause, Urban, Jr. & Stadecker, 2003; Thomas *et al.*, 2004). The fucosylated glycan lacto-*N*-fucopentaose (LNFPIII) that contains Galβ1-4(Fucα1-3)GlcNAc-

(LeX) has often been used as a model-glycan to study the immunomodulatory effects of schistosome glycans. When conjugated to a protein carrier for multivalent presentation it induces Th2-responses in naive mice (Okano *et al.*, 2001; Thomas *et al.*, 2003). Peripheral blood mononuclear cells (PBMC) from humans infected with *S. mansoni*, but not of uninfected individuals, respond to the LNFP-III-HSA conjugate by increased proliferation and IL-10 production (Velupillai *et al.*, 2000). A synthetic LDN-DF conjugate has been shown to be a potent cytokine inducer on PBMCs of naive human donors (Van der Kleij *et al.*, 2002b). Moreover, in the induction of liver granulomas in *in vivo* mouse models, the glycosylation of egg antigens appears to play a crucial role (Weiss *et al.*, 1987; El Ridi, Velupillai & Harn, 1996; Jacobs *et al.*, 1998; Jacobs *et al.*, 1999).

The expression of (fucosylated) glycans on glycoproteins or glycolipids is developmentally regulated (Nyame, Yoshino & Cummings, 2002) but intense cross-reactivity occurs between the various schistosome stages based on the shared glycan elements (Strand, McMillan & Pan, 1982; Weiss *et al.*, 1986; Dunne & Bickle, 1987a; van Remoortere *et al.*, 2000). These glycan motifs may be present on many different lipid and protein carriers (Weiss & Strand, 1985; Weiss, Magnani & Strand, 1986; van Dam *et al.*, 1993a; Deelder *et al.*, 1996; van Remoortere *et al.*, 2000). In this study, the expression profiles of five major fucosylated epitopes: Fucα1-3GalNAcβ1-4GlcNAc (F-LDN), Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F), GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F), Galβ1-4(Fucα1-3)GlcNAc (Lewis X), and GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF) (Table 1) were mapped utilizing defined mAbs that specifically recognised these epitopes. *S. mansoni* cercarial, adult worm and egg preparations were analysed combining several techniques; glycoproteins were analysed on Western blot, simultaneously glycolipids were analysed using the HPTLC-overlay technique as well as ELISA and additionally localization of the epitopes was studied using fluorescence microscopy.

MATERIALS AND METHODS

Antigens and antibodies

The life cycle of *S. mansoni* parasites was maintained using Golden hamsters (HsdHan-Aura). Worms were collected by perfusion 7 weeks after infection with 1200 cercariae. Eggs were isolated from livers as previously described (Dalton *et al.*, 1997). Proteins were extracted from cercariae, adult worms and eggs by heating for 10 min at 95°C in 0.1M Tris-HCl, pH 6.8, containing 20% glycerol, 2% SDS and 2% 2-mercaptoethanol. *S. mansoni* glycolipids were prepared as described previously (Wuhrer *et al.*, 1999). The prestained, broad range

precision protein standard from BioRad (Veenendaal, The Netherlands) was used and the globoside standard was purchased from Matreya (State College, PA).

Monoclonal antibodies (mAbs) were produced as described previously (van Dam *et al.*, 1993a; Nourel Din *et al.*, 1994a; Nibbeling *et al.*, 1998a). Monoclonal antibody 291-4D10-A is specific for Galβ1-4(Fucα1-3)GlcNAc (Lewis X), mAb 290-2E6-A recognizes GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F), mAbs 290-4A8-A and 114-5B1-A bind to GalNAcα1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF) (van Remoortere *et al.*, 2000), mAb 291-5D5-A recognizes Fucα1-3GalNAcβ1-4GlcNAc (F-LDN) and mAb 128-1E7-C is specific for Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F) (C.H. Hokke, A. van Remoortere, H.J.Vermeer and A.M. Deelder, *unpublished observations*). For glycoprotein analysis, the murine infection serum (MIS) was taken from a 15 weeks-infected outbred Swiss mouse that had been exposed to 70 cercariae and the human infection sera (HIS) originated from heavily infected schistosomiasis patients from Senegal (Stelma *et al.*, 1993). For glycolipid analysis, different MIS and HIS were used (Kantelhardt *et al.*, 2002).

Table 1. An overview of the carbohydrate structures included in this study giving the abbreviations and the defined mAbs that recognise these glycan epitopes (van Remoortere et al., 2000; C.H. Hokke, A. van Remoortere, H.J. Vermeer and A.M. Deelder, unpublished observations).

Abbreviation	Carbohydrate structure	Defined mAb
Lewis X	Galβ1-4(Fucα1-3)GlcNAcβ1	291-4D10-A
LDN-F	GalNAcβ1-4(Fucα1-3)GlcNAcβ1	290-2E6-A
LDN-DF	GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1	290-4A8-A & 114-5B1-A
F-LDN	Fucα1-3GalNAcβ1-4GlcNAcβ1	291-5D5-A
F-LDN-F	Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAcβ1	128-1E7-C

Western blotting

Approximately 1µg protein extracts from *S. mansoni* cercariae, adult worms and eggs were subjected to SDS-PAGE on 10% gels using the Mini-Protean 3 Cell system (Bio-Rad, Veenendaal, the Netherlands). The proteins were transferred onto a nitrocellulose membrane (Schleicher & Schuell BioScience GmbH, Dassel, Germany) in a Bio-Rad Criterion Blotter system according to the manufacturer's instructions. The protein blots were blocked in phosphate-buffered saline/5% BSA (PBS-B) at 4°C, o/n. The blots were washed with

PBS/0.5%BSA/0.05%Tween (PBS-BT) and subsequently incubated with the primary anti-carbohydrate mAb, or with infection serum, for 1h at room temperature (RT). Hybridoma culture supernatant was used 1:2 diluted in PBS-BT, infection serum was used in a 1:1000 dilution in PBS-BT. After extensive washing, the blots were incubated with AP-labelled goat anti-mouse Ig (1:2000) (Zymed Laboratories, San Francisco, CA) in the case of primary incubation with mAbs or murine infection serum. For detection of Ig in human infection serum we combined biotinylated goat anti-human IgG and –IgM (1:1000) (Nordic Immunological Laboratories, Tilburg, The Netherlands) with Streptavidin-AP (1:2000) (Zymed). Finally, the blots were washed with PBS and stained using X-phosphate/5-Bromo-4-chloro-3-indolyl-phosphate (BCIP) (Roche Diagnostics, Mannheim, Germany) and 4-nitro blue tetrazolium chloride (NBT) (Roche).

High performance TLC (HPTLC) and ELISA

S. mansoni glycolipids were separated by HPTLC using chloroform/methanol/0.25% aqueous KCl (50:40:10, by volume) as running solvent, followed by either orcinol/H_2SO_4 staining or HPTLC immunostaining (Wuhrer *et al.*, 1999). The primary incubation step was carried out using mAbs (hybridoma culture supernatant 1:25 in PBS-BT) or human and murine infection sera (1:1000 in PBS-BT). ELISA with *S. mansoni* glycolipids (10 ng carbohydrate/well) as adsorbed antigens and KLH (10 µg / 100 µl per well) as an inhibitor was performed as outlined previously (Wuhrer *et al.*, 2000a). As secondary antibodies for both HPTLC and ELISA, alkaline-phosphatase-conjugated goat anti-mouse Ig (Dako Diagnostics, Hamburg, Germany) and goat anti-human Ig (Dianova, Hamburg, Germany) were used, diluted 1:1000.

Immunofluorescence assay (IFA)

Immunofluorescence assays were performed on frozen gut sections (6µm-thick) of *S. mansoni* infected hamsters as reported previously (van Dam *et al.*, 1993a). Briefly, slides were fixed in ice-cold aceton and dried. To remove glycolipids, slides were incubated for 30 min in chloroform/methanol 1:1 and dried again. Subsequently slides were incubated with a mAb (undiluted culture supernatant) or with 1:10 diluted murine or human infection serum for 1 h at 37°C. In KLH-inhibition studies, mAbs or infection sera were incubated with KLH (Sigma-Aldrich, The Netherlands) 1mg/ml, for 2 h at RT, prior to incubation with the slides. Subsequently, the slides were washed and incubated with FITC labelled goat anti-mouse Ig (1:40), or swine anti-human Ig (Nordic, The Netherlands) in a conjugate solution containing Evans blue. Para-phenylenediamine was used as anti-fading and slides were analysed using a

Leica DMRA microscope equipped with a Hamamatsu Orca-ER digital camera and Improvision software.

RESULTS

Fucose containing glycans are differentially expressed on schistosomal glycoproteins or glycolipids.

To survey the developmental expression of antigenic fucosylated glycan motifs in schistosomes (Table 1), we have analysed glycoproteins and glycolipids of *S. mansoni* cercariae, adult worms and eggs by Western blotting, HPTLC overlays and IFAs using previously characterised anti-carbohydrate mAbs (van Remoortere *et al.*, 2000),(C.H. Hokke, A. van Remoortere, H.J. Vermeer and A.M. Deelder, *unpublished observations*) or infection sera. Staining with mAb 291-4D10-A showed that the Lewis X epitope was expressed on glycoproteins of all three examined life cycle stages, but most extensively on egg-glycoproteins (Figure 1A). Lewis X was also expressed on cercarial and adult worm glycolipids, but could hardly be detected on egg glycolipids (Figure 2A), as shown previously (Kantelhardt *et al.*, 2002; Wuhrer *et al.*, 2002). Like Lewis X, LDN-F was expressed on glycoproteins of all examined stages and highest in the egg-stage (Figure 1B). In contrast to Lewis X however, LDN-F expression on glycolipids was restricted to the adult worm- and egg-stage (Figure 2B) and could not be detected on cercarial glycolipids.

The LDN-DF motif was present in all three examined stages on glycoproteins as well as on glycolipids (Figure 1C,D and 2C,D) and, again, the most intense staining was observed for glycoproteins of the egg-stage. The two anti-LDN-DF mAbs 290-4A8-A and 114-5B1-A revealed distinct band patterns on Western blots and on HPTLC, indicating that these mAbs recognize different natural epitopes. The mAbs 291-5D5-A (anti-F-LDN) and 128-1E7-C (anti-F-LDN-F) yielded rather different staining patterns on HPTLC, but in contrast displayed very similar staining patterns on Western blot. The F-LDN and F-LDN-F epitopes were highly expressed on glycoproteins of eggs and cercariae, but almost entirely absent in the glycoprotein preparation of the adult worm-stage (Figure 1E,F). On glycolipids however, these epitopes were expressed in all three stages (Figure 2E,F). The band pattern on HPTLC obtained with anti-F-LDN-F mAb 128-1E7-C (Figure 2F) showed striking similarity with the band pattern that was observed for anti-LDN-DF mAb 114-5B1-A (Figure 2D).

Staining of Western blots and HPTLC with human- or murine infection sera resulted in different complex band patterns for each serum and each examined life cycle stage. In these patterns some bands recognised by the serum occurred in every stage of the parasite, whereas other (glyco-) proteins were only stage-specifically expressed (Figure 1,G-I). Strikingly, on HPTLC, staining with HIS or MIS resulted in highly comparable patterns (Figure 2L,M), as depicted previously (Kantelhardt *et al.*, 2002).

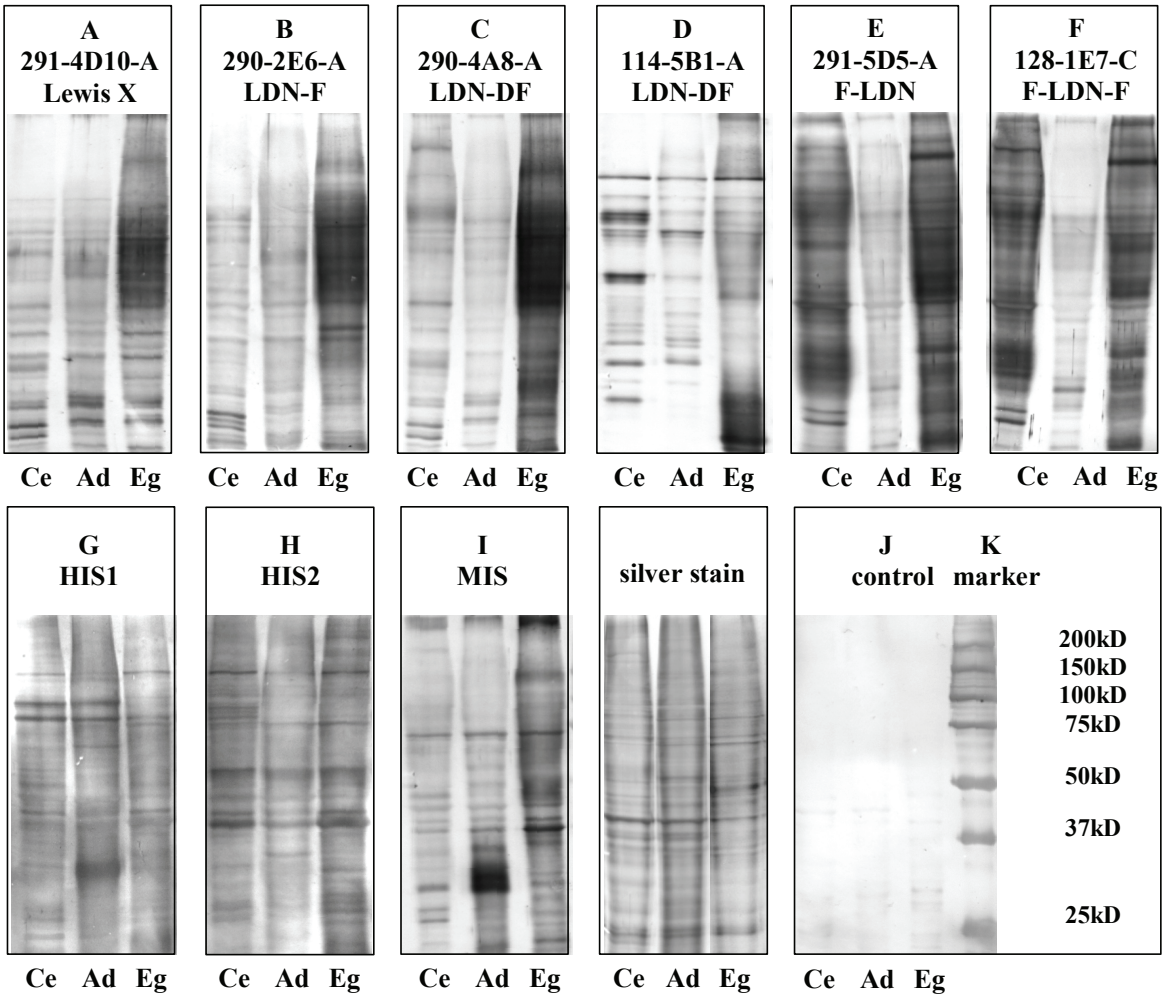

Figure 1. Western blots and silver staining of *S. mansoni* glycoproteins. Cercarial (*Ce*), adult worm (*Ad*) and egg (*Eg*) protein extracts were applied to a 10% gel and visualised by silver staining or immunostaining using various mAbs directed against carbohydrate epitopes (*A-F*) and human- (*HIS1,2*) or murine (*MIS*) infection sera (*G-I*). An isotype control is shown (*J*) along the prestained broad range protein marker (*H*).

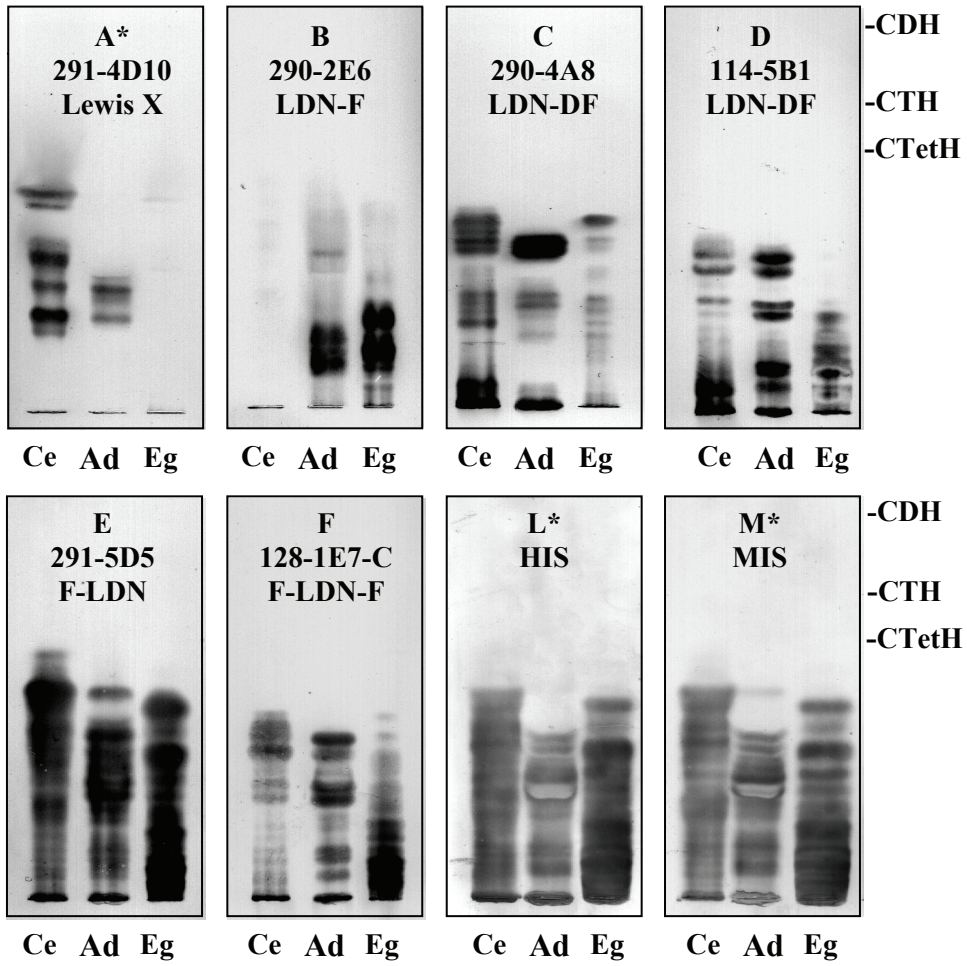

Figure 2. HPTLC-Immunostaining of *S. mansoni* glycolipids. HPTLC-resolved cercarial (*Ce*), adult (*Ad*) and egg (*Eg*) complex glycolipids (50 ng of carbohydrate per lane; 300 ng carbohydrate per lane in E, L and M) were visualised by immunostaining using the same panel of anti-carbohydrate mAbs as was used for the Western blots (*Figure 1*) and human- (*HIS*) or murine (*MIS*) infection sera (*L,M*). The migration positions of orcinol/H2SO4-stained globoside standards (CDH, CTH and CtetH) are indicated. Pictures marked by * have already been published (Kantelhardt et al., 2002).

Cross-reactivity between schistosomes and KLH.

In the paper of Kantelhardt (Kantelhardt *et al.*, 2002) the terminal Fuc(α1-3)GalNAc-motif was found to be the dominant epitope on *S. mansoni* glycolipids that causes cross-reactivity between schistosome infection sera and KLH. In order to see whether other fucosylated epitopes than the Fuc(α1-3)GalNAc-motif are involved in the cross-reactivity between *S. mansoni* and KLH, we have examined the potential of KLH to inhibit the binding of the different mAbs to *S. mansoni* glycolipids in an ELISA experiment. Reactivity of anti-F-LDN mAb 291-5D5-A as well as anti-F-LDN-F mAb 128-1E7-C with *S. mansoni* glycolipids was strongly inhibited by KLH (Figure 3E,F), confirming that F-LDN(-F) epitopes are present on KLH. Reactivity of the anti-Lewis X, anti-LDN-F and anti-LDN-DF mAbs with glycolipids

of all three examined life cycle-stages was unaffected by the presence of KLH (Figure 3A-D), which showed that these carbohydrate motifs were not present on KLH. For Lewis X and LDN-F, the ELISA results confirmed the HPTLC-results regarding stage-specific expression of glycolipids exhibiting Lewis X and LDN-F epitopes.

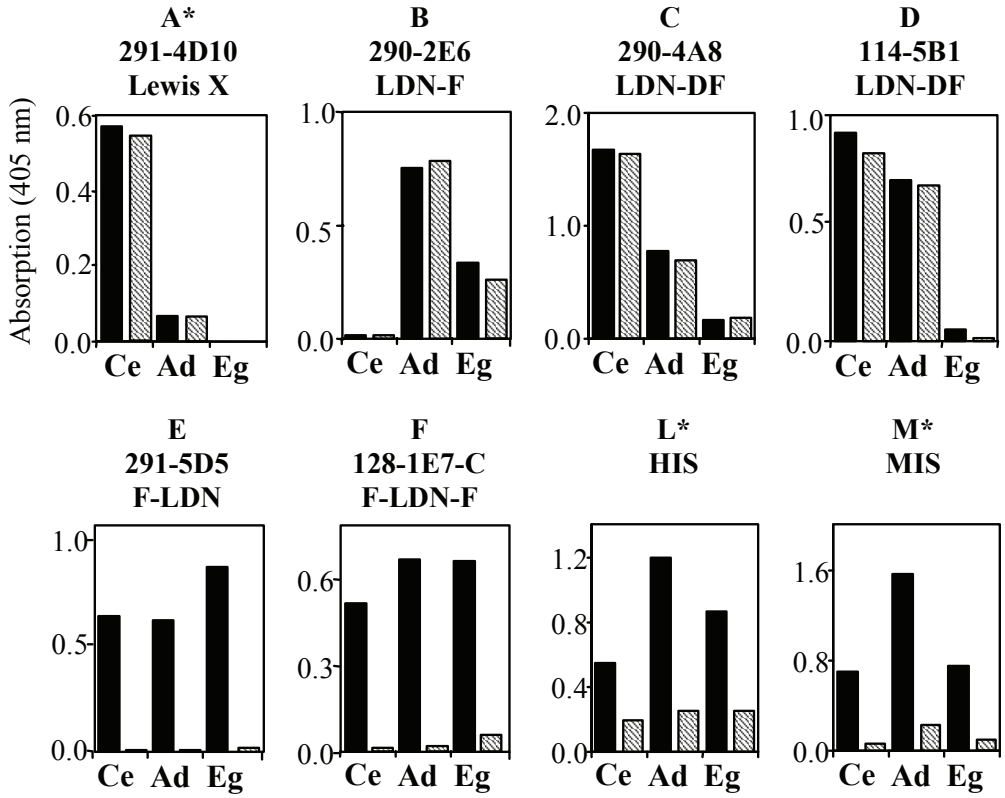

Figure 3. ELISA-Detection of *S. mansoni* glycolipids by various monoclonal antibodies and infection sera in the presence or absence of KLH as an inhibitor. *S. mansoni* glycolipids from cercariae (*Ce*), adults (*Ad*) and eggs (*Eg*) were probed with anti-carbohydrate mAbs (*A-F*) and human- (*HIS*) or murine (*MIS*) infection sera (*L,M*) with (hatched columns) or without (black columns) KLH as an inhibitor. Pictures marked by * have already been published (Kantelhardt *et al.*, 2002).

F-LDN(-F) in S. mansoni adult worms is mainly expressed on glycolipids.

To further examine the expression of the major antigenic F-LDN and F-LDN-F motifs we performed IFA on frozen sections of *S. mansoni* adult worms and eggs. Results are shown for anti-F-LDN-F mAb 128-1E7-C (Figure 4a-d), but staining with anti-F-LDN mAb 291-5D5-A gave the same fluorescence patterns (data not shown). Incubation of slides with mAb 128-1E7-C resulted in fluorescence of spots in the parenchyma of the adult worm and in the secreted egg antigens and eggshell (Figure 4a, c).

Chloroform/methanol treatment of the slides to remove lipids and glycolipids prior to incubation with mAb 128-1E7-C resulted in a complete loss of fluorescence in adult worms (Figure 4a,b). Together with the limited staining of adult worm glycoproteins with the F-LDN(-F) mAbs on Western blots (Figure 1E,F) this indicated that F-LDN(-F) epitopes in adult worms are predominantly glycolipid conjugated. In addition, pre-incubation of mAb 128-1E7-C with KLH before IFA resulted in the same loss of fluorescence in the parenchymal spots of adult worms as caused by chloroform/methanol treatment (data not shown), which is consistent with the finding in ELISA that KLH fully inhibits the binding of the anti-F-LDN(-F) mAbs to schistosome glycolipids. In contrast to our observations in adult worms, in the egg-stage reactivity of mAb 128-1E7-C remained unchanged after chloroform/methanol treatment (Figure 4c,d), which, together with intensive staining of egg-glycoproteins on Western blots, showed that the F-LDN(-F) motif in this life cycle stage is expressed at least for a significant part on glycoproteins.

Immunofluorescence staining with MIS showed fluorescence of the adult worm parenchymal spots, tegument and gut (Figure 4e,g) and of egg-secreted antigens, the miracidium, and the egg shell (data not shown). After chloroform/methanol treatment,reactivity of MIS with the spots in the parenchyma of adult worms and the tegument completely disappeared and only the gut remained brightly positive (Figure 4e,f). This suggests that antigens in the tegument and the parenchymal spots recognized by the serum antibodies are of (glyco-)lipid origin, whereas antigens in the gut are for a significant part of (glyco-)protein origin. Reactivity with the parenchymal spots was also lost when MIS was pre-incubated with KLH (Figure 4g,h). In combination with the observation that the reactivity of MIS to these spots disappears after (glyco-)lipid extraction, this shows that MIS contains antibodies directed against F-LDN(-F) containing worm-glycolipids. In contrast to the findings with adult worms, for eggs and egg secretions no differences could be observed between the MIS fluorescence patterns with or without chloroform/methanol treatment, showing that a substantial part of the antibodies in MIS recognise egg-glycoproteins. Also, no effect of pre-incubation of MIS with KLH was observed for egg fluorescence. Although Western blots, HPTLC overlay data and ELISA show that significant amounts of F-LDN(-F) are produced in schistosome eggs, this suggests that F-LDN(-F) is a less prominent epitope for MIS in eggs than in adult worms.

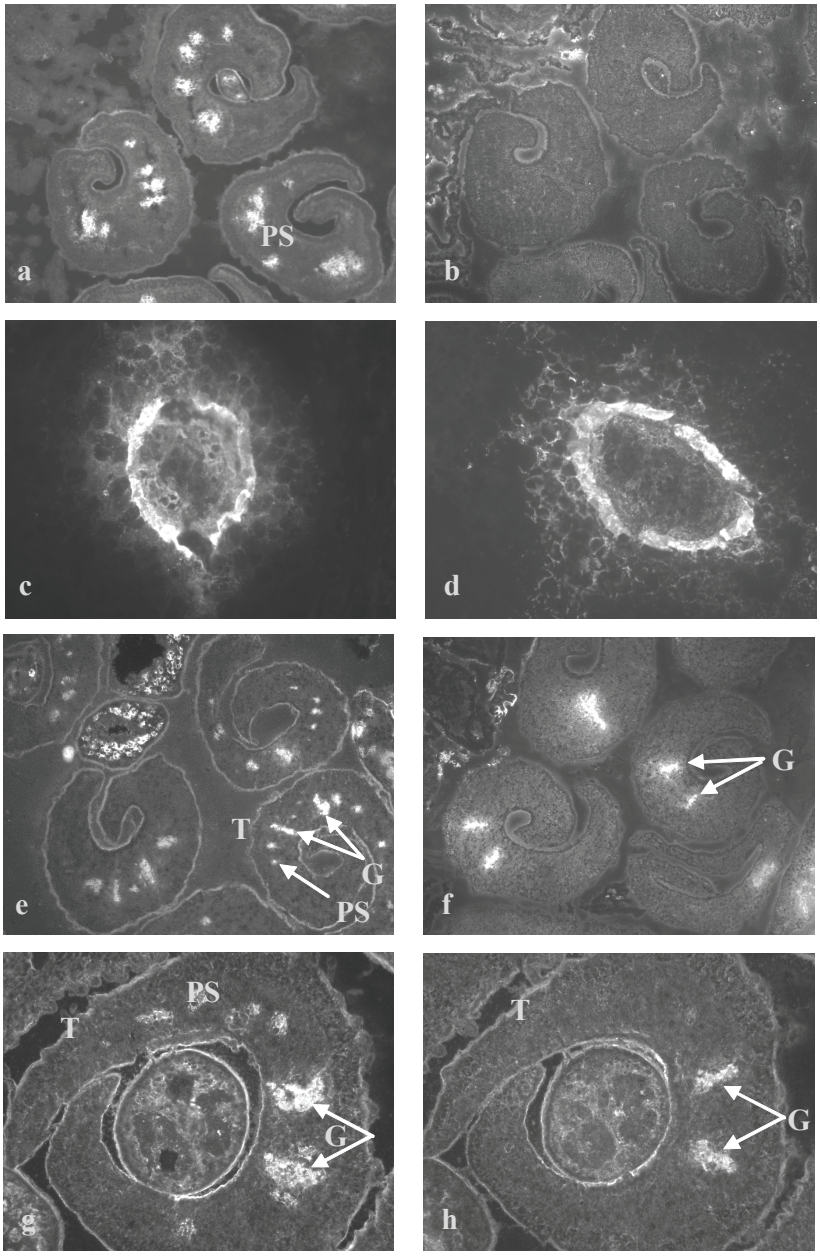

Figure 4. Immuno-fluorescence patterns of *S. mansoni* sections with or without chloroform/ methanol treatment.
Frozen sections of adult worms and eggs were stained using anti-F-LDN-F mAb 128-1E7-C (*a-d*) or murine infection serum (*MIS*) (*e-h*) followed by an FITC labelled anti-mouse Ig conjugate. Use of anti-F-LDN mAb 291-5D5-A gave the same results as mAb 128-1E7-C and an isotype control showed no fluorescence (data not shown). After incubation with mAb 128-1E7-C fluorescence was observed in parenchymal ducts of adult worms (*a*) and in egg-secreted antigens and egg shell (*c*).
After chloroform/ methanol treatment F-LDN-F related fluorescence patterns were lost in adult worms (*b*), but not in eggs (*d*). MIS recognised adult worm tegument (*T*), gut (*G*) and parenchymal spots (*PS*) in adult worms (*e, g*). Only the gut fluorescence stayed after chloroform/methanol treatment (*f*). When MIS was preincubated with KLH (1mg/ml) fluorescence in the parenchymal structures disappeared (*h*).

DISCUSSION

This study on the expression of the fucosylated antigenic glycan motifs F-LDN, F-LDN-F, LDN-F, Lewis X and LDN-DF revealed their prevalence and distribution on proteins and lipids of *S. mansoni* cercariae, adult worms and eggs. The global expression patterns of the glycan motifs observed here were mostly in agreement with earlier mass spectrometry based structural studies performed on isolated glycans of diverse parasite preparations (as reviewed in (Hokke *et al.*, 2001)), showing that the use of defined anti-carbohydrate mAbs provides a convenient alternative to study the expression of specific glycan epitopes in schistosomes.

A major focus of this study were the F-LDN and F-LDN-F glycan motifs, which occur in schistosomes and keyhole limpet hemocyanin, but have never been described in mammals. (Khoo *et al.*, 1995; Khoo *et al.*, 1997a; Kantelhardt *et al.*, 2002; Wuhrer *et al.*, 2002; Geyer *et al.*, 2004). In IFA, incubation with 291-5D5-A (F-LDN) and 128-1E7-C (F-LDN-F) resulted in similar staining of spots in the parenchyma of adult worms (see Figure 4a). These IFA pictures show fluorescence patterns rather similar to those described by Thors and Linder who found that KLH cross-reactive components are present in a schistosome adult worm duct-system (Thors & Linder, 1998; Thors & Linder, 2003), of which the function remains unknown so far. The disappearance of fluorescence after chloroform/methanol treatment of adult worm sections (Figure 4b) together with the faint staining of adult worm glycoproteins on Western blot (Figure 1E,F) indicates that the F-LDN(-F) element in these parenchymal spots, or ducts, is mainly glycolipid conjugated. In contrast to adult worms, cercariae and eggs express F-LDN and F-LDN-F not only on glycolipids, but also extensively on glycoproteins (Figure 1E,F). The Western blotting data suggest that F-LDN and F-LDN-F are present on the same sets of glycoproteins. SDS-PAGE does not easily give rise to the separation of the different glycoforms of the protein, in particular when neutral carbohydrate chains are involved. Notably, the band patterns on 291-5D5-A or 128-1E7-C stained HPTLC overlays differed from each other (Figure 2E,F), showing that these mAbs have different specificities and that the different glycolipids bound by the mAbs are separated by HPTLC. Because F-LDN is a partial structure of F-LDN-F (Table 1) the sets of glycolipids recognised by mAbs 291-5D5-A and 128-1E7-C are partly overlapping. These glycolipid subsets seem to be situated in the same parenchymal spots in the adult worm as no differences in the localisation of the subsets could be observed in our IFA experiments (data not shown).

KLH inhibition experiments in ELISA and IFA (Figure 3E-M, 4g,h) showed that the schistosomal F-LDN(-F) motif is the motif cross-reactive with KLH, which corroborated the earlier findings (Kantelhardt *et al.*, 2002) that Fuc(α1-3)GalNAc- is the major antigenic motif of *S. mansoni* glycolipids implicated in KLH cross-reactivity. This current observation is also in line with the recent report (Geyer *et al.*, 2004) which describes that the anti-schistosomal mAb M2D3H recognises the tetrasaccharide Deoxyhexose-HexNAc-[Deoxyhexose]-HexNAc on KLH. It is conceivable that the complete structure of this tetrasaccharide is Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F).

Another highly antigenic glycan that plays a major role in the immunobiology of schistosomiasis is LDN-DF, against which similar high antibody titres are observed as to F-LDN(-F) (van Remoortere *et al.*, 2003). There are no indications that LDN-DF, or other

known strongly immunogenic glycans like polyLeX (CCA) and CAA occur on KLH: Mabs reactive with these epitopes do not bind to KLH (A. van Remoortere, *unpublished observations*). A previous study has shown that 114-5B1-A and 290-4A8-A bind to synthetic LDN-DF, and both mAbs stain an adult worm excretory system in IFA (van Remoortere *et al*., 2000). The current study shows that the naturally occurring epitopes of these mAbs differ and that they are present on different sets of glycoproteins and glycolipids (Fig 1C,D and 2C,D). To be able to map in more detail the expression of schistosome glycoconjugates it will be of significant interest to deduce what these natural epitopes are.

The antigens recognised by mAb 114-5B1-A (LDN-DF) are localised in a duct- or excretory system in adult worms that has been well-defined in comprehensive microscopic studies (Bogers *et al.*, 1994; Bogers *et al.*, 1995a; van Remoortere *et al.*, 2000). Thors and Linder (2002) suggested that this system was the same as they observed after staining schistosome adult worms with rabbit anti-KLH polyclonal antibodies. This may be the case, since anti-LDN-DF mAb 114-5B1-A and the KLH reactive anti-F-LDN-F mAb 128-1E7-C (Van de Vijver *et al*., 2004) yielded similar reaction patterns in HPTLC immunostainings for the adult worm stage (Figure 2D,F), indicating that the F-LDN-F and LDN-DF motifs occur as combined structural elements on the same glycolipids. In line with this observation, the (Fucα1-3GalNAcβ1-4[Fucα1-2Fucα1-3]GlcNAcβ1-) (F-LDN-DF) sequence that combines the two motifs has previously been identified in *S. mansoni* cercarial glycocalyx (Khoo *et al.*, 1995) and on O-glycans and glycolipids of eggs (Khoo *et al.*, 1997a; Khoo *et al.*, 1997b; Wuhrer *et al.*, 2002). The current results implicate that glycolipids with this structure are also present in adult worms. However, in contrast to the F-LDN motif, in adult worms LDN-DF is not exclusively present on glycolipids, since numerous LDN-DF containing glycoproteins were observed on Western blots of all three examined stages (Figure 1C,D). Moreover, fluorescence patterns of mAb 114-5B1-A in adult worms and eggs were not lost after chloroform/methanol treatment (data not shown), indicating that mainly glycoproteins were recognised.

The expression of Lewis X and LDN-F on schistosome glycoproteins in the cercarial, adult worm, and most abundantly in the egg-stage is in line with the observations of Nyame *et al*. (Nyame *et al.*, 2003), who also found that Lewis X, LDN-F and in addition non-fucosylated LDN are more extensively expressed on egg glycoproteins compared to other developmental stages. However, no detailed comparisons can be made between the band patterns on the Western blots in the two studies since different antibodies and methods for sample preparation were used. Remarkably, the current study shows that on the glycolipid level

Lewis X was hardly detectable in eggs ((Figure 2A) and (Kantelhardt *et al.*, 2002; Wuhrer *et al.*, 2002)). The single Lewis X containing glycolipid observed as a weak band on HPTLC (Figure 2A) is in line with the finding of a Lewis X containing glycolipid in eggs in a previous study (Wuhrer *et al.*, 2002). This would imply that fluorescence observed with anti-Lewis X mAb 291-4D10-A (van Remoortere *et al.*, 2000) of the egg shell and secreted egg antigens in *S. mansoni* is mainly based on glycoprotein conjugated Lewis X.

In the same study of van Remoortere *et al.* (2000), it has been shown that Lewis X is only present in the oral sucker of cercariae, but not on the surface. In total cercarial extracts, we found numerous cercarial glycoproteins and glycolipids that contain Lewis X. Lewis X containing structures appear on the surface after shedding of the cercarial glycocalyx during the process of transformation to schistosomula (Koster & Strand, 1994). Probably, the numerous Lewis X containing glycoproteins and glycolipids we observed are already assembled but not yet expressed on the cercarial surface prior to transformation.

The biosynthesis of the many different fucosylated glycoconjugates in *Schistosoma* is catalysed by various fucosyltransferases, of which the expression is developmentally regulated (Marques Jr *et al.*, 2001). Marques *et al.* found a 50-fold higher total fucosyltransferase-specific activity in egg-extracts than in cercariae and adult worms. In agreement with their study, we observed that the fucosylated epitopes Lewis X, LDN-F, LDN-DF, F-LDN and F-LDN-F were significantly more abundantly expressed on egg-glycoproteins (Figure 1A-F) than on glycoproteins from cercariae and adult worms. This increase in fucosylation in eggs is not clearly visible in case of the glycolipids (Figure 2A-F). Notably, in contrast to egg glycoproteins, the Lewis X epitope is almost entirely absent in glycolipids of eggs. This difference in the expression of the different fuco-oligosaccharides may reflect the specific activities of the different fucosyltransferases involved.

Although several studies on the structural characterisation of schistosome glycans have been reported, only very few studies have addressed identification of the protein backbones that carry the different glycan epitopes so far described. The present study showed that panels of monoclonal anti-glycan antibodies are useful tools for identification of glycosylation patterns. In this respect, the current quick expansion of the schistosome genomic and proteomic databases open up new possibilities for the large-scale identification of antigenic schistosome glycoproteins by targeted approaches using the specific, anti-carbohydrate mAbs described in this and previous studies.

3

Targeted identification of a unique glycan epitope of *Schistosoma mansoni* egg antigens using a diagnostic antibody.

MARJOLEIN L.M. ROBIJN[1], CAROLIEN A.M. KOELEMAN[1], MANFRED WUHRER[1], LOUISE ROYLE[2], RUDOLF GEYER[3], RAYMOND A. DWEK[2], PAULINE M. RUDD[2], ANDRÉ M. DEELDER[1] AND CORNELIS H. HOKKE[1]

1 Department of Parasitology, Centre of Infectious Diseases, Leiden University Medical Centre, P.O. Box 9600, 2300 RC Leiden, The Netherlands; 2 The Glycobiology Institute, Department of Biochemistry, Oxford University, South Parks Road, Oxford, OX1 3QU, U.K.; 3 Institute of Biochemistry, Medical Faculty, University of Giessen, Friedrichstrasse 24, D-35392 Giessen, Germany

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SUMMARY

The eggs of *Schistosoma mansoni* express a plethora of glycoconjugate antigens. A specific subset of these antigens can be detected in the serum or urine of infected individuals by a diagnostic sandwich ELISA using the anti-carbohydrate monoclonal antibody (mAb) 114-4D12-A (Nourel Din *et al.*, (1994) Am J Trop Med Hyg 50: 585-594). We used affinity chromatography to isolate the 114-4D12-binding glycoprotein subset from soluble egg antigens (SEA) of *S. mansoni*. SEA and the isolated SEA-subset (SEA-4D12) were subjected to reductive β-elimination and hydrazinolysis to release intact glycans and glycan fragments, respectively, from the protein backbones. The released glycans were characterised by matrix-assisted laser-desorption-ionisation time-of-flight (MALDI-TOF) mass spectrometry (MS), liquid-chromatography (LC)-MS and gas chromatography (GC)-MS linkage analysis. Glycans released by reductive β-elimination from SEA-4D12 were larger and more heavily fucosylated than glycans released from SEA. Most SEA-4D12 glycans contained a branched O-glycan core structure carrying up to 4 *N*-acetylhexosamines per chain which were substituted with maximum 12 fucose residues. Hydrazinolysis of SEA-4D12 resulted in the release of fucosylated antenna fragments. After 2-aminobenzamide (2AB)-labelling these fragments were subjected to 114-4D12-affinity purification. Normal phase (NP)-LC analysis of the flow-through and retained fractions indicated that the Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1- element forms the epitope of mAb 114-4D12. Most O-glycans from SEA-4D12 contain this structural element. Potential epitope-bearing N-glycans were however hardly detectable. In terms of abundance in total SEA, only a minority of all glycans possesses the epitope. This multifucosylated motif has so far only been found in schistosomes, providing a structural basis for the high specificity of the diagnostic antibody.

INTRODUCTION

Schistosomiasis is one of the major parasitic diseases in the world with an estimated 200 million people infected, a global disease burden of 4.5 million disability-adjusted life years, and an annual mortality of more than 280,000 (Chitsulo *et al.*, 2000; van der Werf *et al.*, 2003). In the case of *Schistosoma mansoni*, the adult worms which live in the portal veins of the human host produce hundreds of eggs per day per worm pair of which about 50% is never excreted with the faeces, but instead gets trapped in the presinusoidal capillaries of the liver. There, through microscopic pores in the egg-shell, antigens are secreted that cause a granulomatous and potentially damaging inflammatory response (Smithers & Doenhoff, 1982; Boros, 1999; Asahi & Stadecker, 2003). A sub-population of these antigens can be detected by ELISA in the circulation or urine of the host using monoclonal antibodies (mAbs) that recognise specific glycan motifs (Nourel Din *et al.*, 1994a; Nourel Din *et al.*, 1994b).

Global glycosylation analyses have revealed that schistosome eggs produce a large variety of complex glycans with highly unusual glycan elements (Hokke *et al.*, 2001; Khoo & Dell, 2001; Wuhrer *et al.*, 2002). The egg stage expresses different fucosylated glycan motifs in relative abundance, such as Galβ1-4(Fucα1-3)GlcNAc (Le^X), GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F), GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF), Fucα1-3GalNAcβ1-4GlcNAc (F-LDN) and Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc (F-LDN-F), although most glycan motifs are also expressed in other developmental stages and organisms (Nyame *et al.*, 2003; Robijn *et al.*, 2005).

It is well established that schistosomal glycans are involved in several aspects of the infection. The start of egg deposition coincides with intense anti-carbohydrate antibody responses (Eberl *et al.*, 2001; van Remoortere *et al.*, 2003). In infected chimpanzees, strongest antibody responses were measured against the non-mammalian F-LDN and LDN-DF elements (van Remoortere *et al.*, 2003). In a cohort of Kenyans migrated into a schistosomiasis area, specific IgG1 antibody responses against F-LDN and LDN-DF were found to be strongly associated with those previously measured to SEA and crude cercarial antigens (Naus *et al.*, 2003). Furthermore, evidence is accumulating that egg-related glycans have immunomodulatory properties. It has been shown that a Le^X-conjugate is capable of inducing IL-10 production by peripheral blood mononuclear cells (PBMCs) of infected individuals (Velupillai *et al.*, 2000), and that Le^X can act on dendritic cells (DC) via toll-like receptor 4 (TLR4) (Thomas *et al.*, 2003). An LDN-DF conjugate induces both pro- and anti-inflammatory cytokines IL-10, IL-6 and TNF-α in naive human PBMCs (Van der Kleij *et al.*, 2002b). Glycosylation also seems to play a major role in the initiation and homeostasis of the granulomas around schistosome

eggs. Using a murine model system, SEA-coated beads induced the formation of granulomas with identical cellular and temporal characteristics as natural granulomas around schistosome eggs, and this activity was harboured in glycans of SEA (Jacobs *et al.*, 1999; Van de Vijver *et al.*, 2004; Van de Vijver *et al.*, 2006).

Some of the glycoprotein antigens excreted by the eggs are detectable in the host's circulation (Ripert *et al.*, 1988; Hassan *et al.*, 1992; Nourel Din *et al.*, 1994b; Shaker *et al.*, 1998), indicating that they are excreted in relatively large quantities. A specific set of such circulating soluble egg antigens can be detected in the serum and urine of *S. mansoni*-infected individuals using an immunodiagnostic sandwich assay employing the anti-carbohydrate monoclonal antibodies (mAbs) 114-4D12-A and 114-5B1-A (Nourel Din *et al.*, 1994b). In immunofluorescence assays (IFAs), both mAbs 114-4D12-A and 114-5B1-A show reactivity with the eggs and with antigens diffusing from the eggs (Nourel Din *et al.*, 1994a). The staining patterns of these two mAbs in IFA are different, which indicates that the epitope recognised by mAb 114-4D12 is different from the epitope recognised by mAb 114-5B1-A. Using a set of synthetic, schistosome-related glycoconjugates it was shown that mAb 114-5B1-A binds to the GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF) sequence (van Remoortere *et al.*, 2000), but no information is yet available on the epitope of mAb 114-4D12.

The current study was undertaken to determine in detail the authentic glycan epitope bound by mAb 114-4D12. To this end, we isolated the 114-4D12-specific glycoprotein subset from SEA by affinity chromatography, analysed the chemically released glycans by MS-based approaches and determined that the 114-4D12 epitope was formed by the schistosome-specific terminal Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc motif in O-glycans that are present on a limited set of egg glycoproteins.

MATERIALS AND METHODS

Antigen preparations and affinity chromatography

The *S. mansoni* SEA preparation was obtained as previously described (Deelder, 1973; Dalton *et al.*, 1997). The monoclonal antibody mAb 114-4D12-A (mouse IgG3) has been described before (Nourel Din *et al.*, 1994a). For coupling to protG Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) mAb 114-4D12-AA (the IgG1 isotype-switch of mAb 114-4D12-A) was used, in this study designated as 114-4D12. The mAb was covalently coupled to the carrier according to the protocol described by Sisson and Castor (Sisson & Castor, 1990) at densities of 12-22 mg mAb/ml wet beads.

For affinity chromatography, the resulting beads were poured into a small column (1 ml), washed with elution buffer (0.05 M glycine-HCl, pH 3) and stabilised with phosphate buffered saline (PBS). SEA (4-6 mg) was applied to the affinity column in PBS/1% sodium chloride, unbound proteins/glycoproteins were washed away with 12 column volumes (CV) of PBS, and bound glycoproteins were eluted with 12 CV of 0.05 M glycine-HCl, pH 3. The elution fraction was immediately neutralised, dialysed against 25 mM ammonium bicarbonate, and finally lyophilised. SEA was repeatedly applied to obtain sufficient SEA-4D12 for glycosylation analysis to a maximum of 9 runs on the same column material.

Affinity chromatography of alkaline borodeuteride-released or hydrazinolysis-released and 2AB-labelled glycans was performed similarly on a small column of 25µl packed beads. The column was pre-treated as described above and the sample was applied in PBS. The flow-through was collected together with the first 10 CV of PBS. Then the column was washed with an additional 10 CV of PBS. Finally, the bound glycans were eluted with 20 CV glycine-HCl, pH 3. Each collected fraction was desalted using a graphitised carbon cartridge as described (Wuhrer *et al.*, 2004b) and freeze-dried, previous to NP-LC or MALDI-TOF MS analysis.

Immunofluorescence assay (IFA)

Immunofluorescence assays were performed as described previously (Robijn *et al.*, 2005). Briefly, slides of frozen liver sections of *S. mansoni*-infected hamsters were incubated with mAb 114-4D12 hybridoma culture supernatant, washed, incubated with FITC-labelled goat anti-mouse Ig and evaluated by fluorescence microscopy.

SDS-PAGE and Western blotting

S. mansoni cercariae, adult worm and egg preparations were separated by SDS-PAGE under reducing conditions on 10% gels and subjected to Western blot as described (Robijn *et al.*, 2005). As primary antibody biotin-labelled 114-4D12 was used, in combination with Streptavidin-AP conjugate (1:2000) (Zymed Laboratories, San Francisco, CA). Staining was afforded by X-phosphate/5-bromo-4-chloro-3'-indolyl-phosphate (BCIP) (Roche Diagnostics, Mannheim, Germany) and 4-nitro blue tetrazolium chloride (NBT) (Roche) as substrates.

MALDI-TOF(/TOF) MS

MALDI-TOF mass spectra were recorded using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) equipped with a LIFT-MS/MS facility. 2,5-Dihydroxybenzoic acid (DHB) (Bruker Daltonics) was used as a matrix, 30 mg/ml in 50%

acetonitrile. Spectra were acquired in the positive ion mode and internally recalibrated after measurement.

Hydrazinolysis treatment and 2AB-labelling

Reducing oligosaccharides were released from SEA and SEA-4D12 by hydrazinolysis according to (Merry *et al.*, 2002). Fetuin was taken as a control. Briefly, samples were incubated with anhydrous hydrazine at 60°C for 6 h. Excess hydrazine was removed by evaporation and the released glycans were re-*N*-acetylated with acetic anhydride in a saturated solution of sodium bicarbonate. After removal of peptides by descending paper chromatography, the obtained glycans were fluorescent labelled with 2AB (Royle *et al.*, 2002; Wuhrer *et al.*, 2004b).

Liquid chromatography of 2AB-labelled glycans

2AB-labelled glycans were fractionated by NP-LC on a TSK-Amide 80 column (4 mm x 250 mm; Tosohaas, Montgomeryville, PA, U.S.A.) as described previously (Wuhrer *et al.*, 2004b). The ÄKTA purifier (Amersham Pharmacia Biotech, Uppsala, Sweden) HPLC system was combined with a YASCO fluorescence detector (FP-1520, Tokyo, Japan) set at 360 nm/ 425 nm (excitation/emission). Fractions were collected manually.

Nano-RP-LC-MS/MS analysis of 2AB-labelled glycans

For separation, detection and fragmentation analysis of isomeric 2AB-labelled glycans, the collected NP-LC fractions were subjected to nano-RP-LC-MS as described (Wuhrer *et al.*, 2006c). Briefly, 2AB-glycans were separated on a Pep-Map column (75µm x 150 mm; Dionex/LC Packings, Amsterdam, the Netherlands) using an Ultimate nano-LC system (LC Packings) and analysed on-line using an Esquire HCT ion-trap mass spectrometer equipped with a nano-electrospray ion-source (Bruker Daltonics). Spectra were recorded in the positive ion mode.

To avoid rearrangement products in the fragmentation spectra of fucosylated glycans after laser- or collision-induced dissociation of protonated precursor ions (Harvey *et al.*, 2002; Wuhrer *et al.*, 2006e) we have exclusively used sodiated precursor ions to record MS/MS spectra. To generate sodiated rather than protonated ions, sodium hydroxide (0.8 mM) was added to the eluent.

Glycan release using reductive β-elimination

S. mansoni SEA was delipidated (Khoo *et al.*, 1997b), incubated with glycosidase (Amyloglycosidase from *Aspergillus niger*, Sigma-Aldrich, Zwijndrecht, The Netherlands) in 200 mM sodium acetate buffer (pH 5) at 56°C and subsequently dialysed to remove small molecular weight components. Affinity chromatography on 114-4D12 was performed as described above. Glycans were released from the glycoproteins in the flow-through, eluate and starting material as oligosaccharide alditols by reductive β-elimination with 1.0 M sodium borohydride or borodeuteride in 0.1 M aqueous sodium hydroxide at 40°C overnight. The samples were neutralised with acetic acid and borates were removed by co-evaporation with 1% acetic acid in methanol. Released oligosaccharides were purified by size exclusion chromatography on a Superdex peptide column (PE 7.5/300, Amersham) eluted with 25 mM ammonium bicarbonate. Collected fractions were analysed by MALDI-TOF MS (not shown) and all glycan-containing fractions were combined and lyophilised.

Linkage analysis

For linkage analysis, 2AB-labelled oligosaccharides were permethylated with methyl iodide after deprotonation with lithium methylsulfinyl carbanion (Paz-Parente *et al.*, 1985). The 114-4D12-purified oligosaccharide alditols used for linkage analysis were obtained from SEA with alkaline sodium borodeuteride. After affinity purification they were permethylated following the sodium hydroxide/dimethylsulfoxide slurry method (Ciucanu & Kerek, 1984). All permethylated oligosaccharide samples were subjected to hydrolysis (4 M aqueous trifluoroacetic acid, 100°C, 4 h), reduction, peracetylation, and analysis by capillary GC-MS as described earlier (Geyer & Geyer, 1994).

Identification of hexose-2AB

To identify the 2AB-linked hexose residue in the hydrazinolysis-released glycan fragments, an aliquot of the major NP-fraction 14 was subjected to hydrolysis in 100 mM TFA for 1 h at 100°C. The glycosidic bond between HexNAc and Hex-2AB appears to be relatively labile and is hydrolysed under these conditions, whereas the fluorescent labelled monosaccharide residues remain largely intact (Wuhrer *et al.*, 2000a). As a control, a sample of 2AB-labelled egg glycolipid-derived glycans (Wuhrer *et al.*, 2002) containing a 2AB-labelled glucose residue was analysed in parallel. Samples were dried and in each case the Hex-2AB residue was collected by preparative NP-LC as described in (Wuhrer *et al.*, 2004b). Subsequently, the Hex-2AB residue was identified by comparative monosaccharide analysis using a standard series of fluorescent labelled hexoses (mannose, galactose, glucose) essentially by the method

of (Anumula, 1994), but in this case adapted for 2AB-labelled monosaccharides. 2AB-hexoses were separated on a Supersphere 100 C-18 RP column (end capped 4 µm, 250x4 mm; Merck, Darmstadt, Germany). Solvent A consisted of 1.0% tetrahydrofuran, 0.5% phosphoric acid and 0.05% butylamine in water. Solvent B consisted of equal volumes of solvent A and acetonitrile. Samples were applied in 100% solvent A and isocratically eluted at 100% solvent A in 10 ml, followed by linear increase to 50% solvent B in 1 ml and a subsequent isocratic elution at 50% solvent B for 5 ml. Fluorescence was detected at 360 nm/ 425 nm.

RESULTS

Immunochemical characterisation of antigens bound by 114-4D12

By IFA (Figure 1A), mAb 114-4D12 was found to bind to antigens of the egg and the miracidium of *S. mansoni*. In addition, 114-4D12 clearly reacted with antigens diffused into the hepatic tissue surrounding the egg. Western blot analysis of total protein extracts showed that 114-4D12 recognised various proteins of eggs, but also distinct sets of proteins of cercariae and adult worms (Figure 1B).

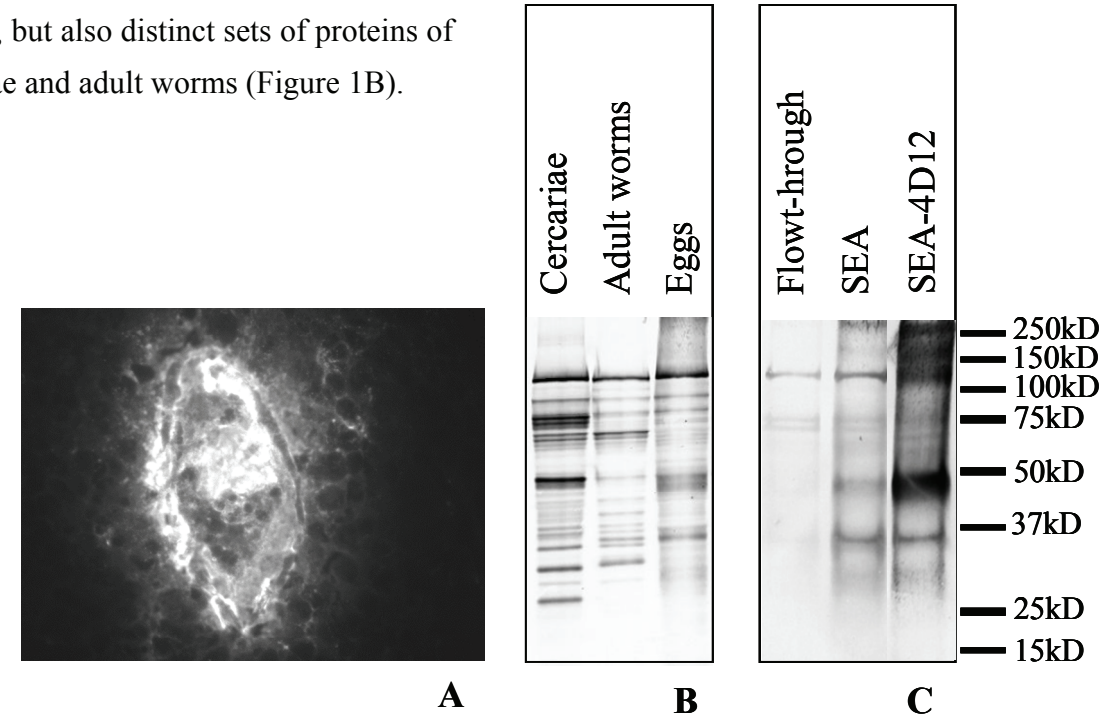


Figure 1. Immunoreactivity of mAb 114-4D12. (*A*) 114-4D12-stained immunofluorescence microscopy image of an *S. mansoni* egg in a frozen hamster liver section, 7 weeks after infection. Parts of the miracidium, the egg-shell and antigens diffused from the egg are visible. (*B*) Western blot analysis showing the stage-specific expression of 114-4D12 glycoprotein antigens in protein extracts of *S. mansoni* cercariae, adult worms and eggs. (*C*) Western blot analysis of the affinity purified 114-4D12 binding subfraction of SEA (SEA-4D12), the flow-through and the starting material SEA. Of the starting material SEA and of the flow-through fraction 6 µg and of the affinity purified SEA-4D12 approximately 1 µg was loaded onto the gel. Masses of a precision protein standard are indicated.

To facilitate the identification and structural characterisation of the 114-4D12-reactive glycans from *S. mansoni* egg glycoproteins, the relevant antigen subset was isolated from SEA by 114-4D12 affinity chromatography. Western blot analysis (Figure 1C) showed that the eluate of the affinity column (SEA-4D12) contained a number of 114-4D12-reactive proteins. Intensely stained bands were observed in the 40-50 kD region, at 35 kD, and in the region above 100 kD. Essentially the same band pattern was observed when the starting material SEA was blotted with 114-4D12, except for the >100 kD region that stained much more intense in the case of SEA-4D12. Virtually no staining was observed for the flow-through, which indicates that the 114-4D12 epitope-containing proteins were successfully captured. With respect to the lower molecular weight region < 50 kD, the Western blot pattern generated from SEA and SEA-4D12 (Figure 1C) largely corresponds to the pattern generated from the total egg protein extract (an extract made with SDS-PAGE loading buffer) (Figure 1B). The latter pattern additionally included several proteins of 60-100 kD that were not visible in the SEA blot. This indicates that these proteins are water insoluble, possibly membrane bound antigens that are therefore not present in the aqueous extract SEA.

Structural characterisation of the glycan epitope of SEA-4D12

Two complementary approaches were followed to identify the epitope of 114-4D12, as well as the structure of the underlying glycans. A reductive β-elimination was carried out to release glycoprotein glycans as oligosaccharide alditols. In parallel, a hydrazinolysis procedure was applied to release oligosaccharides of SEA and SEA-4D12 with a reducing terminus, to render them suitable for fluorescent labelling with 2AB for sensitive detection and multiple chromatographic separations. The hydrazinolysis approach was designed to selectively obtain intact reducing O-glycans, however after comparison with the data obtained by the β-elimination procedure it became clear that an unexpected but apparently selective degradation had occurred during hydrazinolysis which yielded fragments of SEA-4D12 glycans, including their non-reducing termini. We will first present the data obtained by the hydrazinolysis procedure that allowed the identification of the 114-4D12 epitope. Subsequently, we will present the structural aspects of the underlying glycans, based on the analysis of the intact, released oligosaccharide alditols.

Structural analysis of glycan fragments released by hydrazinolysis

The affinity purified fraction SEA-4D12 was treated with anhydrous hydrazine and the resulting reducing oligosaccharides were subsequently reacted with 2AB. MALDI-TOF MS (Figure 2A) of this material (denoted SEA-4D12-H) indicated the presence of highly

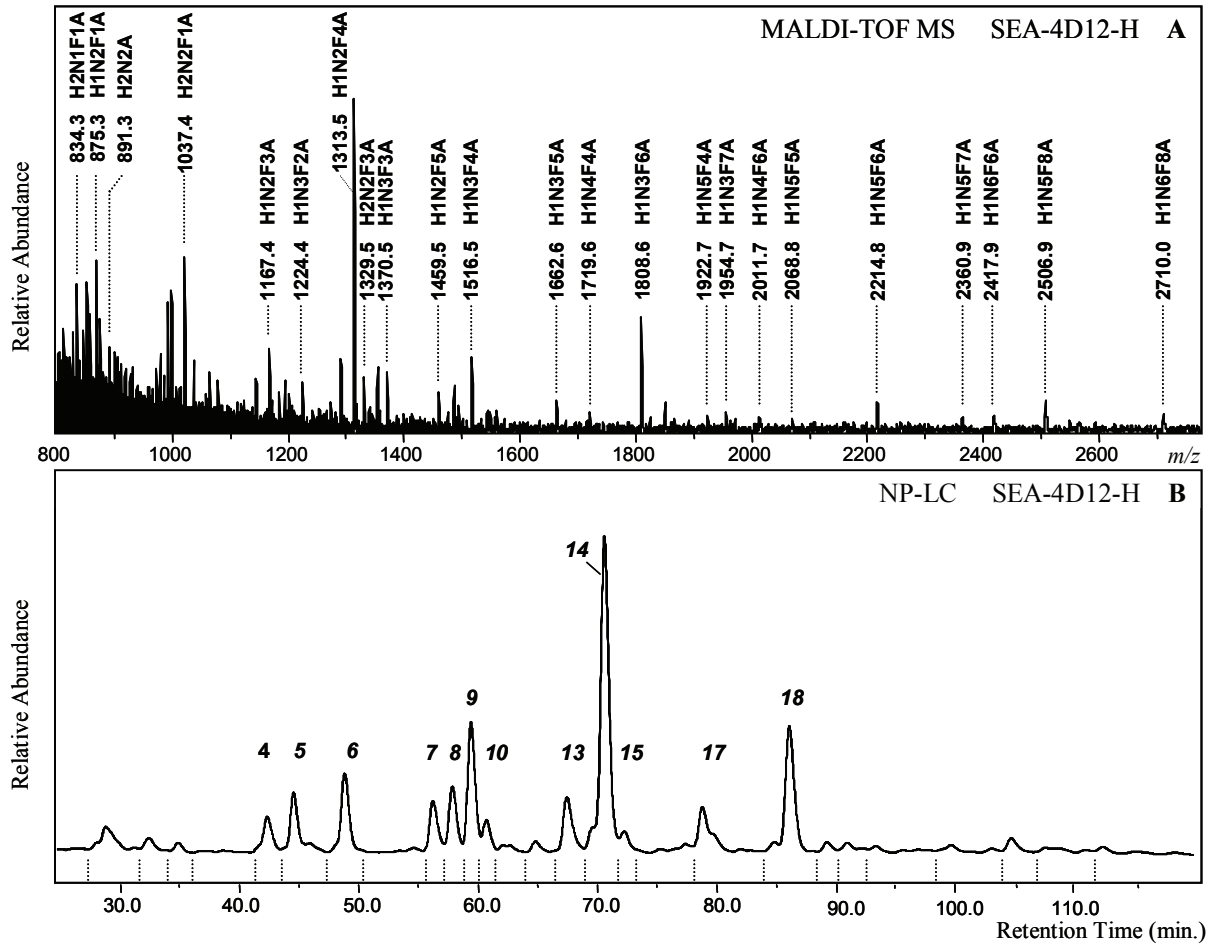


Figure 2. (*A*) MALDI-TOF MS and (*B*) NP-LC profile of the hydrazinolysis-released and 2AB-labelled glycans from SEA-4D12 (SEA-4D12-H). (*A*) The MALDI-TOF MS profile shows monoisotopic masses of the sodiated ions together with the putative compositions. H stands for hexose, N for N-acetylhexosamine, F for fucose, A for 2AB. (*B*) For further analysis, the hydrazinolysis-released glycans were fractionated by NP-LC. Numbers indicate the fractions that contain the major components of SEA-4D12-H (see Table I).

fucosylated glycans, with $H_1N_2F_4$-2AB and $H_1N_3F_6$-2AB as the major species (H, hexose, Hex; N, *N*-acetylhexosamine, HexNAc; F, fucose, Fuc). To afford a more detailed analysis, the SEA-4D12-H oligosaccharide pool was subjected to preparative NP-LC (Figure 2B). Collected fractions were further analysed by MALDI-TOF MS, and by nano-RP-LC-MS to resolve oligosaccharides that co-eluted in the first dimension NP system and to obtain MS/MS fragmentation spectra of the individual compounds. Although most NP fractions appeared to be heterogeneous, the major fraction 14 was found to contain only a single oligosaccharide with composition $H_1N_2F_4$-2AB. In the MS/MS spectrum of the double-charged parent ion of $H_1N_2F_4$-2AB (m/z 668.3 [M+2Na]2+; Figure 3A), a single-charged ion-species appeared at m/z 1313.5 [M+Na]+. From this ion, two Fuc residues were lost prior to the loss of HexNAc,

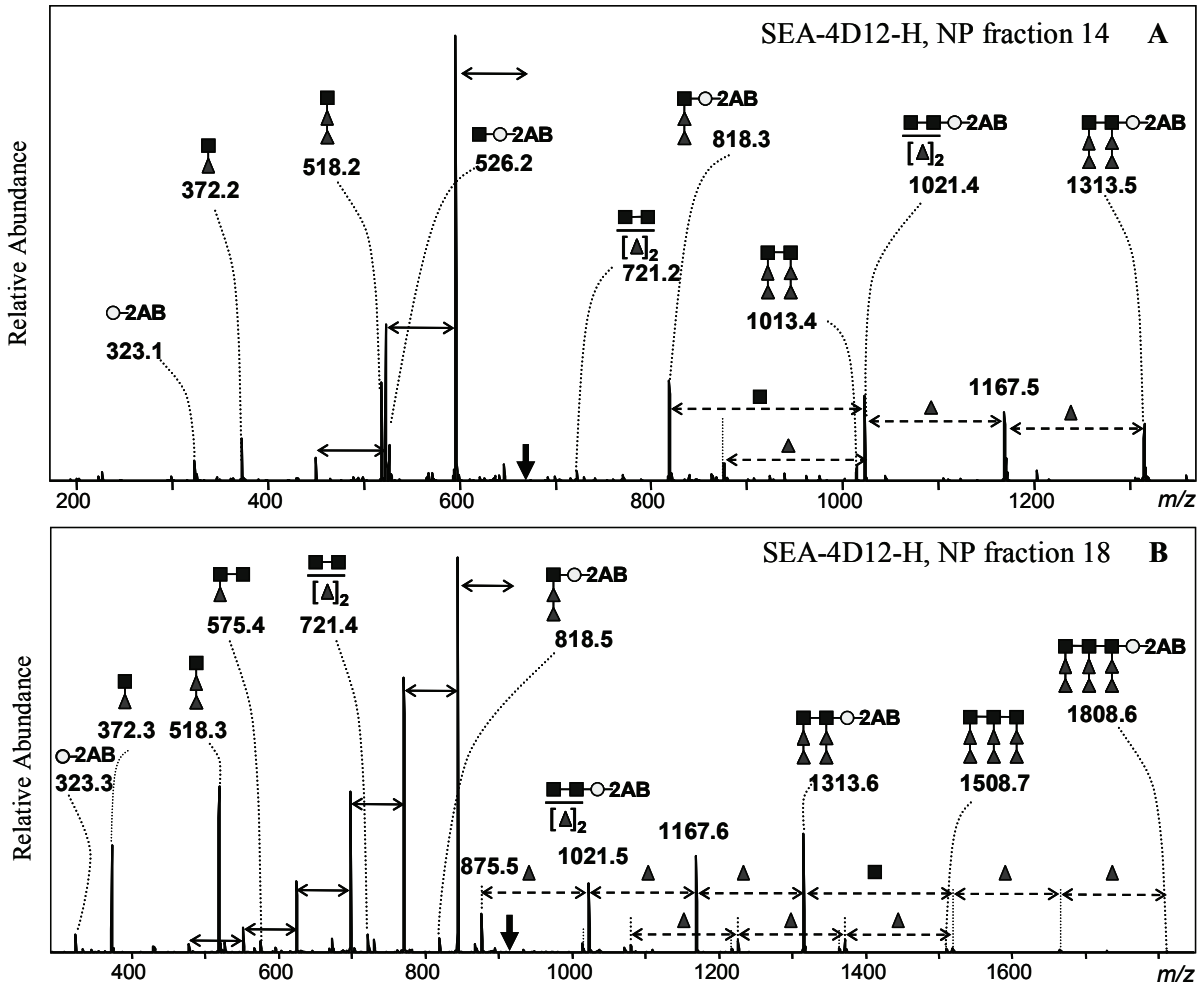


Figure 3. Nano-RP-LC-ESI-MS/MS spectra of the two major components of SEA-4D12-H. The glycans with composition (*A*) $H_1N_2F_4$-2AB and (*B*) $H_1N_3F_6$-2AB were detected in NP-LC fractions 14 and 18, respectively. The measured mono-isotopic masses are indicated together with the symbolic representation of the fragment ions. The indicative ions were single charged [M+Na]+, whereas the double charged ions $[M+2Na]^{2+}$ only showed losses of fucose. Symbols: square, HexNAc; circle, Hex; triangle, Fuc. Double-headed arrows with dashed line and symbols indicate monosaccharide differences. Double-headed arrows with solid line indicate Fuc losses of double charged ions. Closed arrow head indicates the parent ion. For discussion of the spectra and further structural details, see text.

indicating the presence of a terminal N_1F_2 element. This element was detected as a conserved fragment (m/z 518.2). From the resulting $H_1N_1F_2$-2AB fragment (m/z 818.3), again two fucoses and one HexNAc residue were lost, resulting in a H_1-2AB fragment (m/z 323.1). Together with the ion at m/z 1013.4 (N_2F_4), this indicates that the primary sequence of the oligosaccharide present in NP-fraction 14 of SEA-4D12-H is Fuc-Fuc-HexNAc-(Fuc-Fuc-)HexNAc-Hex-2AB.

The fragmentation spectrum of $H_1N_3F_6$-2AB (*m/z* 916.0, $[M+2Na]^{2+}$; Figure 3B), the major compound in NP fraction 18, contained similar indicative ions as found in the MS/MS

spectrum of $H_1N_2F_4$-2AB. The additional signal at *m/z* 1508.7 (single-charged N_3F_6 fragment), however, indicates the presence of an extra N_1F_2 repeat. The subsequent loss of two Fuc residues prior to HexNAc from the molecular ion-species at *m/z* 1808.6 ($H_1N_3F_6$-2AB $[M+Na]^+$) resulted in the fragment ion *m/z* 1313.6 ($H_1N_2F_4$-2AB $[M+Na]^+$). Hence, the proposed sequence of the major compound in NP-fraction 18 is Fuc-Fuc-HexNAc-(Fuc-Fuc-)HexNAc-(Fuc-Fuc-)HexNAc-Hex-2AB. Similarly, putative compositions and primary sequences of the most abundant compounds in the other major NP-fractions were deduced, as summarised in Table I.

To clarify the identity of the constituent monosaccharides of $H_1N_2F_4$-2AB, and their substitution patterns, linkage analysis was performed on an aliquot of NP fraction 14. GC-MS analysis (Table II) of the resulting partially methylated alditol acetates revealed the presence of 3-substituted GalNAc, 3,4-substituted GlcNAc, 2-substituted Fuc and terminal Fuc. Since the 2AB-labelled Hex residue does not appear in the GC-MS analysis, its identity was determined after mild TFA-hydrolysis of an aliquot of NP fraction 14. RP-LC with fluorescence detection showed that the released 2AB-linked monosaccharide residue of $H_1N_2F_4$-2AB was galactose (Figure 4). Taken together, the LC-MS/MS and linkage analysis suggest that the major oligosaccharide released by hydrazinolysis from SEA-4D12 is Fuc1-2Fuc1-3GalNAc1-4(Fuc1-2Fuc1-3)GlcNAc1-Gal.

Identification of the 114-4D12 glycan epitope

To determine which of the different oligosaccharides released by hydrazinolysis from SEA-4D12 actually carry the epitope of 114-4D12, a small portion of the SEA-4D12-H pool was applied to 114-4D12 affinity chromatography. The flow-through and the elution fraction were subsequently analysed by NP-LC (Figure 5). The major peaks 14 and 18, representing $H_1N_2F_4$-2AB [Fuc1-2Fuc1-3GalNAc1-4(Fuc1-2Fuc1-3)GlcNAc1-Gal-2AB] and $H_1N_3F_6$-2AB [Fuc-Fuc-HexNAc-(Fuc-Fuc-)HexNAc-(Fuc-Fuc-)HexNAc-Hex-2AB] respectively, appeared in the chromatogram of the eluate, but completely disappeared from that of the flow-through fraction, indicating that these compounds were bound by mAb 114-4D12. Glycans that did not contain the terminal Fuc-Fuc-HexNAc-(Fuc-Fuc-)HexNAc- sequence (e.g. peaks 6, 7, 8, 9, 13 and 17, see Table I) were not retained on the 114-4D12 matrix and appeared in the flow-through fraction. Together with the MS/MS and linkage analysis data, this identifies the Fuc1-2Fuc1-3GalNAc1-4(Fuc1-2Fuc1-3)GlcNAc1- element as the binding motif of mAb 114-4D12.

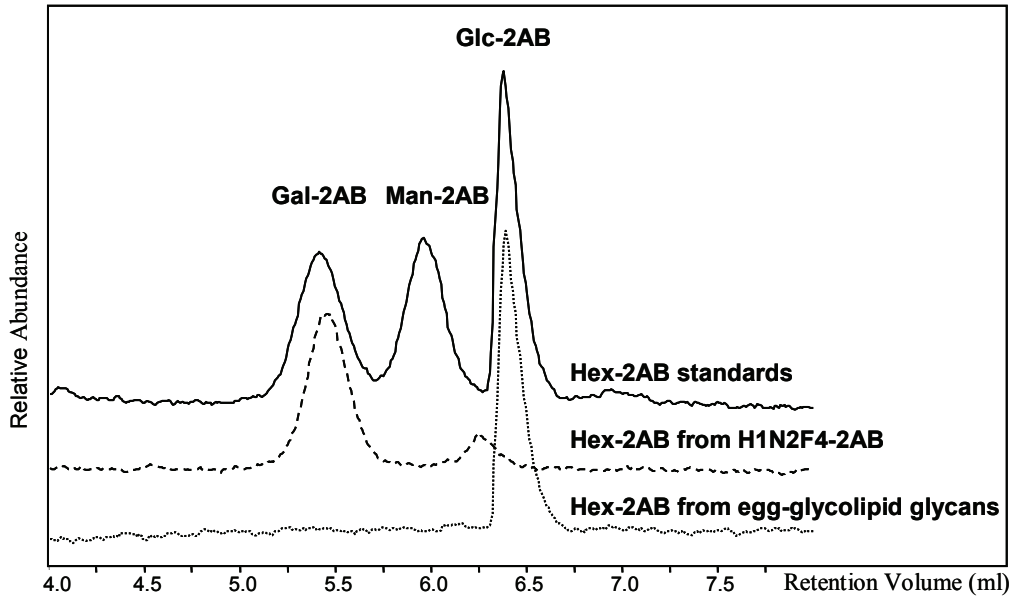


Figure 4. NP-chromatogram of the 2AB-labelled hexose residue from H1N2F4-2AB. After mild TFA hydrolysis the 2AB-labelled hexose of H1N2F4-2AB was identified as galactose (Gal-2AB) (dashed line). As reference, egg-glycolipid glycans that contain fucosylated HexNAc-chains linked to the lipid moiety via a glucose residue (Khoo et al., 1997a; Wuhrer et al., 2002) were analysed and we confirmed the presence of a terminal glucose residue (dotted line). As standard, a mix of Gal-2AB, Man-2AB and Glc-2AB was used (straight line).

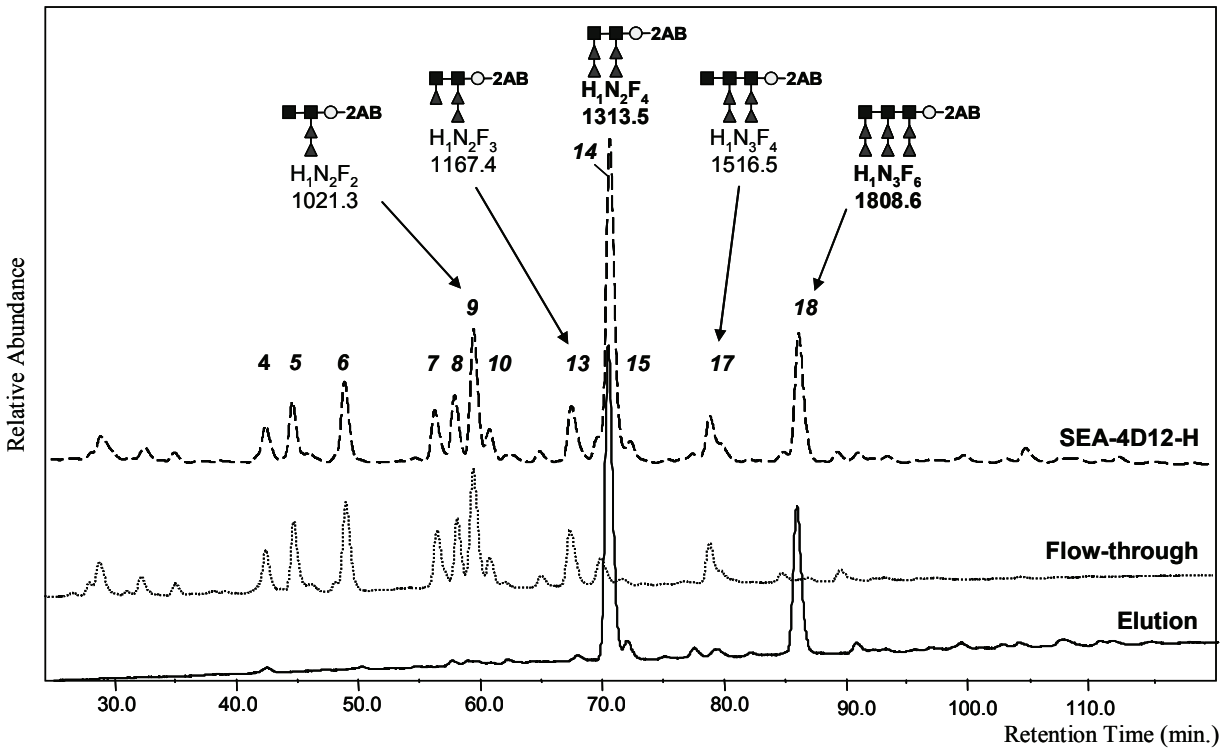


Figure 5. NP-LC analysis of affinity fractionated SEA-4D12-H. SEA-4D12-H glycans (dashed line) were applied to a mAb 114-4D12 affinity column. Compounds that were washed off the column with PBS are indicated as flow-through (dotted line), glycans that were captured by the 114-4D12 column were eluted with 50 mM Glycine-HCl, pH3 (solid line). Numbers indicate the original NP-LC fractions to which the major components of SEA-4D12-H correspond (see also Table I).

Table I. Major components of SEA-4D12-H. An overview is given of the hydrazinolysis-released glycans observed in the major NP fractions from SEA-4D12-H. Structures are arranged according to composition. NP-LC fractions containing sufficient amounts of material were analysed by MALDI-TOF MS, single sodiated ions were observed. Monoisotopic masses are indicated. Fragmentation analysis was performed by nano-RP-LC-ESI-MS/MS on double sodiated ions to prevent rearrangements of the fucoses. The MS/MS fragments indicated are single charged ions [M+Na]+. H stands for hexose, N for N-acetylhexosamine, F for fucose and A for 2AB.

Composition	Detected in NP-fr	$[M+Na]^+/[M+2Na]^{2+}$ calc.	*m/z* MALDI-TOF MS meas.	*m/z* LC-MS meas.	*m/z* Indicative MS/MS fragments $[M+Na]^+$	Proposed sequence
H_1N_2A	4	729.2 / 376.1	729.2	-		-
H_2N_1A	5	688.2 / 355.6	688.2	-		-
H_2N_1A	6	688.2 / 355.6	688.2	-		-
$H_1N_2F_1A$	7	875.3 / 449.1	875.2	-		-
$H_2N_1F_1A$	8	834.3 / 428.6	834.3	-		-
$H_1N_2F_2A$	9	1021.3 / 522.1	1021.4	1021.5	323 (HA), 721 (N_2F_2), 729 (HN_2A), 818 (HN_1F_2A)	N-(FF)N-H-A
$H_2N_1F_2A$	10	980.3 / 501.6	980.4	-		-
$H_1N_2F_3A$	13	1167.4 / 595.2	1167.6	1167.5	323 (HA), 721 (N_2F_2), 729 (HN_2A), 818 (HN_1F_2A), 867 (N_2F_3)	FN-(FF)N-H-A
$H_1N_2F_4A$	14	1313.5 / 668.2	1313.5	668.7	323 (HA), 518 (NF_2), 818 (HN_1F_2A), 1013 (N_2F_4)	FFN-(FF)N-H-A
$H_1N_2F_5A$	15	1459.5 / 741.2	n.d.	741.8	323 (HA), 518 (NF_2), 664 (NF_3), 964 (HNF_3A)	FFN-(FFF)N-H-A
$H_1N_3F_4A$	17	1516.5 / 769.7	1516.8	770.6	323 (HA), 518 (NF_2), 932 (HN_3A), 1313 (HN_2F_4A)	N-(FF)N-(FF)N-H-A
$H_1N_3F_6A$	18	1808.6 / 915.8	1808.8	915.9	323 (HA), 518 (NF_2), 818 (HN_1F_2A), 1013 (N_2F_4), 1313 (HN_2F_4A), 1508 (N_3F_6)	FFN-(FF)N-(FF)N-H-A

Monosaccharide	Elution time (min.)	Relative abundance [A] in: 4D12 bound SEA-B	NP fr 14
term. Fuc	14.92	1.0	+ [B]
2-subst. Fuc	18.93	0.9	+
3-subst. Gal	25.73	0.2	n.d. [C]
term. GlcNAc	36.80	<0.02	n.d.
3,6- subst. GalNAc-ol	39.27	0.03	n.d.
term. GalNAc	39.50	0.04	n.d.
4- subst. GlcNAc	41.51	0.1	n.d.
3- subst. GalNAc	45.13	0.4	+
3,4- subst. GlcNAc	46.46	0.9	+

Table II. Linkage analysis data of the 114-4D12 bound glycan fraction of SEA-B and the hydrazinolysis released SEA-4D12 glycan H1N2F4-2AB from NP-fraction 14. Abundances of the constituent monosaccharides are expressed as relative peak ratios of the corresponding partially methylated alditol acetates observed at specific elution times.

A based on peak height, response factors not determined. *B* detected, but peak height not determined. *C* not determined.

Structural analysis of intact epitope-containing glycans released by reductive β-elimination

As our data indicated that the hydrazine treatment of SEA-4D12 induced a partial glycan degradation (see also below, and Figure 6C) we performed a detailed analysis of the intact glycan structures released by reductive β-elimination from SEA, as well as from the 114-4D12 flow-through (SEA-4D12FT) and bound (SEA-4D12) SEA fractions. The obtained oligosaccharide pools (denoted SEA-B, SEA-4D12FT-B and SEA-4D12-B, respectively) were analysed by MALDI-TOF MS analysis (Figure 6). In SEA-4D12-B (Figure 6C) a limited series of highly fucosylated glycans is heavily enriched, whereas the spectra of SEA-B and SEA-4D12FT-B (Figure 6A,B) are largely identical and suggest the presence of a significant number of different glycan compositions in SEA, in accordance with previous observations (Khoo *et al.*, 1997b). This indicates that the 114-4D12-captured SEA fraction represents a minor, specific subset of glycoproteins from SEA.

To deduce in a direct approach which glycans from SEA contain the 114-4D12 epitope, the alkaline borodeuteride-released glycan pool from SEA (SEA-B) was subjected to 114-4D12 affinity chromatography, and the eluate was analysed by MALDI-TOF MS (Figure 6D). Most of the glycan species that appeared highly enriched in SEA-4D12-B (Figure 6C) through selection at the glycoprotein level clearly emerged as epitope-containing glycan alditols in the spectrum of the specific glycan eluate (Figure 6D).

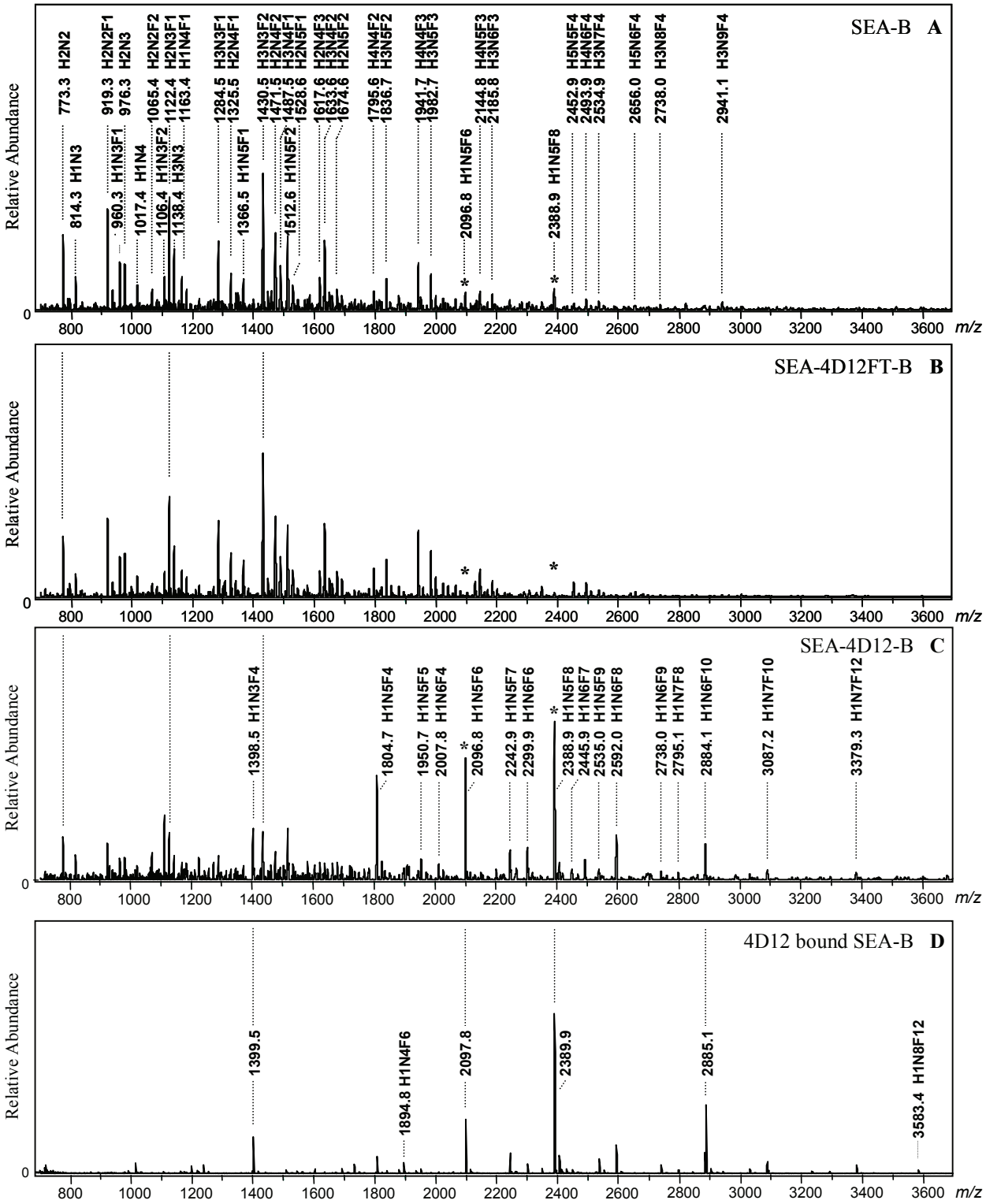


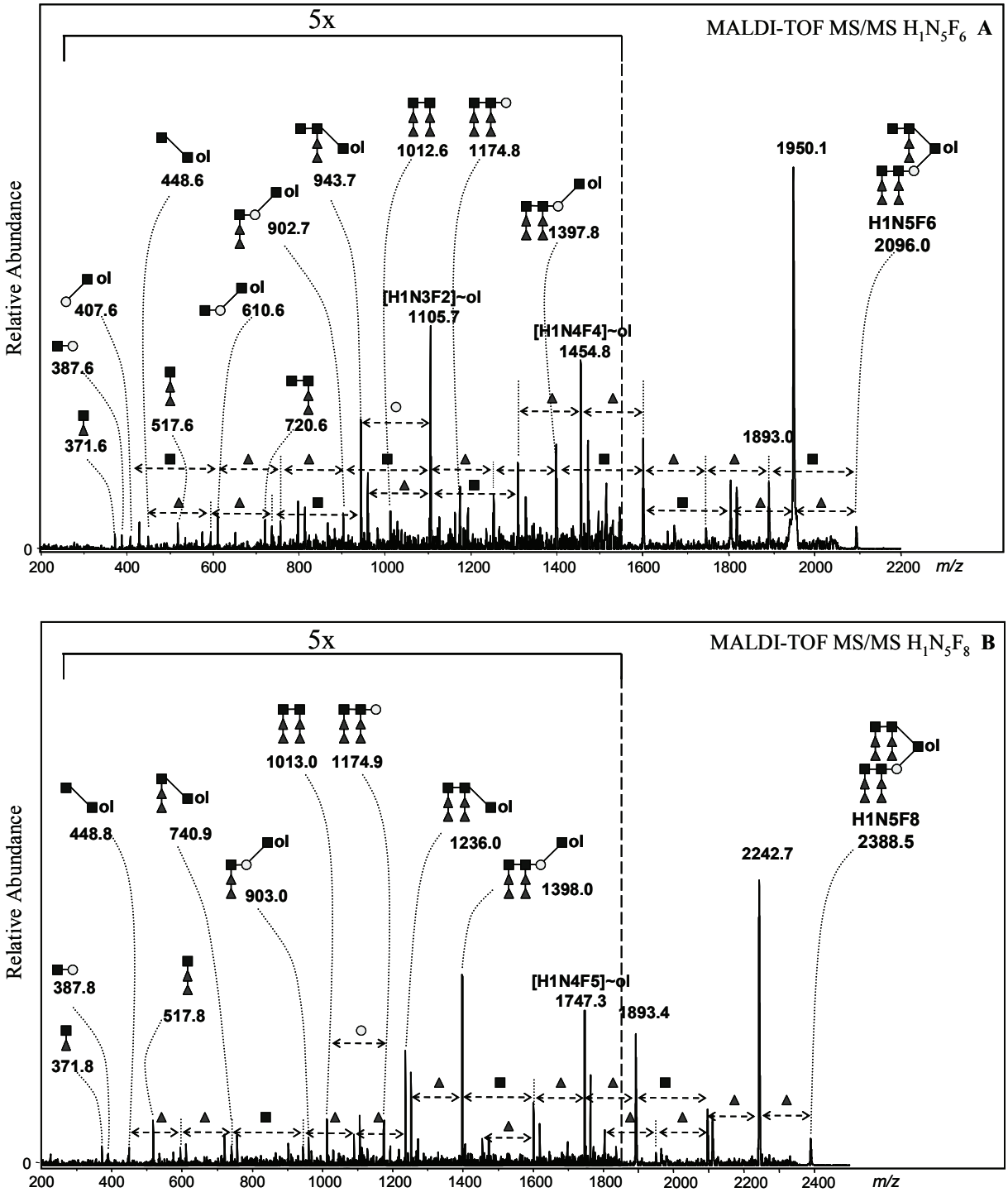
Figure 6. MALDI-TOF MS analysis of glycans released by reductive β-elimination from (*A*) SEA (SEA-B), (*B*) the flow-through fraction of the 114-4D12 affinity column (SEA-4D12FT-B), (*C*) the 114-4D12 affinity purified SEA fraction (SEA-4D12-B) and (*D*) the 114-4D12 glycan eluate from sodium borodeuteride-treated glycans from SEA (m/z +1, compared to the corresponding sodium borohydride-treated glycans in A, B and C). Mono-isotopic masses of the sodiated ions are indicated, together with the putative structural compositions. The major components of SEA-4D12-B that were also visible in SEA-B, but not in SEA-4D12FT-B are indicated by *. H stands for hexose, N for N-acetylhexosamine, F for fucose.

This 114-4D12-binding glycan fraction from SEA-B was further analysed by linkage analysis (Table II, discussed below). For an overview of the proposed structures of the major 114-4D12-binding intact glycans, see Figure 8. Fragmentation spectra of the three major epitope-containing molecular ion species $H_1N_5F_6$-ol (-ol, alditol), $H_1N_5F_8$-ol and $H_1N_6F_{10}$-ol, are shown in Figure 7.

In the MS/MS spectrum of $H_1N_5F_6$-ol (Figure 7A), from the parent ion (*m/z* 2096.0, $[M+Na]^+$) either a Fuc or a HexNAc residue was lost, indicating the presence of a non-substituted terminal HexNAc residue, in addition to terminal Fuc. After the initial loss of one HexNAc (*m/z* 1893.0) subsequent losses of three Fuc-Fuc-HexNAc (N_1F_2) sequences resulted in the fragment ions at *m/z* 1397.8 ($H_1N_3F_4$-ol), at *m/z* 902.7 ($H_1N_2F_2$-ol) and *m/z* 407.6 (H_1N_1-ol). The N_1F_2 element is also observed as a conserved fragment at *m/z* 517.6 or as N_2F_4 dimer at *m/z* 1012.6. This N_2F_4 unit is linked to Hex (*m/z* 1174.8) which indicates that one arm of the glycan is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-Hex. Similarly, the signal at *m/z* 943.7 is indicative for the N_3F_2-ol sequence containing the other arm of the branched structure. Taken together, the proposed structure of $H_1N_5F_6$-ol is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-Hex-[HexNAc(Fuc-Fuc-)HexNAc-]HexNAc-ol.

In the case of $H_1N_5F_8$-ol (Figure 7B), a cascade of Fuc losses can be observed, but not the initial loss of HexNAc, which indicates that only Fuc residues occupy terminal positions. Again, from the parent ion (*m/z* 2388.5, $[M+Na]^+$) we observed subsequent losses of two sequential N_1F_2 elements, which is corroborated by the conserved fragments at *m/z* 517.8 (N_1F_2) and *m/z* 1013.0 (N_2F_4). Similar to the structure of $H_1N_5F_6$-ol, one branch of $H_1N_5F_8$-ol consists of the Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-Hex (*m/z* 1174.9) sequence. However, in this case, the other branch of the structure gives rise to the signal at *m/z* 1236.0 which is indicative for N_3F_4-ol (compare *m/z* 943.7 in Figure 7A). From *m/z* 1236.0, two Fuc residues and one HexNAc residue are lost to form N_2F_2-ol (*m/z* 740.9), and after the subsequent loss of another two Fuc residues, the N_2-ol fragment remains at *m/z* 448.8. Taken together this fragmentation spectrum indicates that the sequence of $H_1N_5F_8$-ol is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-Hex-[Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-]HexNAc-ol.

By a similar reasoning, the sequence of $H_1N_6F_{10}$-ol, which is the second major glycan in the 114-4D12-bound fraction from SEA-B (see Figure 6D), can be deduced (Figure 7C).



Compared to $H_1N_5F_8$-ol, this compound contains an additional N_1F_2 unit in the Hex-containing branch as indicated by the fragment ions at *m/z* 1670.3 and *m/z* 1508.2 (N_3F_6), whereas the other branch is identical (N_3F_4-ol, *m/z* 1237.2). Overall, the MS/MS spectrum of $H_1N_6F_{10}$-ol indicated that the sequence of this species is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-(Fuc-Fuc-)HexNAc-Hex-[Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-]HexNAc-ol.

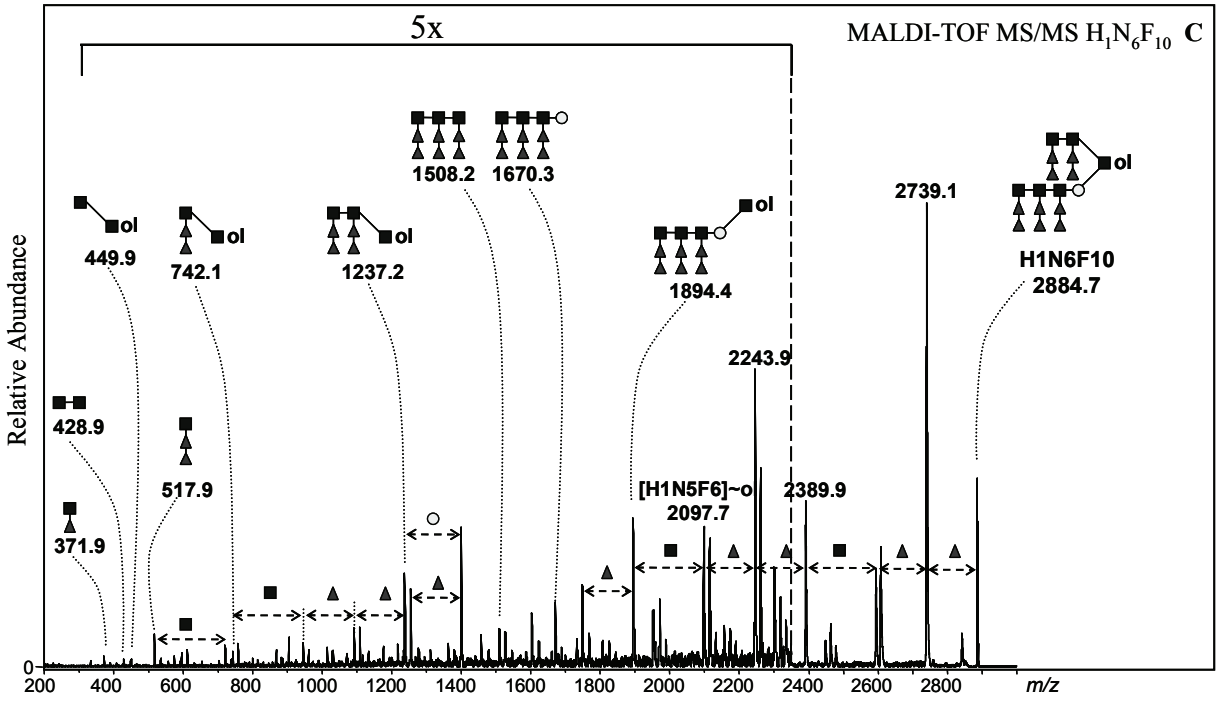


Figure 7. MALDI-TOF/TOF MS/MS spectra of three major glycans released by reductive β-elimination (*A*) H1N5F6 m/z 2096.0, (*B*) H1N5F8 m/z 2388.5 and (*C*) H1N6F10 m/z 2884.7. The MS/MS spectra of (*A*) H1N5F6 and (*B*) H1N5F8 were recorded from SEA-4D12-B (Figure 6C). The MS/MS spectrum of (*C*) H1N6F10 was recorded from the 114-4D12 bound glycans from SEA-B (Figure 6D), which were released using alkaline borodeuteride in stead of alkaline borohydride, giving rise to a m/z +1 shift of the HexNAc-ol containing fragments. Measured monoisotopic masses are indicated. The Y-scale of the lower molecular range of the spectrum was magnified 5x for better visualisation of the peaks. Symbols: square, HexNAc; circle, Hex; triangle, Fuc; -ol, alditol obtained by borohydride (*A, B*) or borodeuteride (*C*) reduction. Double-headed arrows with dashed line and symbols indicate monosaccharide differences. For discussion of the spectra, see text.

Linkage analysis of the 114-4D12-bound glycan fraction of SEA-B (Table II) indicated the presence of 3,6-substituted GalNAc-ol and 3-substituted Gal, which together form the Hex-HexNAc-ol element observed in the MS/MS fragmentation spectra (Figure 7). Terminal Fuc and 2-substituted Fuc were observed in a 1.0:0.9 ratio, indicating that almost all Fuc residues occur as pairs. 3,4-Disubstituted GlcNAc and 4-substituted GlcNAc were found in ratio of 0.9:0.1, indicating that most GlcNAc residues were fucosylated, in addition to being substituted by GalNAc or GlcNAc, and terminal GlcNAc was essentially absent. Also terminal GalNAc was virtually absent, whereas 3-substituted GalNAc was present in a relative amount of 0.4, indicating that for each 3,4-substituted GlcNAc stretch of two residues, on average one fucosylated GalNAc capping residue is present.

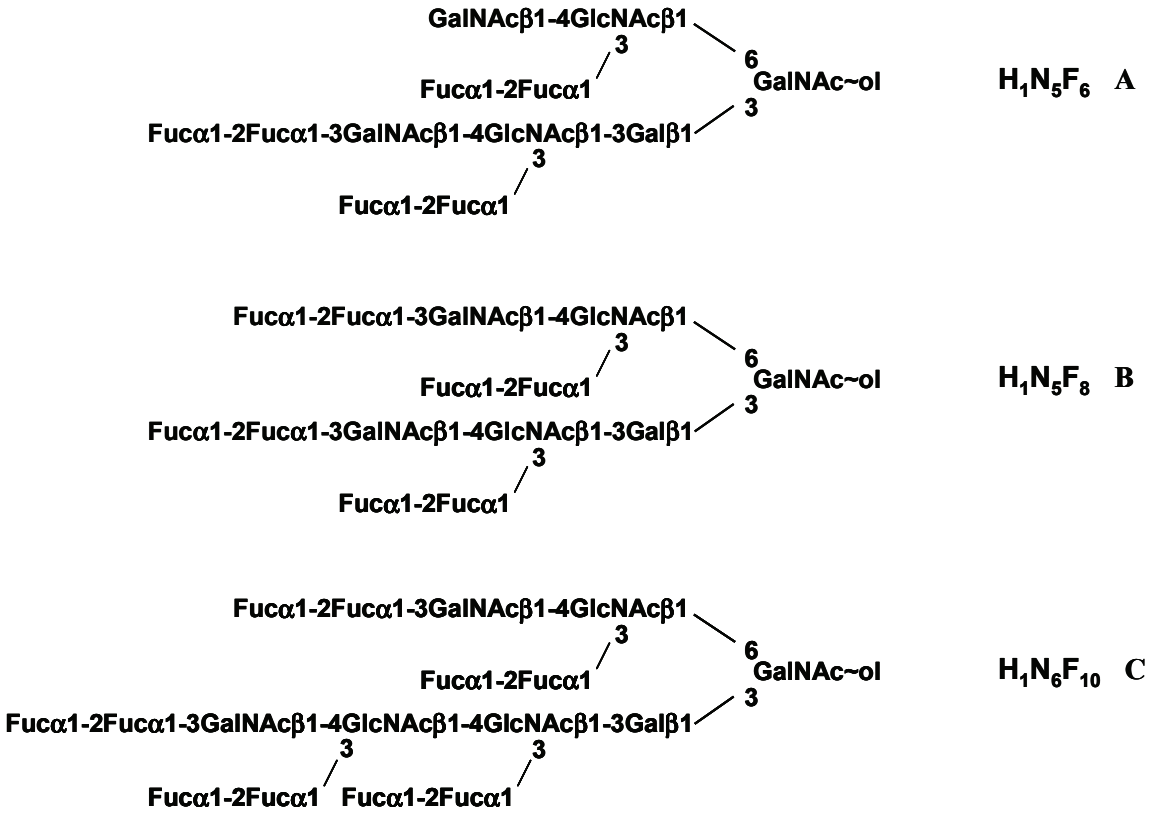


Figure 8. Proposed structures of three major 114-4D12 epitope-containing O-glycans of *S. mansoni* SEA.

Summary of the structural data of the 114-4D12 binding glycans of SEA

In summary, our data indicate that the 114-4D12 epitope is carried by unique branched O-glycans of a specific glycoprotein subset of schistosome eggs (see Figure 8). These glycans are based on a core structure of Gal- and GlcNAc-substituted GalNAc, elongated with stretches of difucosylated GlcNAc residues mostly terminating with a difucosylated GalNAc residue. Although the MS approach that we applied can not determine the anomeric configuration of the constituent monosaccharides, previous structural data of schistosome glycans (Khoo *et al.*, 1995; Khoo *et al.*, 1997a; Khoo *et al.*, 1997b; Hokke *et al.*, 1998; Khoo *et al.*, 2001a; Wuhrer *et al.*, 2002) suggest that all Fuc residues are α–anomers, whereas GalNAc and GlcNAc residues are β–anomeric. The combined data indicate that the epitope of mAb 114-4D12 is formed by the terminal Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1- motif.

DISCUSSION

Schistosomes produce a wide range of unusual glycans (Hokke *et al.*, 2001; Khoo & Dell, 2001; Nyame, Kawar & Cummings, 2004). In particular the egg stage is rich in immunogenic glycoproteins (Hokke *et al.*, 2001; Nyame, Kawar & Cummings, 2004; Robijn *et al.*, 2005), of which the glycans have been implicated in various aspects of schistosomiasis (Hokke *et al.*, 2001; Nyame, Kawar & Cummings, 2004; Robijn *et al.*, 2005), including immunity (MacDonald, Araujo & Pearce, 2002; Capron *et al.*, 2005), immunomodulation (Maizels & Yazdanbakhsh, 2003; Thomas *et al.*, 2004) and granuloma formation (Boros, 1999; Van de Vijver *et al.*, 2004). In the current study, we have analysed the glycosylation of a subset of *S. mansoni* egg glycoproteins (SEA-4D12) bound by an anti-carbohydrate mAb 114-4D12 that is used in a diagnostic circulating egg antigen assay (Nourel Din *et al.*, 1994a; Nourel Din *et al.*, 1994b). Our data indicate that this mAb specifically binds to the unique carbohydrate motif Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GalNAc (DF-LDN-DF) in the context of a glycan or glycoprotein that contains one or more copies of this epitope.

Structural aspects

The majority of glycans released by reductive β-elimination from 114-4D12-bound egg glycoproteins (Figure 6C) are O-glycans that contain the terminal epitope motif in at least one of two branches on a 3,6-disubstituted GalNAc core residue. These glycans were hardly detectable in the pool of glycans released from total SEA (Figure 6A), indicating that the epitope-carrying SEA-4D12 glycoproteins form a minor subset of SEA. Whereas the MALDI-TOF MS data suggest that the major glycans from SEA are 700-1700 Da in size, and consist mainly of mono- or difucosylated $Hex_{1-3}/HexNAc_{1-5}$ combinations, the glycans of SEA-4D12 are significantly richer in highly fucosylated species of 1700 - 3000 Da. Apparently, these glycans are specifically present on the targeted subset of SEA-4D12 glycoproteins. In a previous global fast atom bombardment mass spectrometry (FAB-MS) based mapping study on the N- and O-glycans of total *S. mansoni* egg glycoproteins, also indications for the presence of a minor amount of terminal multi-fucosylated $HexNAc_{1-3}$ elements were found (Khoo *et al.*, 1997b).

Although the conditions applied for reductive β-elimination release N-glycans as well as O-glycans, composition and fragmentation analysis of the SEA-4D12 glycans by MALDI-TOF(/TOF) MS(/MS) indicated the presence of O-glycans mainly. Moreover, among the affinity-purified epitope-containing glycans from SEA (Figure 6D) only O-glycans were found by MALDI-TOF(/TOF) MS(/MS) analysis. These O-glycans also formed the major peaks in the MALDI-TOF MS profile of the pool of glycans released from SEA-4D12 (Figure

6C), which further supports the notion that the major glycans carried by the SEA-4D12 glycoprotein subset carry the 114-4D12 epitope, and that they are O-glycans. It is clear, however, that a certain proportion of the SEA-4D12 glycans visible in the 700-1700 Da range in MALDI-TOF MS (Figure 6C) exhibit compositions that exclude the presence of the epitope. These glycans which, based on their monosaccharide composition, include O- as well as N-glycans almost completely disappear in the specific eluate (Figure 6D). Such a display of heterogeneity of glycosylation of specific (subsets of) glycoproteins is a common feature that underlines the complexity of protein glycosylation in schistosomes and in general.

In parallel to the alkaline borohydride/borodeuteride procedure which creates oligosaccharide alditols by reductive β-elimination, we performed a mild hydrazinolysis on SEA-4D12 under conditions previously developed to selectively release reducing O-glycans (Royle *et al.*, 2002; Merry *et al.*, 2002). Surprisingly, this procedure did not yield intact O-glycans, but gave rise to the formation of linear, reducing-end galactose-terminating structures most apparantly derived from the branched O-glycans that could be detected in the β-elimination released material. In combination with the previous data of egg O-glycosylation by Khoo *et al*. (Khoo *et al.*, 1997b) we conclude that the original putative 1-3-linkage between the Gal and GalNAc core residues was cleaved during the hydrazinolysis procedure. Apparently, a reducing Gal residue was created which reacted with 2AB, whereas the other part of what was most probably a type 2 O-glycan core gave rise to fragments that could not be reacted with 2AB and therefore were not visible the subsequent analysis. Since sialylated Galβ1-3GalNAc O-glycans were released selectively, and fully intact, under the same conditions in a control experiment on bovine fetuin (data not shown), it is not clear why in the case of SEA-4D12 such massive degradation occurred. Fortunately, the short, linear, epitope-containing 2AB-labelled sequences created by hydrazinolysis did give us the opportunity to determine the exact, monovalent binding motif of 114-4D12 in an affinity chromatography experiment (Figure 5).

The Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1- sequence present on the SEA-4D12 O-glycans harbours characteristic elements that occur also in other types of *S. mansoni* glycans. Of the glycosphingolipid-derived glycans from *S. mansoni* eggs, those containing internal difucosyl GlcNAc residues capped with mono- or difucosylated GalNAc (Khoo *et al.*, 1997a; Wuhrer *et al.*, 2002) are structurally similar to the O-glycans found here. Furthermore, the Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1- epitope appears to be abundantly present also in the glycocalyx of *S. mansoni* cercariae (Khoo *et al.*, 1995). In that case, however, the underlying glycan backbone consists of -3GalNAcβ1-

4(±Fucα1-2Fucα1-2Fucα1-3)GlcNAcβ1-3Galα1- repeats, instead of the non-repetitive element observed here. It should be mentioned, particularly in relation to the diagnostic use of mAb 114-4D12, that the epitope sequence so far has not been detected in any other organism than schistosomes, nor by ELISA, nor by biochemical methods.

The 114-4D12-bound antigens from SEA formed a specific subset of several glycoproteins (Figure 1B, C). The epitope-containing glycans released from the protein backbones could be isolated directly on the same affinity column as the glycoproteins (Figure 5, 6D). Lectins or anti-carbohydrate antibodies generally recognise individual oligosaccharides with low affinity (Hindsgaul & Cummings, 1999). In the case of 114-4D12, the affinity appears to be sufficient to also specifically pull out free glycans that contain monovalent epitopes from a complex mixture.

Biological aspects

Although minor in terms of abundance, the specific egg protein glycosylation analysed in this study may be highly relevant to schistosome egg biology and immunology, and the data presented here give a structural basis to previous observations made with mAb 114-4D12. First of all, the mAb and its antigens have been extensively studied by microscopic methods. Bogers *et al*., (Bogers *et al.*, 1996) observed immunoreactivity with the primary granules of eosinophilic granulocytes, spleen macrophages and Kupffer cells surrounding eggs trapped in the liver. This strongly suggests that the studied egg antigens are excreted and were subject to phagocytosis, and that these glycoproteins were initially bound through recognition by host lectin receptors, perhaps such as DC-SIGN, galectin-3, or macrophage galactose-type lectin (MGL) (van Die *et al.*, 2003; van den Berg *et al.*, 2004; Van Vliet *et al.*, 2005). It remains to be investigated however, which known or unknown host lectins might bind the specific difucosylHexNAc element shown to be present on soluble SEA-4D12 glycoproteins. In any case, excretory/secretory egg antigens including those in SEA-4D12 seem to interact with different cells of the host immune system.

Detailed microscopy studies have in addition shown that the epitope of mAb 114-4D12 is present at the outer surface of the parasite at different moments in the life cycle. The egg-shell was recognised by mAb 114-4D12-A, next to the soluble egg antigens surrounding the egg and the glandular structures in the miracidium (Bogers *et al.*, 1994). In worms, mAb 114-4D12-A recognised the male testes and a canal-system, and the whole surface of the cercariae is stained. In view of the structural data obtained in the current study it is not surprising that 114-4D12 binds to the cercariae, since a subset of O-glycans from the glycocalyx of *S.*

mansoni has been shown to carry the terminal multifucosylated LDN epitope (Khoo *et al.*, 1995; Huang *et al.*, 2001). Clearly, also adult worms contain glycoconjugates with this epitope, but no structural data on adult worm oligosaccharides have been reported so far to substantiate this.

Most importantly, mAb 114-4D12-A has been used as a diagnostic mAb to detect schistosome antigens in the circulation (serum, urine) of the host in a sandwich ELISA (Nourel Din *et al.*, 1994b). Optimal performance of the ELISA was achieved when 114-4D12-A was combined with mAb 114-5B1-A, a mAb that stains similar but not identical patterns in IFAs (Bogers *et al.*, 1994; Nourel Din *et al.*, 1994a). It has been shown that 114-5B1-A binds preferentially to GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (LDN-DF) (van Remoortere *et al.*, 2000) and this epitope was also found to occur on the SEA-4D12 glycoprotein subset isolated in this study (Figure 5, fraction 9; Table I). The present findings provide a structural explanation for the earlier suggestion that mAb 114-4D12 and 114-5B1-A recognise similar but not identical populations of antigens in serum and urine (Nourel Din *et al.*, 1994a). It is also suggested by the Western blot patterns of *S. mansoni* extracts stained with mAb 114-4D12 (Figure 1) and mAb 114-5B1-A (Figure 1D in (Robijn *et al.*, 2005)) that some specifically stained glycoproteins contain both epitopes.

The diagnostic urine assay based on 114-4D12-A and 114-5B1-A is strongly correlated with the faecal egg output of *S. mansoni*-infected individuals (Nibbeling *et al.*, 1998b). In a murine model, antigens can only be detected after the onset of egg laying (Nourel Din *et al.*, 1994a) and adult worms do not secrete any antigens *in vitro* that could be detected by 114-4D12 (Thesis Bogers J.P.M., Immunohistochemical localisation studies in *Schistosoma mansoni* and *Schistosoma*-induced lesions. University of Leiden. 1996; 113-122). To investigate the clearance and degradation of circulating egg antigens, possibly in relation to diagnosis, assessment of morbidity and the effect of chemotherapy (Nibbeling, van Lieshout & Deelder, 1998), it would be of interest to determine what the exact chemical structure of antigens detected in urine is.

Finally, fucosylation is an important factor in the activity of various helminth-derived glycans so far shown to be immunologically active (Velupillai *et al.*, 2000; Van der Kleij *et al.*, 2002b; Thomas *et al.*, 2003; van Remoortere *et al.*, 2003). It remains to be investigated if the glycans detected in this study, which appear to form the most highly fucosylated glycans from schistosome eggs, play a role in the immunomodulatory properties attributed to SEA (Jacobs *et al.*, 1999; Okano *et al.*, 1999; Asahi *et al.*, 2003; Van de Vijver *et al.*, 2004).

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4

Schistosoma mansoni eggs excrete specific free oligosaccharides that are detectable in the urine of the human host.

MARJOLEIN L.M. ROBIJN, CAROLIEN A.M. KOELEMAN,
CORNELIS H. HOKKE AND ANDRÉ M. DEELDER
Department of Parasitology, Centre for Infectious Diseases, Leiden University Medical Centre,
P.O. Box 9600, 2300 RC Leiden, The Netherlands

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SUMMARY

In infections with *Schistosoma mansoni* the paired adult worms produce hundreds of eggs daily, of which many get trapped in various organs of the human host. The eggs produce complex and unique protein- and lipid-linked glycans, which are important activators and modulators of the host's immune response. The same parasite-derived glycoconjugates are also attractive immunodiagnostic targets in enzyme-linked immunosorbent assays (ELISAs), which detect circulating antigens in serum or urine of the host. Here, we report for the first time that in addition to glycoprotein and glycolipid antigens, schistosome eggs also excrete unique unconjugated oligosaccharides. Employing the schistosome-specific anti-carbohydrate monoclonal antibody 114-4D12 in an affinity purification approach, a specific set of free oligosaccharides was detected by matrix-assisted laser-desorption-ionisation time-of-flight mass spectrometry (MALDI-TOF MS) in human *S. mansoni* infection urine as well as in egg-incubation medium, but not in worm-culture medium.

Nano-scale reverse-phase liquid-chromatography mass spectrometry (nanoRP-LC-MS) analysis of the purified egg-derived oligosaccharides indicated that the captured compounds form a series of multi-fucosylated multimeric *N*-acetylhexosamine chains with a non-reducing terminal Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1- (DF-LDN-DF) sequence which forms the epitope of mAb 114-4D12. Since fucosylated (egg) glycoconjugates have been shown to harbour immunogenic properties, we anticipate that these unconjugated oligosaccharides also play a role in the immunobiology associated with schistosome eggs. Moreover, our data indicate that mass spectrometric detection of a set of signature molecules in urine has potential as a new approach for the diagnosis of schistosomiasis and possibly other helminth infections.

INTRODUCTION

Schistosomiasis mansoni is a chronic and debilitating disease, which still affects more than 200 million people mainly in the developing world. The eggs, of which several hundreds are produced daily by the adult worm-pairs, are generally the main cause of pathology. About half of the eggs migrate through the intestinal wall to be excreted with the faeces, but the other half are taken by the blood flow until they become lodged in mainly the liver, where they induce granulomatous inflammatory responses. In severe infections this may lead to fibrosis, portal hypertension and oesophageal varices, which may rupture and cause fatal bleedings (Jordan, Webbe & Sturrock, 1993).

In the last 10-15 years it has become clear that glycans play an important role at the schistosome-host interface. Schistosomes express numerous complex glycoproteins and glycolipids that contain unique glycan elements such as Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1-3Galα1- (DF-LDN-DF) (Robijn *et al.*, manuscript submitted) (Khoo *et al.*, 1995; Khoo *et al.*, 1997a; Khoo *et al.*, 1997b), $[(GlcA\beta1\text{-}3)GalNAc\beta1\text{-}6]_n$ (Bergwerff *et al.*, 1994) and Fucα1-3Galβ1-4(Fucα1-3)GlcNAc (pseudo-LeY) (Wuhrer *et al.*, 2000b). In addition, more common patterns also occurring in other helminths, plants or mammals, like core β1-2Xyl, core α1-3Fuc, GalNAcβ1-4GlcNAc (LDN), GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) and Galβ1-4(Fucα1-3)GlcNAc (Lewis X, Le^X) (Cummings & Nyame, 1999; Hokke *et al.*, 2001; Khoo & Dell, 2001) have been found in schistosomes. Several schistosome-derived glycans have been shown to possess immunomodulatory properties. Le^X and LDN-DF conjugates can induce the production of typical immune system mediators such as IL-10 by B-cells or peripheral blood mononuclear cells (PBMC) (Velupillai & Harn, 1994; Velupillai *et al.*, 2000; Van der Kleij *et al.*, 2002b), and Le^X conjugates can act as an innate Th2 promoter on dendritic cells via a Toll-like receptor (TLR)-4 dependent mechanism (Thomas *et al.*, 2003). Glycoprotein glycans from eggs are involved in the formation of hepatic granulomas (Weiss *et al.*, 1987; Van de Vijver *et al.*, 2004; Van de Vijver *et al.*, 2006). Furthermore, antibodies are produced against LDN, LDN-F, Fucα1-3GalNAcβ1-4GlcNAc (F-LDN), Fucα1-2Fucα1- (DF), mono- and multimeric Le^X and against core-substituted N-linked glycans in natural and experimental schistosomiasis (Nyame *et al.*, 1996; Nyame *et al.*, 1999; van Die *et al.*, 1999; Nyame *et al.*, 2000; Eberl *et al.*, 2001; Naus *et al.*, 2003; van Remoortere *et al.*, 2003; van Roon *et al.*, 2004).

Anti-carbohydrate monoclonal antibodies (mAbs) derived from schistosome-infected or immunised mice have been used for the immunodiagnosis of schistosome infections by sandwich ELISAs based on the detection of parasite-derived glycoconjugates present in the

blood or urine of infected individuals. In particular the detection of the adult worm gut-associated circulating anodic antigen (CAA) and circulating cathodic antigen (CCA) and of circulating soluble egg antigens (CSEA), have proven valuable in various epidemiological studies and routine settings (Hassan *et al.*, 1992; van Lieshout *et al.*, 1993; Nourel Din *et al.*, 1994b; Polman *et al.*, 1995; van Lieshout *et al.*, 1997; Polman *et al.*, 1998; Nibbeling *et al.*, 1998b).

A particular mAb, 114-4D12-A has been used in a diagnostic sandwich ELISA for CSEA that showed a good correlation with *S. mansoni* egg output (Nourel Din *et al.*, 1994b). The concentration of antigens detected by 114-4D12-A in CSEA is 40.000 times higher than in soluble adult worm antigens (Nourel Din *et al.*, 1994a). In the preceding elaborate mass spectrometry-based study we determined by analysis of *S. mansoni* egg glycoproteins that 114-4D12 binds to the terminal DF-LDN-DF sequence present on O-glycans of a glycoprotein subset of soluble egg antigens (SEA) (Robijn *et al.*, manuscript submitted).

Employing the same mAb 114-4D12 in an affinity purification approach, we now show that specific highly fucosylated unconjugated oligosaccharides with the same epitope are present in the urine of *S. mansoni*-infected subjects. Furthermore, we determined that these oligosaccharides are excreted by *S. mansoni* eggs, indicating that they are novel potential markers of infection and morbidity.

MATERIALS AND METHODS

S. mansoni urines and incubate medium

A pool of urine samples (20 ml) from Senegalese *S. mansoni*-infected individuals excreting over 1000 eggs per gram (epg) faeces (Stelma *et al.*, 1993), was centrifuged, filtered (0.45 µm) and buffered (17.5 mM sodiumphosphate, pH 7.6). Additionally, sodium chloride was added to 150 mM. A similarly treated urine pool (20 ml) from schistosomiasis-negative Senegalese people served as control (Polman *et al.*, 2000).

S. mansoni worms were collected by perfusion of golden hamsters (HsdHan-Aura) 7 weeks after infection with 1200 cercariae. Fifty worm-pairs were incubated in 4 ml complete RPMI medium (Gibco-Invitrogen, Breda, The Netherlands) containing 2.0 g/l sodiumhydrogencarbonate, 10 µg/ml Ciproxin antibiotics (Bayer, Mijdrecht, The Netherlands), 200 mM L-glutamine, 100 mM pyruvate and 10% heat-inactivated fetal calf serum (FCS). After 4 hours, the supernatant was harvested and stored at –20 ^{o}C. Adult worm

gut-derived antigens CAA and CCA levels in the supernatant were determined as described before (Deelder *et al.*, 1989; De Jonge *et al.*, 1990).

Eggs were isolated from hamster livers as previously described (Dresden & Payne, 1981). Eggs (50,000) were washed with sterile 0.85% sodium chloride solution, and incubated in 4 ml complete RPMI medium to which 1.7% sodium chloride was added to prevent the eggs from hatching (Xu & Dresden, 1990). After 4 hours, supernatant was collected by centrifugation, filtrated on a 1.2 µm filter (Sartorius, Nieuwegein, The Netherlands) and immediately frozen. Incubations were carried out in duplicate.

Monoclonal antibody and affinity procedure

The monoclonal antibody 114-4D12-AA (mouse IgG1, isotype-switch from mAb 114-4D12-A, IgG3) (Nourel Din *et al.*, 1994a) was coupled to ProtG Sepharose (Amersham Pharmacia Biotech AB, Uppsala, Sweden) according to the protocol described by Sisson and Castor (Sisson & Castor, 1990). In this study mAb 114-4D12-AA is designated as 114-4D12. For glycan affinity capturing, both urine pools were incubated with 200µl (packed) mAb-coupled ProtG Sepharose beads (12mg mAb/ml), and each egg incubate supernatant was incubated with 85µl (packed) beads (22mg mAb/ml). Before use, the immuno-affinity beads were washed with 5 packed-bead volumes (BV) phosphate buffered saline (PBS), 5 BV acidic eluent (formic acid, 0.04-2%, pH<3; Fluka, Buchs SG, Schweiz) and 5 BV PBS, respectively. Then, beads were added to the urine pools or egg-incubate supernatants and incubated O/N at 4°C under continuous mixing. Beads were spun down (50 x *g*) and washed with 3x10 BV of PBS. Bound components were eluted with 3x10 BV formic acid (0.04%-2%, pH<3; Fluka). The pH of the eluates was immediately neutralised with ammonium hydroxide solution (Fluka). Each eluate was lyophilised and an aliquot was analysed by MALDI-TOF MS.

MALDI-TOF MS

MALDI-TOF mass spectra were recorded on an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). As matrix, 6-aza-2-thiothymine (ATT) (Sigma-Aldrich, Zwijndrecht, The Netherlands) was used, 5 mg/ml in water. Spectra were acquired in the positive-ion mode.

Carbon purification and 2-aminobenzamide labelling

The affinity-captured oligosaccharides (eluates) and unbound oligosaccharides (flow-throughs) were desalted using a graphitised carbon cartridge as described previously (Wuhrer

et al., 2004b). Prior to carbon purification, the unbound fraction was treated with cold (4°C) acetonitrile (v/v 60%) to remove proteins by precipitation.

Purified oligosaccharides were fluorescent labelled with 2-aminobenzamide (2AB) as described previously (Wuhrer *et al.*, 2004b).

Preparative normal phase liquid chromatography (NP-LC) and reverse phase (RP)-LC-ESI-MS/MS

2AB-labelled oligosaccharides were fractionated by NP-LC as outlined (Wuhrer *et al.*, 2004b). Briefly, glycans were fractionated on a TSK-Amide 80 column (4 mm x 250 mm; Tosohaas, Montgomeryville, PA, U.S.A.) combined with an ÄKTA Purifier (Amersham Pharmacia Biotech, Uppsala, Sweden) HPLC system. Fluorescence was detected at 360 nm/425 nm (excitation/emission) using a YASCO fluorescence detector (FP-1520, Tokyo, Japan). Collected NP-fractions were desalted using C18 ZipTips (Millipore, Amsterdam, The Netherlands). After extensive washing with water, 2AB-labeled oligosaccharides were eluted with 30% acetonitrile directly onto a polished stainless steel MALDI target plate in 1 µl ATT and subjected to MALDI-TOF MS. Fragmentation spectra of the major compounds were obtained by nano-RP-LC-ESI-MS analysis as described (Wuhrer *et al.*, 2004b). Briefly, 2AB glycans were separated on a Pep-Map column (75 µm x 150 mm; Dionex/LC Packings, Amsterdam, the Netherlands) using an Ultimate nano-LC system (LC Packings) and analysed on-line with an Esquire HCT ion-trap mass spectrometer equipped with a nano-electrospray ion-source (Bruker). Spectra were recorded in the positive-ion mode. Since major fucose rearrangements have been observed in the fragmentation spectra of protonated precursor ions of fucosylated glycans (Harvey *et al.*, 2002; Wuhrer *et al.*, 2006e). After sample injection the column was run isocratically with eluent A [0.8 mM sodium hydroxide, 0.1% formic acid in water/acetonitrile, 19:1 (v/v)] for 5 min, followed by a linear gradient to 30% eluent B [0.1% formic acid in water/acetonitrile, 1:19 (v/v)] in 15 min and a final wash with 100% solvent B for 5 min.

RESULTS

Urine of S. mansoni-*infected individuals contains free oligosaccharides bound by mAb 114-4D12*

A pool of urine of *S. mansoni*-infected individuals was incubated with Sepharose beads coupled with mAb 114-4D12, an antibody that binds to the terminal motif Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1- (Robijn *et al.*, manuscript submitted). From the

114-4D12-affinity purified fraction of infection urine (SmUrine-4D12), MALDI-TOF MS generated a peak-pattern (Figure 1A) in the mass region between *m/z* 1300 and 3300, with the major peak at *m/z* 2443.9. No peaks were observed in the mass spectrum of the eluate of mAb 114-4D12-coupled beads incubated with the urine pool of endemic schistosome-negative individuals (NegUrine-4D12) (Figure 1B). Putative compositions based on the observed ions indicated that a series of reducing oligosaccharides had been captured from the *S. mansoni* infection urine. The overall compositions suggested the presence of oligosaccharides consisting of one hexose (Hex, H) and variable numbers (3-7) of *N*-acetylhexosamines (HexNAc, N) and (4-10) deoxyhexoses (deoxyHex, F), as indicated in figure 1A. In view of the preceding study (Robijn *et al.*, manuscript submitted) in which we deduced that mAb 114-4D12 is specific for terminal DF-LDN-DF and previous structural studies on schistosome oligosaccharides by other groups (as reviewed by (Hokke *et al.*, 2001)), we assume that all deoxyHex residues found here are fucoses (Fuc, F).

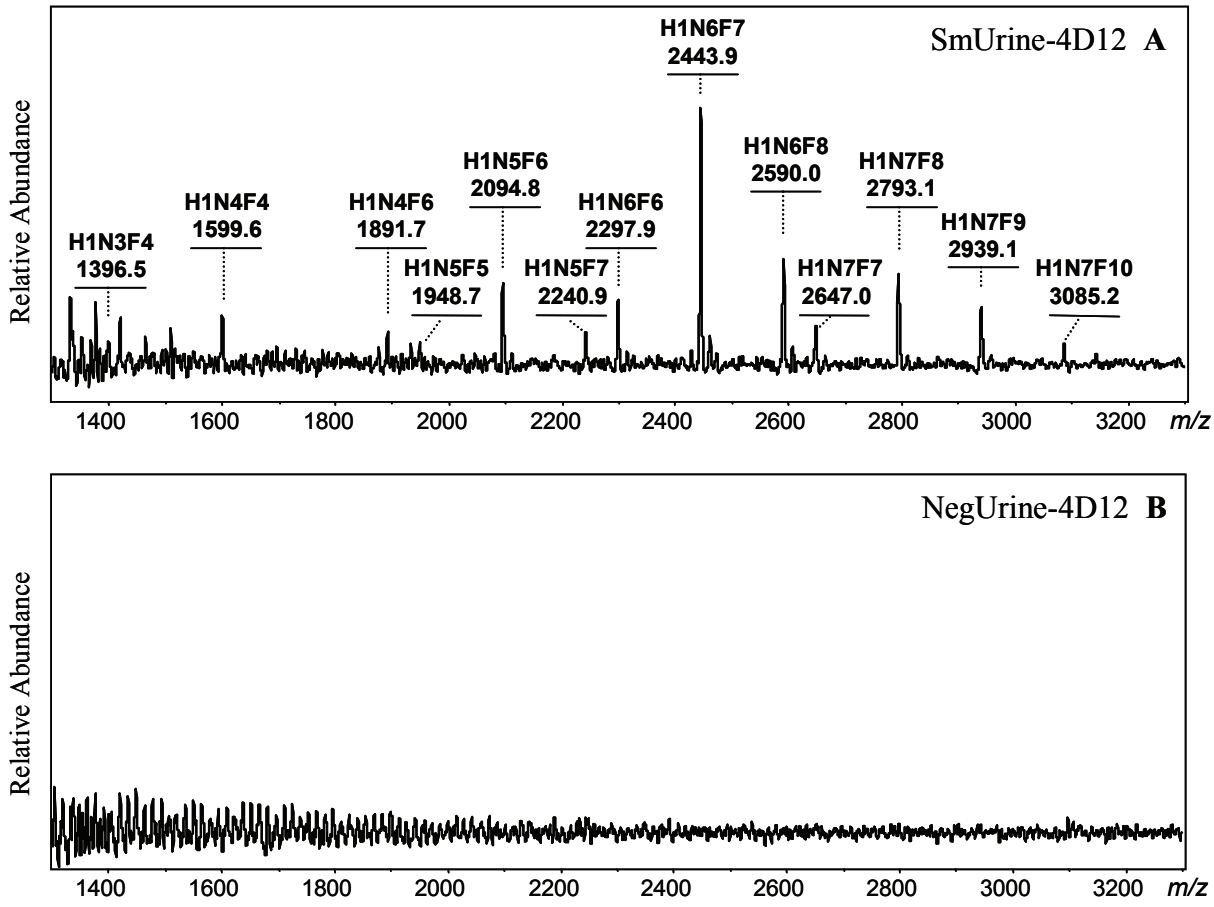


Figure 1. MALDI-TOF MS-spectra of the mAb 114-4D12 affinity purified free oligosaccharides from (*A*) S. mansoni positive (SmUrine) and (*B*) negative (NegUrine) urine pools. Mono-isotopic masses of the sodiated ions are indicated together with the putative compositions. H stands for Hex, N for HexNAc and F for Fuc.

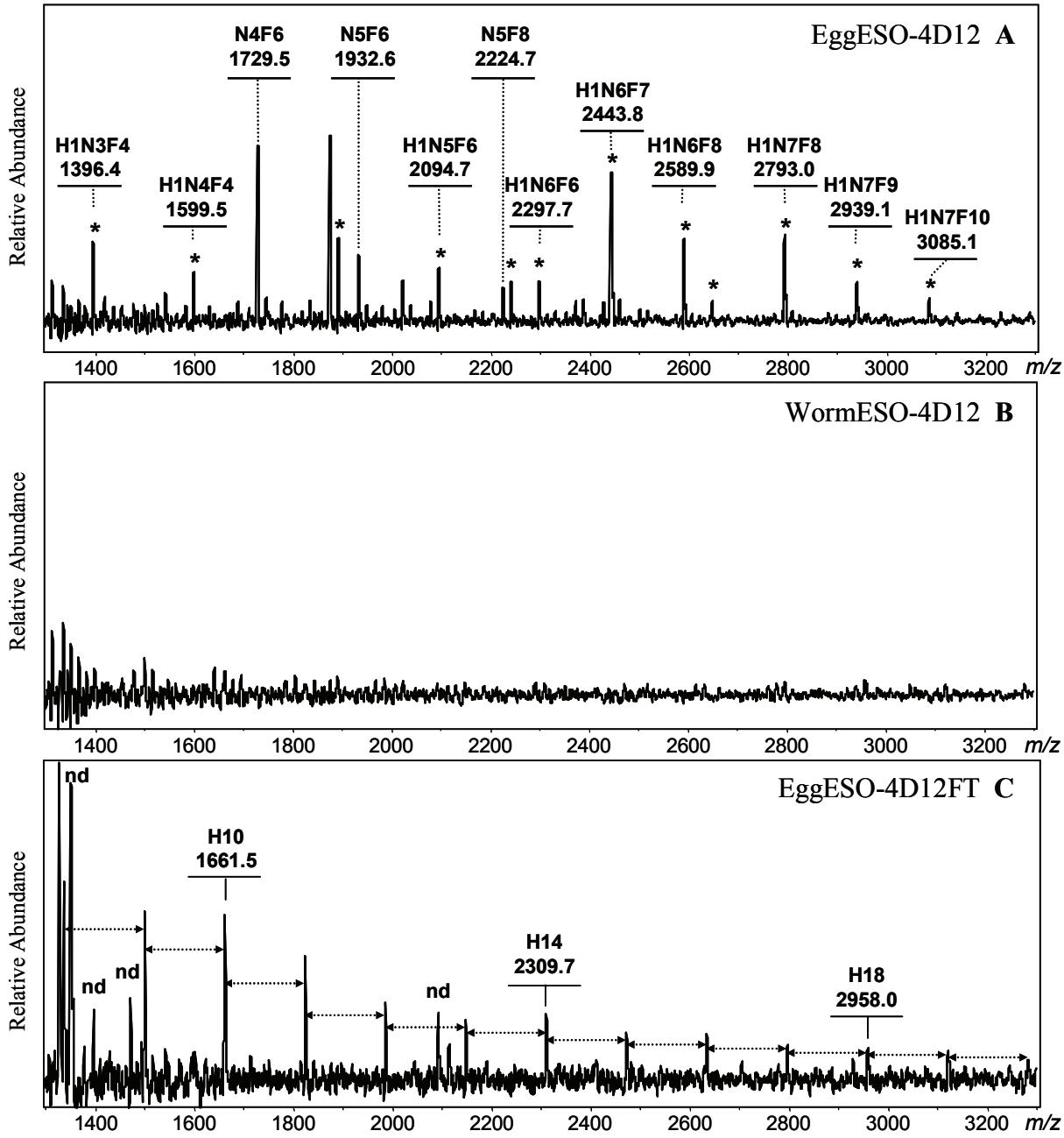


Figure 2. MALDI-TOF MS-spectra of egg- or worm- excreted free oligosaccharides. Incubation medium of *S. mansoni* eggs or worms was collected. MALDI-TOF MS analysis was performed on the 114-4D12 affinity purified fraction of (*A*) egg excreted/secreted oligosaccharides (ESO) (eggESO-4D12), (*B*) worm ESO (wormESO-4D12) and (*C*) the 114-4D12 unbound eggESO fraction (eggESO-4D12FT, flow-through). Mono-isotopic masses of the sodiated ions are indicated together with the putative compositions. No specific peaks were observed in the mass spectrum of wormESO-4D12. The unbound eggESO glycan fraction mainly contained hexose polymer fragments up to H20. H stands for Hex, N for HexNAc and F for Fuc. N.d. is not determined. Components in eggESO-4D12 that were also detected in SmUrine-4D12 are indicated with a * (see also Figure 1 and Table I). Double-headed arrows indicate a difference equivalent to one hexose.

Free oligosaccharides are secreted by S. mansoni *eggs*

The finding of oligosaccharides in schistosomiasis urine that can be captured with a schistosome-specific mAb raised the question whether these oligosaccharides are secreted as such by the schistosome or not. To this end, we incubated live *S. mansoni* worms and eggs in RPMI and subjected the resultant supernatants to 114-4D12-affinity purification. The MALDI-TOF mass spectrum of the mAb 114-4D12 eluate of *S. mansoni* egg excreted/secreted oligosaccharides (eggESO-4D12) contained the same peak-pattern as SmUrine-4D12, considering the observed *m/z* values as well as the relative peak intensities (Figure 2A, peaks assigned with *). In addition, in the mass spectrum of eggESO-4D12 a second series of peaks was observed that corresponds to oligosaccharides with compositions of $N_{3\text{-}6}F_{4\text{-}9}$ (Figure 2A).

In the 114-4D12 eluate of the worm-incubate supernatant (wormESO-4D12), no specific peaks were observed by MALDI-TOF MS. As a reference for the metabolic activity of the worms, the CAA and CCA production was determined. The incubated worms secreted on average 0.7 ng CAA and 1.2 ng CCA per worm per hour, similar to earlier measurements (van Dam *et al.*, 1996), indicating that the worms were metabolically active during the incubations.To investigate whether the egg-incubate contained oligosaccharides that were not bound by mAb 114-4D12, the flow-through fraction of the eggESO was purified on a carbon cartridge and examined by MALDI-TOF MS. The spectrum indicated the presence of mainly hexose polymer fragments up to H_{20} (Figure 2C).

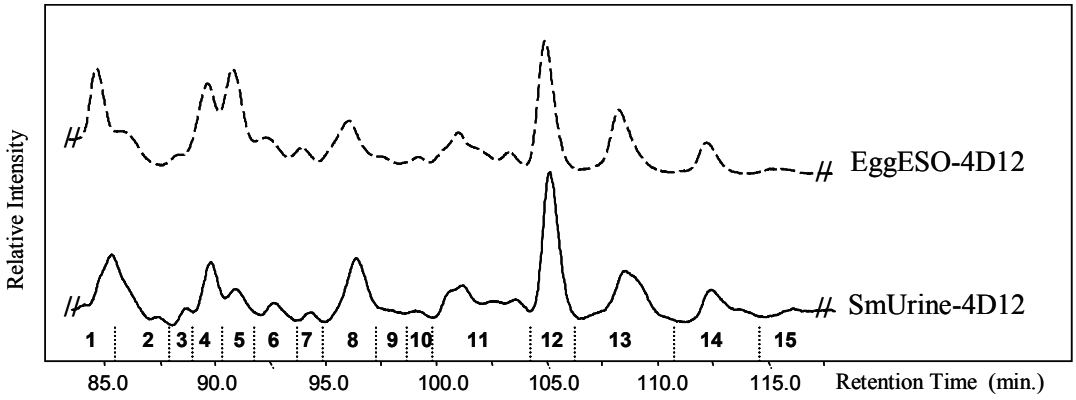


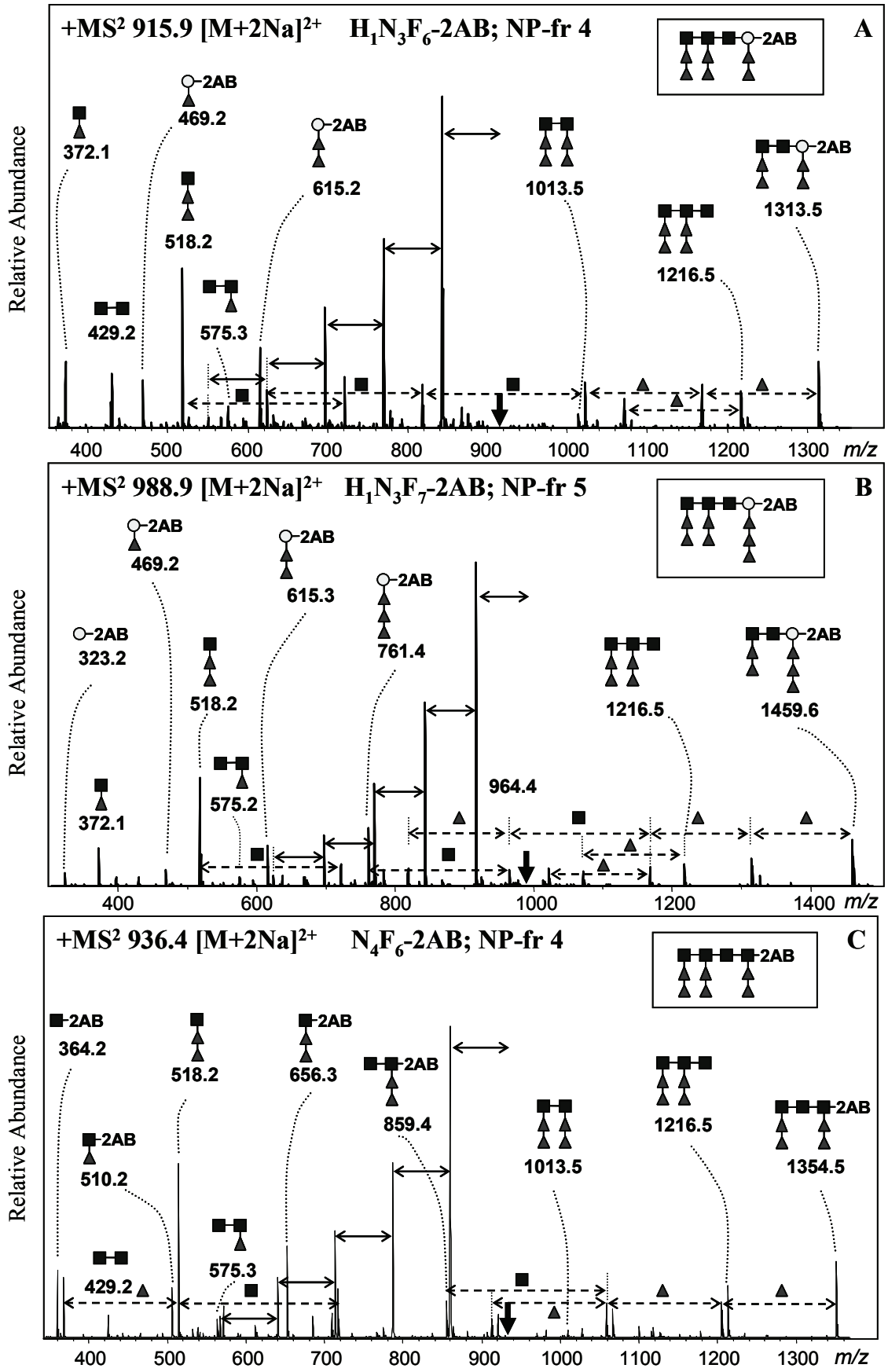
Figure 3. NP-LC chromatograms of 114-4D12 affinity purified oligosaccharides after 2AB-labelling. The (*A*) eggESO-4D12 and (*B*) SmUrine-4D12 glycans were 2AB-labelled and preparatively fractionated by NP-LC. A selected region of the NP-chromatogram is shown, in which the specific, fucosylated glycans were detected. The chromatograms were normalised on the major peak for easy comparison. Collected fractions were numbered as indicated. See Table I for further information on the contents of these fractions.

Table I. Overview of 114-4D12 affinity-purified free oligosaccharides from *S. mansoni* infection urine and/or eggs. All oligosaccharides detected in eggESO-4D12 and SmUrine-4D12 (see Figures 2A and 1A, respectively) are listed and arranged according to the Hex and HexNAc content of the putative compositions based on the m/z [M+Na]+ value observed by MALDI-TOF MS. Mono-isotopic masses are indicated. Only those compounds were included that were found in both of the duplicate experiments, showed clear isotope patterns and occurred as part of a larger series. Fragmentation analysis by nano-RP-LC-ESI-MS/MS was performed on the major NP-fractions of the purified and 2AB-labelled eggESO-4D12 (see also Figure 3 and 4).

Composition [A]	eggESO-4D12 relative amount	SmUrine-4D12 relative amount	MALDI-TOF MS *m/z* obs. (*m/z* calc.) $[M+Na]^+$		MALDI-TOF MS *m/z* obs. (*m/z* calc.) $[(M+2AB)+Na]^+$		LC-MS *m/z* obs. (*m/z* calc.) $[(M+2AB)+2Na]^{2+}$		LC-MS *m/z* obs. (*m/z* calc.) $[(M+2AB)+3Na]^{3+}$		NP-fraction [B]	Proposed sequence [A C]
H1N3F4	+++	+	1396.4	(1396.5)	1516.9	(1516.5)	769.8	(769.3)			-	FFN-(FF)N-N-H-A
H1N3F5	++	-	1542.5	(1542.6)	1662.5	(1662.6)	-	(842.3)			-	
H1N3F6	++	-	1688.6	(1688.6)	1808.5	(1808.6)	915.9	(915.3)			**4**, (1)	FFN-(FF)N-N-(FF)H-A
H1N3F7	++	-	1834.6	(1834.7)	1954.7	(1954.7)	988.9	(988.3)			**5**	FFN-(FF)N-N-(FFF)H-A
H1N3F8	+	-	1980.7	(1980.8)	2100.5	(2100.8)	-	(1061.4)			6	
H1N4F3	+	-	1453.5	(1453.5)	1573.4	(1573.5)	-	(797.8)			1	
H1N4F4	++	++	1599.5	(1599.6)	1719.5	(1719.6)	871.3	(870.8)			**1**	FFN-(FF)N-N-N-H-A
H1N4F5	++	+	1745.6	(1745.7)	1865.5	(1865.7)	-	(943.8)			6, (3)	
H1N4F6	+++	+	1891.6	(1891.7)	2011.5	(2011.7)	1017.4	(1016.9)			**5**, 6, 7	FFN-(FF)N-[N2F2]-H-A
H1N4F7	+	-	2037.6	(2037.8)	2157.6	(2157.8)	-	(1089.9)			6, 7	
H1N4F8	+	-	2183.7	(2183.8)	2303.7	(2303.8)	-	(1162.9)			15, (11)	
H1N5F4	+	-	1802.6	(1802.7)	1922.6	(1922.7)	-	(972.3)			6	
H1N5F5	+	+	1948.7	(1948.7)	2068.6	(2068.7)	-	(1045.4)			6, 7	
H1N5F6	++	++	2094.7	(2094.8)	2214.6	(2214.8)	-	(1118.4)			10, (6, 7)	
H1N5F7	++	+	2240.8	(2240.9)	2360.5	(2360.9)	-	(1191.4)			11	
H1N5F8	+	-	2386.8	(2386.9)	2506.6	(2506.9)	-	(1264.5)			11	
H1N5F9	+	-	2532.9	(2533.0)	2652.7	(2653.0)	-	(1337.5)			11	
H1N6F4	+	-	2005.7	(2005.8)	2125.5	(2125.8)	-	(1073.9)			9	
H1N6F5	+/-	-	2151.7	(2151.8)	-	(2271.8)	-	(1146.9)			-	
H1N6F6	++	++	2297.8	(2297.9)	2417.6	(2417.9)	-	(1219.9)			11	
H1N6F7	+++	+++	2443.9	(2443.9)	2563.7	(2563.9)	-	(1293.0)	869.7	(869.9)	**12**	FFN-(FF)N-[N3F3]-N-H-A
H1N6F8	++	++	2589.0	(2590.0)	2709.8	(2710.0)	-	(1366.0)	918.3	(918.7)	**12**, (13)	FFN-(FF)N-[N3F4]-N-H-A
H1N6F9	+/-	-	2736.0	(2736.1)	-	(2856.1)	-	(1439.0)			-	

H1N7F7	+	+	2647.0	(2647.0)	2766.8	(2767.0)	-	(1394.5)	13	
H1N7F8	++	++	2793.1	(2793.1)	2912.8	(2913.1)	-	(1467.5)	13	
H1N7F9	++	+	2939.2	(2939.1)	2058.9	(3059.1)	-	(1540.6)	(13)	
H1N7F10	+	+	3085.3	(3085.2)	-	(3205.2)	-	(1613.6)	-	
N4F4	+	-	1437.4	(1437.5)	-	(1557.5)	-	(789.8)	-	
N4F5	++	-	1583.5	(1583.6)	1703.5	(1703.6)	-	(862.8)	2	
N4F6	+++	+	1729.6	(1729.7)	1849.4	(1849.7)	936.4	(935.8)	**4**, (5)	FFN-(FF)N-N-(FF)N-A
N4F7	+++	+	1875.6	(1875.7)	1995.3	(1995.7)	-	(1008.9)	6, (5)	
N4F8	++	-	2021.7	(2021.8)	2141.6	(2141.8)	-	(1081.9)	7	
N4F9	+	-	2167.7	(2167.8)	-	(2287.8)	-	(1154.9)	-	
N4F10	+	-	2313.8	(2313.9)	-	(2433.9)	-	(1227.9)	-	
N5F4	+/-	-	1640.7	(1640.6)	-	(1760.6)	-	(891.3)	-	
N5F5	+	-	1786.6	(1786.7)	-	(1906.7)	-	(964.3)	-	
N5F6	+++	+	1932.7	(1932.7)	2052.5	(2052.7)	1037.8	(1037.4)	**4**	FFN-(FF)N-(FF)N-N-N-A
							1037.9	(1037.4)	**5**	FFN-(FF)N-N-(FF)N-N-A
									2	
N5F7	++	-	2078.7	(2078.8)	2198.6	(2198.8)	-	(1110.4)	6	
N5F8	++	-	2224.8	(2224.9)	2344.6	(2344.9)	-	(1183.4)	11, 9, (7)	
N5F9	+	-	2370.9	(2370.9)	2490.6	(2490.9)	-	(1256.5)	11, (15)	
N5F10	+	-	2517.0	(2517.0)	-	(2637.0)	-	(1329.5)	-	
N6F7	+/-	-	2281.8	(2281.9)	2401.7	(2401.9)	-	(1211.9)	9	
N6F8	+	-	2427.8	(2427.9)	2547.7	(2547.9)	-	(1285.0)	9, 11	
N6F9	+/-	-	2573.9	(2574.0)	-	(2694.0)	-	(1358.0)	-	

[A] H stands for Hex, N for HexNAc, F for Fuc and A for 2AB. [B] For each composition, the NP-fraction(s) are given in which the corresponding 2AB-labelled glycan was detected by MALDI-TOF MS as a single sodiated ion [(M+2AB)+Na]+. When isomeric structures were detected in multiple NP-fractions in a significantly different ratio, the fraction in which a minority was found is indicated between parentheses. The NP-fractions containing an oligosaccharide of which an MS-MS fragmentation spectrum has been recorded resulting in the proposed sequence presented in this table are indicated in bold. [C] The proposed sequences are based on fragmentation analysis by nano-RP-LC-ESI-MS/MS and are schematically depicted. When the exact fucose positions could not be determined the composition of this part of the structure is shown between brackets.


+MS² 915.9 [M+2Na]²⁺ H1N3F6-2AB; NP-fr 4
A
2AB
372.1
469.2
518.2
615.2
429.2
575.3
1013.5
1216.5
1313.5
Relative Abundance
m/z
+MS² 988.9 [M+2Na]²⁺ H1N3F7-2AB; NP-fr 5
B
323.2
615.3
761.4
964.4
1459.6
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C
364.2
656.3
859.4
510.2
1354.5

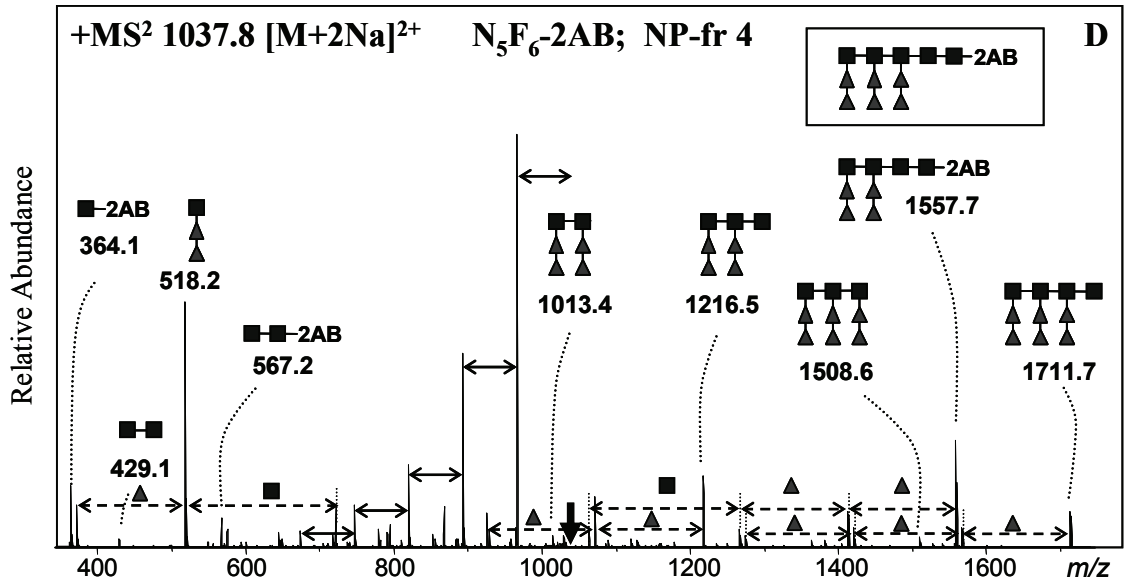

Figure 4. RP-LC-ESI-MS/MS spectra of 114-4D12 affinity purified oligosaccharides after 2AB-labelling. The NP-LC fractions (see Figure 3) containing the 2AB-labelled eggESO-4D12 and SmUrine-4D12 glycans were subjected to RP-LC-ESI-MS. Representative MS/MS spectra of double-sodiated parent ions are shown. Mono-isotopic masses are indicated together with the symbolic interpretation of the indicative fragment ions. Symbols: square, unspecified HexNAc; circle, unspecified Hex; triangle, Fuc. Double-headed arrows with dashed line and symbols indicate monosaccharide differences. Double-headed arrows with solid line indicate Fuc losses of double charged ions. The closed arrow head indicates the parent ion. For further discussion of the spectra, see text.

Structural characterisation of the free oligosaccharides

After carbon purification and 2AB-labelling, eggESO-4D12 and SmUrine-4D12 were preparatively sub-fractionated by NP-LC (Figure 3). The NP-chromatograms of the two eluates were highly similar, in parallel to the corresponding MALDI-TOF MS patterns (see Figure 1A and 2A). Some of the major fractions, eggESO NP-fraction 1, 4, 5 and 12, were subjected to nano-RP-LC-ESI-MS to generate MS/MS spectra of individual glycans for structural characterisation. These MS/MS spectra (Figure 4) confirmed the putative compositions given in Figure 2A and examples are discussed in detail below. The remaining fractions were subjected to MALDI-TOF MS analysis. Most *m/z* species initially detected by MALDI-TOF MS immediately after the capturing procedure (see figure 2A) appeared as 2AB-derivative in one or more of the NP-fractions. Table I shows an overview of all oligosaccharides detected in eggESO and SmUrine. Oligosaccharides with the same putative compositions were found in multiple NP-fractions, indicating the occurrence of isomeric

structural variants. Furthermore, most NP-fractions contained multiple glycans that were separated in the second dimension by nano-RP-LC-ESI-MS.

To illustrate the different structural characteristics and substitution patterns of eggESO-4D12 glycans, MS/MS spectra of three different oligosaccharides detected in NP-fraction 4, namely, $H_1N_3F_6$-2AB (*m/z* 915.9, $[M+2Na]^{2+}$), N_4F_6-2AB (*m/z* 936.4, $[M+2Na]^{2+}$) and N_5F_6-2AB (*m/z* 1037.8, $[M+2Na]^{2+}$) are shown in Figure 4 (A, C, D, respectively; see also Table I). In the MS/MS spectrum of $H_1N_3F_6$-2AB (Figure 4A), the largest (single sodiated) fragment ion observed ($H_1N_2F_4$-2AB, *m/z* 1313.5) results from the initial loss of the terminal N_1F_2 moiety, which is also detected as a conserved fragment (*m/z* 518.2). From *m/z* 1313.5 two repetitive losses of fucoses are observed, as indicated by red intersected lines with solid arrows, prior to the loss of two HexNAc residues, indicated by blue, irregularly intersected lines with solid arrows, resulting in the core H_1F_2-2AB fragment (*m/z* 615.2). Together with the indicative ions at *m/z* 1013.5 (N_2F_4) and *m/z* 1216.5 (N_3F_4) representing the non-reducing end motif, this suggests that the sequence of $H_1N_3F_6$-2AB is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-HexNAc(Fuc-Fuc-)Hex-2AB.

Similar indicative fragment ions were observed for N_4F_6-2AB (*m/z* 936.4, $[M+2Na]^{2+}$; Figure 4C), the only difference being that the internal 2AB-linked monosaccharide was HexNAc. The initial loss of the terminal N_1F_2 moiety (*m/z* 518.2) results in the fragment ion N_3F_4-2AB (*m/z* 1354.5). Here, the initial loss of two fucoses prior to a HexNAc can also be deduced from the double sodiated fragment ion-pattern (indicated by open arrows). Then, from *m/z* 1354.5 three repetitive losses of fucoses are observed. Alternatively, after the loss of two fucoses a HexNAc residue was lost, resulting in N_2F_2-2AB (*m/z* 859.4). The remaining fucoses were substituted to the internal HexNAc, as indicated by the N_1F_2-2AB fragment (*m/z* 656.3). Altogether this indicates that the sequence of N_4F_6-2AB is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-HexNAc(Fuc-Fuc-)HexNAc-2AB

The fragmentation spectrum of the third component in NP-fraction 4, N_5F_6-2AB (*m/z* 1037.8, $[M+2Na]^{2+}$; Figure 4D), also shows the initial loss of the terminal N_1F_2 moiety (*m/z* 518.2), which resulted in a major single sodiated fragment ion at *m/z* 1557.7. From there, again two fucoses were lost prior to a HexNAc residue. However in N_5F_6-2AB, in contrast to the previously described glycans, the six fucoses were arranged in three pairs linked to three adjacent HexNAc residues, which resulted in the conserved fragment ion N_3F_6 (*m/z* 1508.6). This indicates the following composition Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc(Fuc-Fuc-)HexNAc-HexNAc-HexNAc-2AB.

In the consecutive NP-fraction 5, an isobaric compound with the same composition N_5F_6-2AB was detected (see Table I, spectrum not shown), which differed from the above described glycan structure with respect to the fucose substitutions. Just like for N_5F_6-2AB from NP-fraction 4 (Figure 4D), the single sodiated terminal fragments *m/z* 518.2 (N_1F_2), *m/z* 1013.4 (N_2F_4) and *m/z* 1216.5 (N_3F_4) were present, but in this case, *m/z* 1508.6 (N_3F_6) was absent. Instead, an intense single sodiated fragment ion at *m/z* 859.4 was observed, representing N_2F_2-2AB. The subsequent loss of two fucoses resulting in N_2F_1-2AB (*m/z* 713.3) and N_2-2AB (*m/z* 567.3) respectively, together with the absence of a fragment ion at *m/z* 656.3 (N_1F_2-2AB), indicates the position of the third fucose-pair on the sub-internal HexNAc. Together these fragmentations indicate that the sequence of N_5F_6-2AB from NP-fraction 5 is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-HexNAc(Fuc-Fuc-)HexNAc-HexNAc-2AB.

Apart from N_5F_6-2AB, two other major glycans were detected in NP-fraction 5: $H_1N_4F_6$-2AB (*m/z* 1017.4 $[M+2Na]^{2+}$) and $H_1N_3F_7$-2AB (*m/z* 988.9, $[M+2Na]^{2+}$). The MS/MS spectrum of the latter structure is shown in Figure 4B, as an example of the highly unusual hexose-fucosylation that was found in the eggESO-4D12 oligosaccharides. After the initial loss of the terminal N_1F_2 motif resulting in the single sodiated molecular ion-species at *m/z* 1459.6 ($H_1N_2F_5$-2AB), the subsequent loss of two fucoses prior to a HexNAc leaves a fragment ion at *m/z* 964.4 ($H_1N_1F_3$-2AB). Surprisingly, from this fragment ion a HexNAc residue is lost resulting in H_1F_3-2AB (*m/z* 761.4). Then, three fucoses are lost as indicated by the molecular ions at *m/z* 615.3 (H_1F_2-2AB), *m/z* 469.2 (H_1F_1-2AB) and finally *m/z* 323.1 (H-2AB). Therefore, the proposed structure of $H_1N_3F_7$-2AB is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-HexNAc(Fuc-Fuc-Fuc-)Hex-2AB.

The larger glycans, for example $H_1N_6F_7$-2AB present in NP-fraction 12, were detected by RP-LC-ESI-MS as triple sodiated glycans. Their MS/MS fragmentation spectra therefore show mainly triple and double sodiated fragment ions, which represent less indicative fragmentations than the single sodiated fragment ions, from which the complete sequences of the smaller glycans could be deduced. For $H_1N_6F_7$-2AB (*m/z* 869.7, $[M+3Na]^{3+}$; see Table 1, spectrum not shown) the double sodiated peak-pattern indicates the loss of a terminal N_1F_2 motif, which is also present as a single sodiated conserved fragment (*m/z* 518.2). In addition fragmentation is observed between the H-2AB core (*m/z* 323.1) and the fucosylated HexNAc-chain (N_6F_7, *m/z* 1143.4). The absence of H_1F_1-2AB (*m/z* 469.2) indicates that the internal 2AB-linked hexose is not fucosylated, and most likely the sub-internal HexNAc also remained unfucosylated since a relatively intense signal is observed for H_1N_1-2AB (*m/z* 526.2), but $H_1N_1F_1$-2AB (*m/z* 672.3) was absent. Unfortunately, not enough material was

available for MS^3 experiments to further deduce the location of the fucose residues. Because the terminal DF-LDN-DF must be present due to binding to mAb 114-4D12, the proposed sequence of $H_1N_6F_7$-2AB is Fuc-Fuc-HexNAc(Fuc-Fuc-)HexNAc-$(HexNAc)_3(Fuc)_3$-HexNAc-Hex-2AB.

DISCUSSION

Employing an affinity purification method based on the anti-schistosomal mAb 114-4D12 specific for the terminal DF-LDN-DF element (Robijn *et al.*, manuscript submitted), we detected free parasite-derived oligosaccharides in a urine pool of *S. mansoni*-infected individuals. The same subset of unique oligosaccharides was found to be excreted by live *S. mansoni* eggs. These findings suggest that the urinary oligosaccharides are derived from eggs present in the infected human host. Interestingly, incubated worms did not release these free oligosaccharides.

Although a glycogen-like hexose polymer has previously been found in a soluble egg antigen (SEA) preparation (Khoo *et al.*, 1997b), to our knowledge it has never been reported that live schistosome eggs excrete specific fucosylated oligosaccharides such as presented here (see Fig 2A, Table I). As expected, all oligosaccharides found in the affinity purified fractions contained the terminal DF-LDN-DF motif. Since the hexose polymer was a major constituent of egg excretory/secretory (ES) oligosaccharides, this may have obscured other oligosaccharides in the MALDI-TOF-MS spectrum of the 114-4D12 flow-through fraction (see Fig 2C). It can therefore not be excluded that also other free glycans than those found here are present in the egg ES fraction.

The oligosaccharides determined here contained multiple fucoses (F_{4-10}) on a linear HexNAc-backbone chain (N_{3-7}) with either a hexose or a HexNAc at the reducing end (see Table I). Previously, a number of studies have addressed the structure of lipid- and protein-linked glycans from schistosome eggs, mainly in specific glycolipid extracts (Khoo *et al.*, 1997a; Wuhrer *et al.*, 2002) or SEA preparations (Khoo *et al.*, 1997b; Huang *et al.*, 2001; Robijn *et al.*, manuscript submitted). The fact that the terminal DF-LDN-DF motif is present also on schistosome egg glycolipids and on N- and O-glycans (Khoo *et al.*, 1997a; Khoo *et al.*, 1997b) raises the question whether the free oligosaccharides are a product of an endogenous enzymatic activity that cleaves the glycans from a protein or lipid backbone.

Clearly, the MALDI-TOF MS profile of SmUrine-4D12 oligosaccharides (Fig 1A) is almost identical to that of the previously studied glycans released from *S. mansoni* egg glycosphingolipids, which were also based on an $H_1N_mF_n$ overall composition with $H_1N_6F_7$ as

the major species (Wuhrer *et al.*, 2002). Correspondingly, Khoo *et al.* (Khoo *et al.*, 1997a) detected *S. mansoni* glycosphingolipid-derived glycans with compositions $H_1N_4F_4$, $H_1N_4F_6$, $H_1N_5F_{6/7}$ and $H_1N_6F_7$, which seem to be some of the major free oligosaccharides detected here (Table I). In addition, the MALDI-TOF MS spectra of DF-LDN-DF containing O-glycans released from egg glycoproteins (Robijn *et al.*, manuscript submitted) show a completely different picture, which makes it highly likely that the egg-excreted oligosaccharides are glycolipid-derived. It could be hypothesised that the HexNAc-Hex element that the urinary oligosaccharides contain is in fact -3GalNAc(β1-4)Glc, the so-called glycolipid derived schisto-core (Makaaru *et al.*, 1992). Unfortunately, due to limitations in the amount of material available we have not been able to confirm the putative identity of the terminal reducing hexose.

Compared to the previously described glycolipid-glycans (Khoo *et al.*, 1997a; Wuhrer *et al.*, 2002) we find an interesting novel element: up to 3 Fuc linked to the reducing Hex. This element has not been detected in the ceramidase-released glycolipid glycans from *S. mansoni* eggs (Wuhrer *et al.*, 2002), nor in any other schistosome or non schistosome-derived material. It might be that the ceramidase used *in vitro* to cleave off glycans from schistosome egg glycosphingolipids (Wuhrer *et al.*, 2002) does not act on fucosylated Glc-Cer, whereas a putative endogenous ceramidase responsible for cleavage of Glc-Cer in schisto-eggs *in vivo* would.

The series of oligosaccharides terminating with HexNAc instead of Hex was clearly present in eggESO-4D12 but could hardly be detected in SmUrine-4D12. This HexNAc-series possibly represents free oligosaccharides that originally contained an unsubstituted Hex at the reducing terminus that were degraded by an endo-hexosaminidase apparently present in the egg culture. Alternatively, the material found in the synchronised egg incubate is not entirely representative for the material accumulated in urine, which is derived from eggs that may vary in age from freshly laid to dead, when trapped in the tissues of the host.

The possible biological and immunological effects of these highly fucosylated free oligosaccharides remain to be determined. The free oligosaccharides may serve as nutrients for the miracidium living inside the egg, identical to the unspecific glucose polymer, which apparently is synthesised in relatively large amounts at this stage of development. Alternatively, these free oligosaccharides may have a role in the strategies applied by the schistosome to escape or suppress the host immune system. The schistosome eggs strongly polarise the host immune response towards Th2. Egg antigens lose this Th2-polarising ability

when they are chemically deglycosylated or periodate-treated (Okano *et al.*, 1999). Furthermore, neoglycoconjugates containing different fucosylated glycans, such as lacto-*N*-fucopentaose (LNFPIII; a pentasaccharide terminating in Le^X) and LDN-DF, have been shown to stimulate host immune cells (Okano *et al.*, 2001; Van der Kleij *et al.*, 2002b; Thomas *et al.*, 2003).

Intriguingly, free unconjugated LNFPIII inhibited SEA-induced IL-10 production by peripheral blood mononuclear cells (PBMCs) when the PBMCs were pre-treated with LNFPIII prior to stimulation with SEA (Velupillai *et al.*, 2000). An interesting hypothesis would be that the free fuco-oligosaccharides found here similarly contribute to the inhibition of immunostimulatory effects of egg-derived multivalent glycoconjugates such as fucosylated glycoproteins. Immunological functions have also been ascribed to the free fucosylated oligosaccharides found in human breast milk and seminal plasma (Chalabi *et al.*, 2002; Eiwegger *et al.*, 2004; Morrow *et al.*, 2005; Deters, Lengsfeld & Hensel, 2005). In addition, free (fucosylated) oligosaccharides may protect the egg/miracidium against host immune attack through binding and neutralisation of harmful antibodies. Antibodies to the characteristic Fucα1-2Fucα1-3 element are abundantly produced in the mammalian host (Naus *et al.*, 2003; van Remoortere *et al.*, 2003).

To conclude, our data show that *S. mansoni* eggs excrete free oligosaccharides similar to those that appear in the urine of infected humans. Our findings indicate that mass-spectrometric profiling of free oligosaccharides excreted by schistosomes has potential as a new approach for monitoring schistosome infections. Since the eggs are causing the main pathology in schistosomiasis, these results may have important implications for the use of schistosome glycans as morbidity markers. Future studies should address the quantitative relationship between urinary oligosaccharides and the individual egg-load.

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5

Mass spectrometric detection of urinary oligosaccharides as markers of *Schistosoma mansoni* infection.

MARJOLEIN L.M. ROBIJN, JOOST PLANKEN, DIEUWKE KORNELIS,
CORNELIS H. HOKKE AND ANDRÉ M. DEELDER
Department of Parasitology, Centre for Infectious Diseases, Leiden University Medical Centre,
P.O. Box 9600, 2300 RC Leiden, The Netherlands

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SUMMARY

Current diagnosis of schistosomiasis is still not ideal. In the present study we have evaluated a targeted affinity approach using monoclonal antibody 114-4D12, reactive with a unique *Schistosoma mansoni*-specific glycan epitope, combined with matrix-assisted laser-desorption-ionization time-of-flight (MALDI-TOF) mass spectrometry. For nine out of eleven urines (1ml) of Senegalese individuals with different intensities of infection, a characteristic MALDI-TOF mass spectrum was observed which represents a series of fuco-oligosaccharides that are produced by schistosome eggs. The identification of these small molecule markers may lead to a new egg load-related assay for light infections in schistosomiasis.

INTRODUCTION

Schistosomiasis is a major human parasitic disease, affecting more than 200 million people mainly in sub-Saharan Africa (Gryseels *et al.*, 2006). Already for decades, there is an obvious need for improvement of diagnosis of schistosomiasis. In brief, egg detection by microscopy is specific but lacks sensitivity and suffers from the highly fluctuating egg output, antibody-based diagnosis is sensitive but fails to reliably identify active infections, and antigen-detection assays have a number of advantages but fail to detect very light infections. The development of more optimal diagnostic methods for the detection of human schistosome infections has recently become even more topical in view of the mass therapy programmes now underway in sub-Saharan Africa. To assess the effect of large scale schistosomiasis treatment programmes, sensitive and specific diagnostic assays should be available in particular to be able to detect remaining infections after treatment, as light infections significantly contribute to the overall morbidity burden associated with schistosomiasis (King *et al.*, 2005).

Antigen detection assays for schistosomiasis are in general sandwich-immunoassays which detect parasite-derived molecules in the blood or urine of the host. Due to the nature of these assays only analytes with at least two sterically available epitopes can be detected, while at a certain concentration a cut-off level has to be determined due to the fact that a (specific) signal can no longer be differentiated from (non-specific) noise. For this reason these assays lack the sensitivity required to detect the light infections in areas where due to extensive treatment overall infection intensities are low. Similarly, sandwich-immunoassays can not reliably detect the generally light infections in travelers from non-endemic areas (van Lieshout, Polderman & Deelder, 2000).

From the early days of antigen-based diagnosis onwards there has been discussion about the potential of specifically detecting small molecules released by the parasite. Mass spectrometry (MS) has become an indispensable tool in studies aiming at the identification of biomarkers in many human diseases and may be ideally suited for the purpose of detecting schistosome-derived molecules in host body fluids with both high specificity and sensitivity. We have recently discovered that in *Schistosoma mansoni* infection it is possible to detect urine oligosaccharides that have been excreted by eggs (Robijn *et al.*, 2007b). These oligosaccharides were revealed after affinity purification using monoclonal antibody (mAb) 114-4D12 coupled to agarose beads followed by MS detection. Previously, mAb 114-4D12 was used in a diagnostic sandwich ELISA to detect *S. mansoni* circulating soluble egg antigen (CSEA) in sera of infected mice (Nourel Din *et al.*, 1994a) and serum and urine of infected

humans (Nourel Din *et al.*, 1994b). MAb 114-4D12 recognizes the Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1- (DF-LDN-DF) terminal carbohydrate motif (Robijn *et al.*, 2007a), which so far appears to be unique to *S. mansoni*.

The objective of this study was to investigate whether the characteristic egg-derived oligosaccharides (Robijn *et al.*, 2007b) could be specifically detected in urine samples from individuals with different infection levels, as an initial step towards a new approach for schistosomiasis diagnosis.

Table 1. Combined intensity measures (CIM), corresponding faecal egg counts and urinary circulating cathodic antigen (CCA) concentrations for each urine sample.

Urine sample	CIM[A]	Egg output (epg)	CCA[B]
A	1620	593	3113
B	1607	717	2698
C	1603	740	2615
D	1510	463	3174
E	1174	976	599
F	1169	387	2371
G	499	160	1027
H	440	153	869
I	355	70	863
J	322	163	483
K	216	67	451

A CCA*0,33+egg output. *B* Levels of CCA were expressed in ng/ml of the trichloroacetic acid-solube fraction of adult worm antigen (AWA-TCA).

MATERIALS AND METHODS

Patient material

Eleven previously collected urine-samples (1ml), denoted A to K, of *S. mansoni*-infected individuals from Egypt (van Lieshout *et al.*, 1992) were tested for the presence of egg-derived free oligosaccharides (Figure 1). The egg output of the included subjects ranged from 67 eggs per gram (epg) to 976 epg as the mean of three consecutive duplicate 25-mg Kato Katz slides (van Lieshout *et al.*, 1992). Because egg output is an inconsistent measure for infection intensity (Wilson *et al.*, 2006a), epg values were combined with urinary circulating cathodic antigen (CCA) levels (van Lieshout *et al.*, 1992) affording a more accurate relative ranking of infection intensities (Table 1). Levels of CCA were expressed in ng/ml of the trichloroacetic

acid (TCA)-soluble fraction of adult worm antigen (AWA) and ranged from 451 to 3174 ng/ml. We ranked the samples allowing for equal weight of the two parameters as follows. First, the average ratio of egg output and CCA level was calculated to be 0.33. Subsequently, for each sample the CCA level was multiplied by 0.33 to normalize, and added to the egg output value. For example: for sample A (see Figure 1A) the egg output value was 593 epg and the urinary CCA was determined at 3113 ng /ml. The calculation 3113*0.33 + 593 results in a combined intensity measure (CIM) of 1620.

As a positive control 1ml of a pool of urine from heavily *S. mansoni* infected (>1000 epg) individuals from Senegal in which the specific oligosaccharides were originally detected (Robijn *et al.*, 2007b) was used. As negative control samples, a pool of urine from Senegalese controls (1ml) and nine urine samples (1ml) from individuals from Burundi were used (Polman *et al.*, 2000). Informed consent was obtained from the individuals who donated urine, or their parents.

Analytical procedure

The capturing procedure was performed using the anti-carbohydrate mAb 114-4D12-AA, as described previously (Robijn *et al.*, 2007b). MAb 114-4D12-AA (mouse IgG1) is an isotype switch of mAb 114-4D12-A (mouse IgG3), which was previously used for the diagnostic ELISA (Nourel Din *et al.*, 1994a; Nourel Din *et al.*, 1994b). Both mAbs are designated in this paper as 114-4D12. Briefly, to each urine sample 40µl of packed 114-4D12-coupled (22mg mAb/ml) Prot G Sepharose beads (Amersham Pharmacia Biotech AB, Uppsala, Sweden) were added and incubated under continuous rotation, overnight at 4°C. After thorough washing, bound components were eluted with 0.04% formic acid (Fluka, Buchs Sg, Switzerland). The eluates were lyophilized and reconstituted with 20µl water, of which 1µl was analyzed by matrix-assisted laser-desorption-ionization time-of-flight (MALDI-TOF) MS using an Ultraflex TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany), as described before (Robijn *et al.*, 2007b). Spectra were acquired for the 1000-4000 m/z range and normalized on the major peak at m/z 2443.9.

To quantify the captured oligosaccharides, known amounts (62.5, 125 and 250 fmol) of a standard oligosaccharide (di-antennary glycan, m/z 1663.6 [M+Na]+) were added directly to the mAb 114-4D12-eluates. Peak-intensities were compared assuming equal ionization efficiency of the oligosaccharide species.

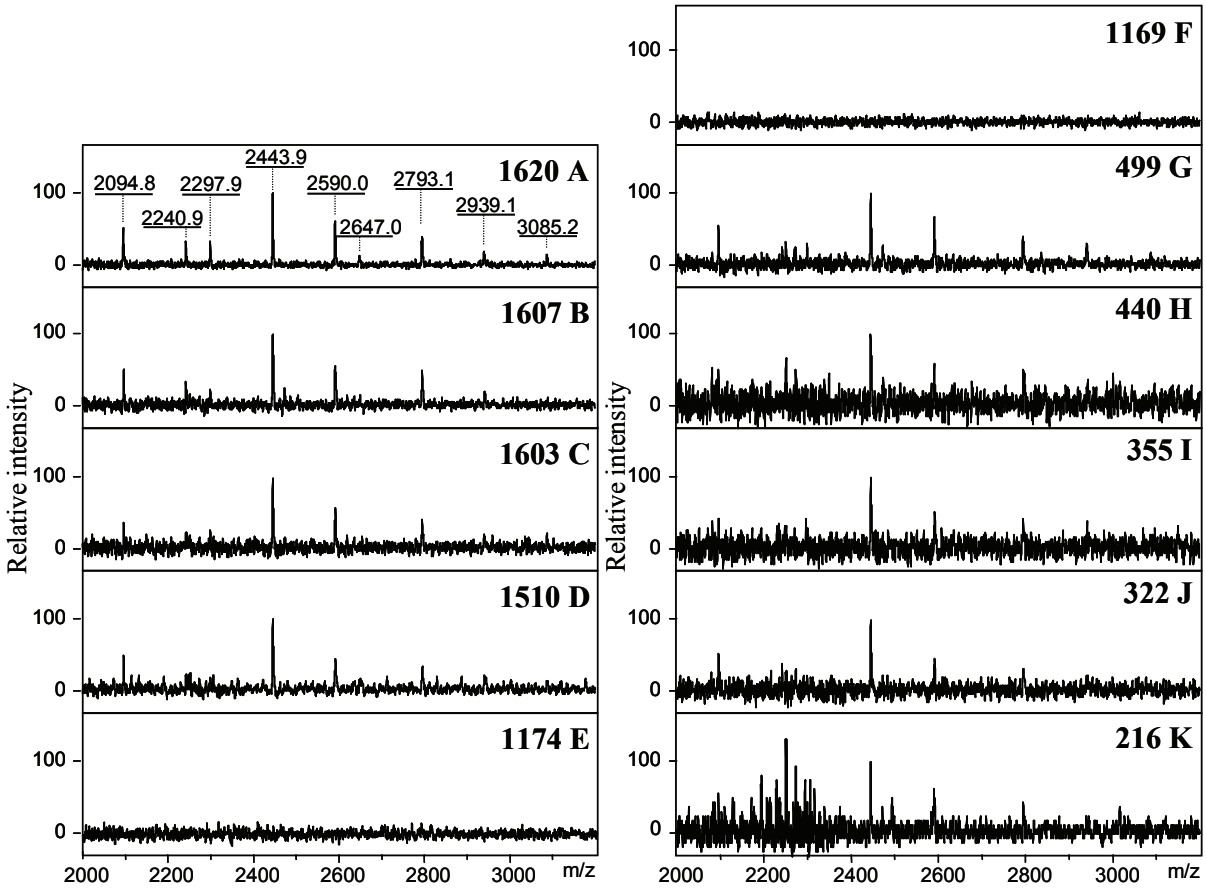

Figure 1. MALDI-TOF MS spectra of mAb 114-4D12-affinity-purified marker oligosaccharides from individual *S. mansoni* infection urines. Monoisotopic masses are indicated (*A*). The spectra are ranked to (decreasing) infection intensity (A-K; see also Table 1) of the corresponding urines. For further explanation see Results.

RESULTS

S. mansoni infection urines and controls were incubated with mAb 114-4D12-coupled agarose beads. Eluted oligosaccharides were analyzed by MALDI-TOF MS. Characteristic peak profiles were observed for nine out of eleven infection urines (Figure 1). Ions were detected at m/z 2094.8, 2240.9, 2297.9, 2443.9, 2590.0, 2647.0, 2793.1, 2939.1 and 3085.2 [M+Na]+, of which those at 2094.8, 2443.9, 2590.0 and 2793.1 were most prominent. In general, the signal-to-noise ratio was higher for the more heavily infected individuals (Figure 1A-D) than for the individuals with lower infection intensity (Figure 1G-K). No peaks were observed in two of the infection urines (Figure 1E, F).

The m/z values of the observed ions indicated that a series of fucosylated unconjugated oligosaccharides had been captured, as described before (Robijn *et al.*, 2007b). The overall compositions show the presence of one hexose (Hex, H) and variable numbers (5-7) of N-

acetylhexosamine (HexNAc, N) and (6-10) deoxyhexose (deoxyHex, F) residues, as indicated in figure 2A. These compositions are in line with the previous observation that each compound bound by mAb 114-4D12 contains a terminal DF-LDN-DF element (Robijn *et al.*, 2007a).

No mAb 114-4D12-binding oligosaccharides were available as internal standard. As an alternative measure for quantification, the mAb 114-4D12-eluate of the positive control urine pool was spiked with the di-antennary N-glycan standard H5N4. Assuming equal ionization of the schistosome glycans and the standard glycan, the major peak at m/z 2443.9 with composition H1N6F7 corresponded to an amount between 62.5 and 125 fmol (Figure 2B-C). Since only 5% of the eluate of 1ml of the urine pool was used for MALDI-TOF MS we estimate that of the H1N6F7 oligosaccharide at least 1.25 pmol/ml was present in this urine sample.

For the pooled negative control samples from Senegal (Polman *et al.*, 2000) (Figure 2D), as well as for the nine individual control urines from Burundi (Polman *et al.*, 2000) (data not shown), no peaks were observed in the MALDI-TOF MS profiles of the corresponding eluates.

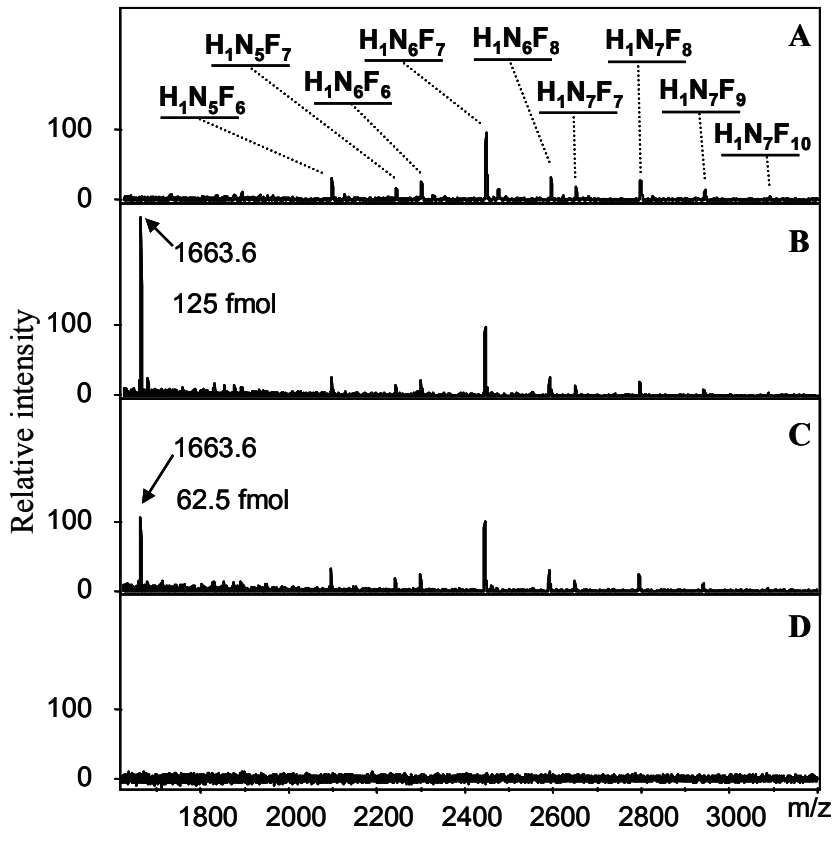

Figure 2. MALDI-TOF MS spectra of mAb 114-4D12-affinity-purified marker oligosaccharides from a pool of urine from heavily *S. mansoni*-infected individuals (*A-C*) and non-infected controls (*D*). The compositions of the marker oligosaccharides are indicated (*A*). H stands for hexose, N for N-acetylhexosamine, F for fucose. For quantification, the eluate of the *S. mansoni* infection urine was spiked with 125 fmol (*B*) and 62.5 fmol (*C*) of the di-antennary N-glycan standard, H5N4 (m/z 1663.6, [M+Na]+). For further explanation see Results.

DISCUSSION

In the present study we have evaluated whether enrichment using a schistosome glycan-specific mAb combined with the high sensitivity and mass accuracy of high-end MALDI-TOF MS for detection would allow identification of novel markers for schistosomiasis.

For the affinity purification method we used the anti-carbohydrate mAb 114-4D12 that recognizes the terminal DF-LDN-DF-carbohydrate motif (Robijn *et al.*, 2007a). The DF-LDN-DF motif has been demonstrated in *S. mansoni* glycoconjugates, but it has never been found in any other organism, including *S. japonicum* (Khoo *et al.*, 1997b; Robijn *et al.*, 2007b). Moreover, urine from *S. haematobium*-infected individuals as well as *S. haematobium* soluble egg antigens (SEA) were not reactive in the mAb 114-4D12-based sandwich ELISA (Nourel Din *et al.*, 1994a). This strongly suggests that also *S. haematobium* glycans are devoid of the DF-LDN-DF element, in which case the targeted 114-4D12 affinity approach described here would be highly specific for *S. mansoni*. However, future studies involving urines from (heavily) infected *S. haematobium* individuals would have to be included to offer final proof for this hypothesis.

The characteristic oligosaccharide markers were detected in the urine of nine out of eleven *S. mansoni* infected individuals of different infection intensities (Figure 1). In the two remaining infection urine samples (Figure 1E,F) no peaks were observed, which may have several reasons. Previously, mAb 114-4D12 was used in a sandwich ELISA to detect *S. mansoni* CSEA in sera of infected mice (Nourel Din *et al.*, 1994a) and serum and urine of infected humans (Nourel Din *et al.*, 1994b). This implies that in urine antigens are present that contain multiple DF-LDN-DF epitopes, which would be a requirement for the detection of these antigens by a sandwich-type ELISA. However, it is highly unlikely that the relatively small, linear free oligosaccharide antigens presented here (see also (Robijn *et al.*, 2007b)) can be detected in a sandwich ELISA as these molecules do not contain more than one epitope. Thus, larger components presenting multiple DF-LDN-DF epitopes in all probability compete with the free oligosaccharides for binding to the mAb, which might (in part) explain the false negative results. Moreover, these false negatives may reflect individual differences in efficiency of antigen clearance. The LDN-DF motif, which is included in the DF-LDN-DF element carried by the oligosaccharides detected here, is antigenic and induces antibody responses in the human host (Naus *et al.*, 2003). Moreover, reactivity of mAb 114-4D12 in immunofluorescence microscopy assays suggests cellular uptake of DF-LDN-DF containing antigens by phagocytic cells of *S. mansoni*-infected hosts (Bogers *et al.*, 1996). Therefore it is

also possible that the circulating oligosaccharides are cleared from the circulation by the host's immune system.

The technology presented here has several advantages. Detection by MALDI-TOF MS is highly accurate, and with current technical developments the accuracy is still increasing. The mass-accuracy results in a high certainty regarding the presence or absence of the characteristic peak(s). This is an important advantage compared to the diagnostic sandwich ELISA, in which antigens are captured 'blindly' and thus always a carefully set cut-off value is needed to eliminate false positive results due to non-specific background signals (Polman *et al.*, 2000). Using the here presented method at least 1.25 pmol of the major H1N6F7 oligosaccharide was detected in 1ml of a urine pool from heavily infected (>1000 epg) Senegalese subjects, which is sufficient for the recording of several good quality MALDI-TOF mass spectra. Since the method is based on affinity-purification, analysis of larger volumes of urine (e.g. 100ml) which are easily and non-invasively obtained is feasible, allowing detection of much lighter infections (Nibbeling *et al.*, 1997).

Interestingly, the oligosaccharides detected in infection urine were shown to be excreted by live *S. mansoni* eggs and not by worms (Robijn *et al.*, 2007b), which suggests that the urinary oligosaccharides are related to egg-load. Since the induction of granulomas around eggs retained in the tissues of the host is the main cause of pathology and morbidity in schistosomiasis, egg-derived oligosaccharides might have potential as a marker for morbidity. Previously, it was suggested that CSEA in urine, as detected by sandwich ELISA, is primarily derived from viable eggs in the tissues (Nibbeling, van Lieshout & Deelder, 1998). Hassan et al. (1999) who detected circulating egg antigens in a sandwich ELISA using a different mAb (128C3/3/21), observed a direct correlation between the antigen level and disease severity as monitored by ultrasonography (Hassan *et al.*, 1999).

The presented capturing procedure has substantial potential to improve diagnosis of *S. mansoni* infections. Presently used diagnostic tests remain inadequate for detection of very light infections, such as of tourists. Our method could be the basis for a diagnostic test with significantly enhanced detection limits by application of larger urine samples. In addition, a quantitative technique could be developed for example by derivatization of the glycans with a fluorophore and subsequent highly sensitive and quantitative analysis by capillary electrophoresis with fluorescence detection. Further improvements might be based on the combined use of multiple specific anti-glycan mAbs for the capturing step, pretreatment of the urine samples e.g. by size exclusion or TCA to dissociate immune-complexes or to

remove interfering proteins, or the addition of a synthetic DF-LDN-DF glycan variant as internal marker.

From the present study it is obvious that a novel technological approach allows identification of new small molecule markers in schistosomiasis. The fact that the employed method shows significant potential for improvement with regard to the detection level, while still retaining “absolute” specificity due to the mass-accuracy of MS, offers the prospect of a new, highly sensitive and egg-load related assay for light infections in schistosomiasis.

6

A novel Gal(β1-4)Gal(β1-4)Fuc(α1-6)-core modification attached to the proximal N-acetylglucosamine of keyhole limpet hemocyanin (KLH) N-glycans

MANFRED WUHRER[1,2], MARJOLEIN L.M. ROBIJN[1], CAROLIEN A.M. KOELEMAN[1], CRINA I.A. BALOG[1], RUDOLF GEYER[2], ANDRÉ M. DEELDER[1], CORNELIS H. HOKKE[1]

1 Department of Parasitology, Center of Infectious Diseases, Leiden University Medical Center, P.O. Box 9600, 2300 RC Leiden, The Netherlands; 2 Institute of Biochemistry, Medical Faculty, University of Giessen, Friedrichstrasse 24, D-35392 Giessen, Germany.

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SUMMARY

Keyhole limpet hemocyanin (KLH), the oxygen-carrying molecule of the marine snail *Megathura crenulata*, is often used as an adjuvant or as a hapten carrier for immunisations with peptides, oligosaccharides or other low-molecular mass organic compounds. KLH exhibits several carbohydrate determinants, at least some of which are immunogenic: It shares an antigenic Fuc(α1-3)GalNAc- determinant with schistosomes and contains unique Gal(β1-6)Man- structural motifs on its N-glycans. This study reveals the presence of N-glycans with unusual ±Gal(β1-4)Gal(β1-4)Fuc- units (α1-6)-linked to the reducing end *N*-acetylglucosamine residue. The following novel structures of KLH N-glycans were deduced by linkage analysis, exoglycosidase digestion, MALDI-MS(/MS) and nano-LC-ESI-ion trap-MS: Man(α1-6)[±Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Fuc(α1-6)]GlcNAc and Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Gal(β1-4)Fuc(α1-6)]GlcNAc. The Gal(β1-4)Fuc- and Gal(β1-4)Gal(β1-4)Fuc- core-modifications are expected to be immunogenic, like other non-mammalian-type core-modifications, and to contribute to the immunostimulatory properties of KLH.

INTRODUCTION

Hemocyanins serve as oxygen-carrying proteins in the hemolymph of many molluscs (van Holde, Miller & Decker, 2001). Whilst the mammalian oxygen-carrier hemoglobin exhibits heme-complexed iron and is packed into red blood cells, hemocyanins contain copper in their oxygen-binding sites and often form oligomers up to the million dalton range.

The most intensely studied hemocyanin is that of the marine snail keyhole limpet (*Megathura crenulata*) (Harris & Markl, 1999). Most work dealing with keyhole limpet hemocyanin (KLH) does not address its role as an oxygen-transporting molecule, but relates to the properties of KLH as a potent immunostimulant and hapten carrier (Harris & Markl, 2000), which is reflected by several thousands of biomedical publications. KLH has, for example, been used as a hapten carrier for covalently attached peptides (Gilewski *et al.*, 2000; Melzer *et al.*, 2002) and carbohydrate-based antigens (Wang *et al.*, 2000; Danishefsky & Allen, 2000; Holmberg & Sandmaier, 2001; Irazoqui *et al.*, 2002; Ilyas, Chen & Prineas, 2002), mostly with the scope of developing anti-cancer therapy. Likewise, glycolipids have been adsorbed to KLH for immunizations (Chapman *et al.*, 2000). Furthermore, KLH has been shown to be a potent adjuvant that stimulated peptide-specific cellular responses (Millard *et al.*, 2003) and has been tested as an adjuvant in immunotherapy against primary liver cancer (Iwashita *et al.*, 2003). In addition, KLH in its natural, i. e., unconjugated form has been shown to be effective against bladder cancer (Kamat & Lamm, 2001; Riggs *et al.*, 2002).

Concerning the glycosylation of this protein, two structural motifs have recently been described: (I.) KLH exhibits a variety of uniqe N-glycans with Gal(β1-6)Man- structural units (Kurokawa *et al.*, 2002); and (II.) a Fuc(α1-3)GalNAc- antigenic determinant found in glycoconjugates of the parasite *Schistosoma mansoni* is also present on KLH and seems to be responsible for cross-reactivity of schistosome infection sera with KLH (Kantelhardt *et al.*, 2002). The other way round, immunization of rabbits with KLH leads to high titres of antibodies cross-reacting with schistosomal antigens (Wuhrer *et al.*, 2000a). Indications for further unusual carbohydrate epitope(s) on KLH were given by the detection of 4-substituted fucose in linkage analysis of total KLH glycans (Kurokawa *et al.*, 2002). A recent study has shown in an *in vivo* model for granuloma formation that induction of a cellular immune response by KLH is dependent on its glycosylation (Koen Van de Vijver, personal communication). Hence, knowledge of specific structural features of KLH glycosylation is needed to be able to study the contribution of these glycanic determinants to KLH immunogenicity.

In this study, we describe a group of unusual KLH N-glycans characterized by a 4-substituted core fucose. As KLH glycans with an internal fucose could not be released in reasonable amounts by PNGase F treatment in the former study (Kurokawa *et al.*, 2002), we here followed a different protocol for KLH denaturation and enzymic deglycosylation, which allowed the isolation and structural characterization of novel KLH N-glycans containing core-fucose 4-substituted by a single β-galactose or, alternatively, by a digalactosyl unit.

MATERIALS AND METHODS

Release and purification of N-glycans

To aliquots (900 µl) of a solution of KLH (100 mg in 18 ml phosphate-buffered saline; Sigma), 90 µl of 10 % SDS and 4.5 µl 2-mercaptoethanol were added. The samples were incubated for 10 min at 100ºC, allowed to cool down to room temperature, and 9 mg of CHAPS was added. After incubation with 5 mU PNGase F (Roche Diagnostics, Mannheim, Germany) overnight at 37ºC, samples were fractionated by gel filtration on a Superdex 75 HiLoad column (16 x 600 mm; Amersham Biosciences, Uppsala, Sweden) at a 1 ml/min flow of 25 mM NH_4HCO_3, pH 8.0. Five ml fractions were collected and released glycans were detected by carbohydrate constituent analysis. Fractions containing released glycans were pooled and applied to a self-packed porous graphitized carbon column (8 x 50 mm; Carbograph; Alltech, Deerfield, Il). After washing the carbon column with water, glycans were eluted with 25 % aqueous acetonitril. Fractions of 1 ml were collected and assayed for the presence of oligosaccharides by MALDI-MS. Glycan-containing fractions were pooled and lyophilised. Fractions from gel filtration which contained protein were pooled, lyophilized, taken up in 50 mM ammonium bicarbonate and trypsinized. Resulting (glyco)peptides were desalted using a RP-cartridge as described earlier (Kurokawa *et al.*, 2002), taken up in 50 mM ammonium acetate, pH 5, and subjected to PNGase A treatment (Roche Diagnostics, Mannheim, Germany). The sample was again applied to a RP-cartridge, and possibly released N-glycans were collected as the flow-through, subjected to 2AB-labeling, and analyzed by analytical normal-phase HPLC using fluorescent-detection for oligosaccharides. The total glycan release procedure with successive PNGase F and PNGase A treatment was performed in parallel on horseradish peroxidase (Sigma), which served as a positive control to confirm PNGase A activity.

Labelling and fractionation of N-glycans

Of a mixture of 30 µl glacial acetic acid and 100 µl dimethylsulfoxide, 100 µl were used to dissolve subsequently 4.8 mg 2-aminobenzamide (2AB; Sigma) and 6.3 mg $NaCNBH_3$

(Fluka). This mixture (100 µl) was added to 300 µg of KLH glycans and incubated for 2 h at 65ºC. The sample was then applied to a reversed-phase cartridge (500 mg Bakerbond Octadecyl; Baker, Phillipsburg, NJ). After a 5 ml wash with water, 2AB-labelled glycans were eluted with 2 ml of 50% methanol and dried under N_2. 2AB-Glycans were fractionated by normal phase HPLC on a TSK-Amide 80 column (4 x 250 mm; Tosohaas, Montgomeryville, PA) at 0.4 ml/min. Solvent A

was 50 mM formic acid adjusted to pH 4.4 with ammonia solution. Solvent B was 20% solvent A in acetonitrile. The following gradient conditions were used: t = 0 min, 100% solvent B; t = 152 min, 52.5% solvent B; t = 155 min, 0% solvent B; t = 162 min, 0% solvent B; t = 163 min, 100% solvent B. The total run time was 180 min. Samples were injected in 80% acetonitrile (Garner *et al.*, 2001). Fluorescence was detected at 360 nm/ 425 nm.

For 2-dimensional separation, KLH 2AB-labelled glycans were first separated on a CarboPac PA-100 (4 x 250 mm (Dionex, Sunnyvale, CA) at 1 ml/min. Solvent A was 500 mM NaOH, solvent B was 500 mM sodium acetate, and solvent C was water. Starting conditions were 25% solvent A, 0% solvent B. After 10 min of isocratic run, a 80 min linear gradient was applied to 25% solvent A, 20% solvent B. Oligosaccharides were detected amperometrically and collected manually. Fractions were re-chromatographed on a reversed-phase column (Sephasil Peptide C18, 5 µm; 4.6 x 100 mm; Amersham Biosciences) at 1 ml/min. Solvent A was water, solvent B was 20% aqueous acetonitrile. The column was equilibrated at 5% solvent B, and a 35 min gradient to 35% solvent B was applied. Oligosaccharides were detected by fluorescence (360 nm/ 425 nm) and collected manually.

Nano-liquid chromatography ESI-ion trap (IT)-MS

2AB-glycans were separated on a Pep-Map column (75 µm x 100 mm; Dionex/LC Packings, Amsterdam, The Netherlands) using an Ultimate nano-LC system (LC Packings). The system was directly coupled with an Esquire 3000 ESI-IT-MS (Bruker Daltonik, Bremen, Germany) equipped with an on-line nanospray source operating in the positive-ion mode. For electrospray (1200-2500 V), capillaries (360 µm OD, 20 µm ID with 10 µm opening) from New Objective (Cambridge, MA, USA) were used. The solvent was evaporated at 150ºC with a nitrogen stream of 6 l/min. Ions from *m/z* 50 to *m/z* 3000 were registered. The column was equilibrated with eluent A (H_2O/acetonitrile 20:80, v/v, containing 0.1% formic acid) at a flow rate of 100 nl/min. After injecting the sample, the column was run isocratically for 5 min, followed by a linear gradient to 30% eluent B (H_2O/acetonitrile 95:5, v/v, containing

0.1% formic acid) in 15 min and a final wash with 100% B for 5 min. The eluate was monitored by absorption at 320 nm.

Exoglycosidase treatment

2AB-oligosaccharides (2-20 pmol) were treated with α-mannosidase from jack beans (100 mU; Sigma), α-fucosidase from bovine kidney (20 mU; Sigma) or β-galactosidase from Streptococcus 6646K (2 mU; Seikagaku, Falmouth, MA) in 50 µl 50 mM sodium acetate buffer, pH 5.0. 2AB-oligosaccharides were extracted by usage of a Zip-Tip (Millipore, Billerica, MA), eluted with 50% methanol onto the target plate and analysed by MALDI-MS, nano-LC-ESI-IT-MS or normal-phase HPLC.

MALDI-TOF MS(/MS)

MALDI-TOF MS(/MS) data were obtained using an Ultraflex time-of-flight mass spectrometer (Bruker) equipped with a LIFT-MS/MS facility. 6-Aza-2-thiothymine (5 mg/ml; Sigma) was used as a matrix, spectra were aquired in the positive-ion mode. For fragment ion analysis in the tandem time-of-flight (TOF-TOF) mode, precursors were accelerated to 8 kV and selected in a timed ion gate. Fragment ions generated by laser-induced decomposition of the precursor were further accelerated by 19 kV in the LIFT cell, and their masses were analyzed after the ion reflector passage. In order to obtain proton adducts besides the routinely observed sodium adducts, 10 nmol of ammonium bicarbonate was added per spot.

Carbohydrate constituent analysis

Samples were hydrolyzed in 20 µl of 4 M aqueous trifluoroacetic acid (Sigma) at 100ºC for 4 h, and dried under a stream of nitrogen. Monosaccharides were converted into their anthranilic acid derivatives by reductive amination, resolved by RP-HPLC and detected by fluorescence (Anumula, 1994).

Methylation analysis

2AB-glycans were permethylated and hydrolyzed (4 M trifluoroacetic acid, 4h, 100°C). Partially methylated alditol acetates obtained after sodium borohydride reduction and peracetylation were analyzed by capillary GLC-MS using electron-impact ionization (Geyer *et al.*, 1994).

RESULTS

Analysis of the entire N-glycan pool

KLH was heat-denatured after addition of reducing agent and detergent, followed by PNGase F-release of N-glycans. Oligosaccharides were purified by gel filtration, and glycan-containing fractions were identified by carbohydrate constituent analysis and pooled. Retrieved protein fractions were trypsinized, and resulting glycopeptides were subjected to PNGase A treatment, which did not release N-glycans in detectable amounts (data not shown). Oligosaccharide pools obtained by PNGase F treatment were applied to a carbon column and eluted in 25% acetonitrile. Linkage analysis of this pool revealed the presence of 4-substituted fucose (data not shown), which has been observed in KLH samples before (Wuhrer *et al.*, 2000a; Kurokawa *et al.*, 2002). Furthermore, the following *N*-acetylhexosamine species were detected (relative abundance given in parenthesis): terminal GlcNAc (0.1), 4-substituted GlcNAc (1.0), 3-substituted GlcNAc (0.2), 3-substituted GalNAc (0.3), 3,4-disubstituted GlcNAc (0.9), and 4,6-disubstituted GlcNAc (0.5).

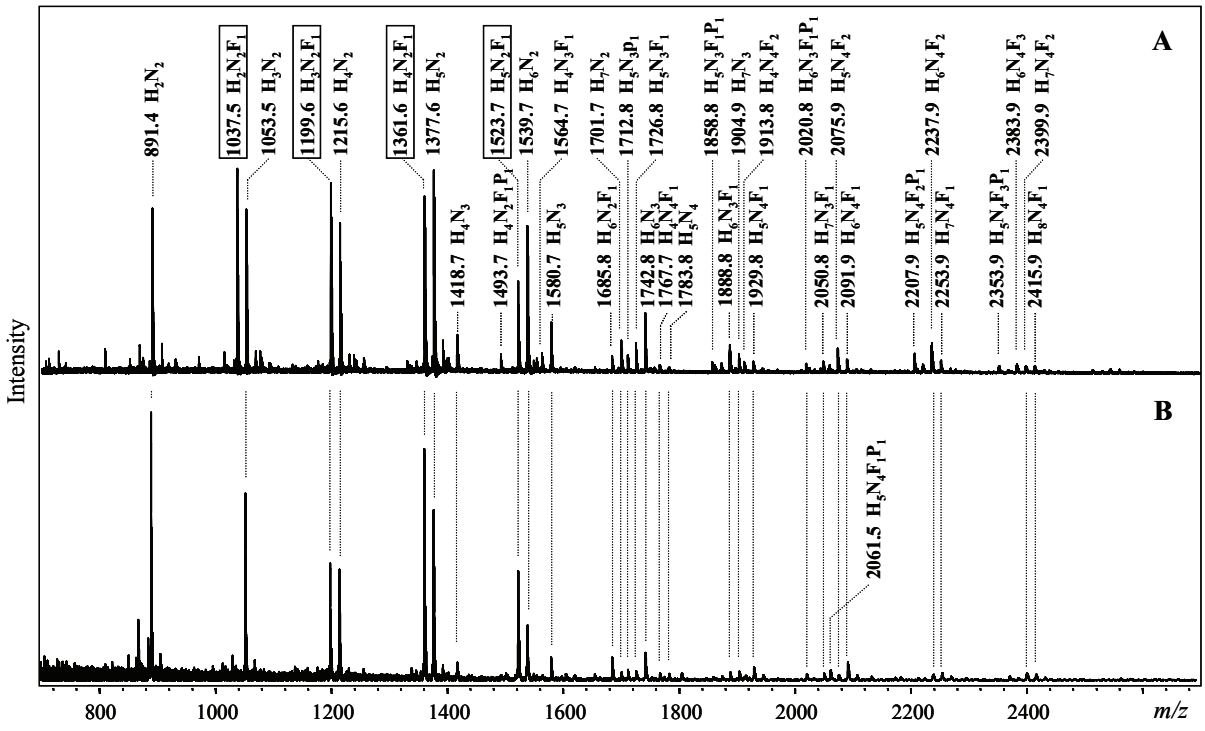

Figure 1. MALDI-MS of the total pool of PNGase F-released KLH N-glycans before and after α–fucosidase treatment. PNGase F-released, AB-labelled N-glycans of KLH were registered as sodium adducts before (*A*) and after (*B*) treatment with α–fucosidase from bovine kidney. Peaks are marked with measured mass and deduced glycan composition. Of the four major fucose-containing species (highlighted by boxes) only the species at m/z 1037 completely disappeared upon α-fucosidase treatment, while the others were largely resistant to enzymic defucosylation (*B*). H: hexose, N: N-acetylhexosamine, F: fucose, P: pentose.

This pattern of *N*-acetylhexosamine species resembled that observed for total KLH (glycopeptides) as well as hydrazinolysis-released glycans (Wuhrer *et al.*, 2000a; Kantelhardt *et al.*, 2002), suggesting an efficient release of KLH N-glycans. PNGase F-released oligosaccharides were then labelled with 2-aminobenzamide (2AB) and analysed by MALDI-MS (Figure 1A). In the mass range up to 1600 Da, high intensity signals were obtained for species with deduced compositions of $H_{2\text{-}6}N_2F_{0\text{-}1}$, while the mass range up to 2500 Da displayed a variety of minor, mainly fucosylated structures, some of which contained a pentose residue.

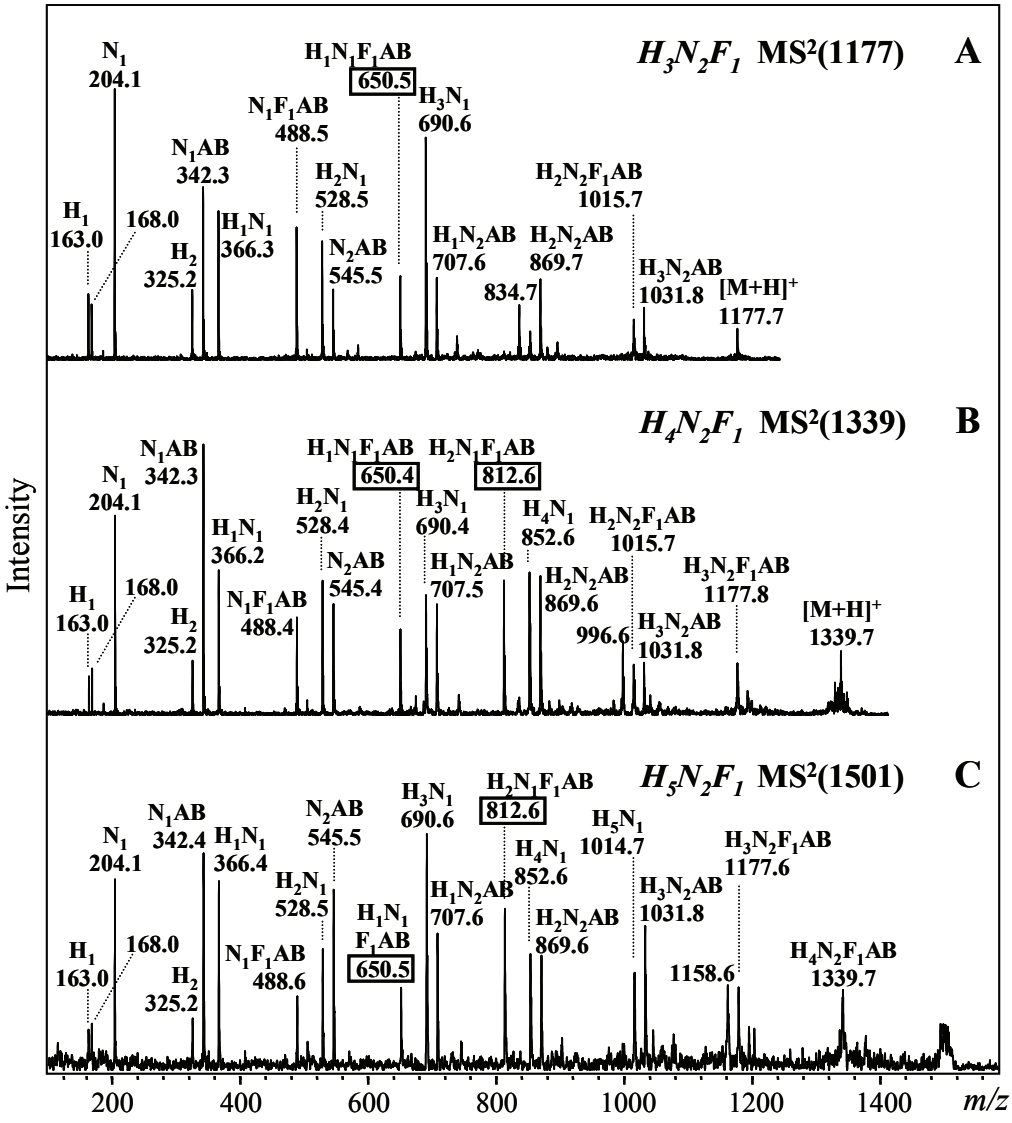

Figure 2. MALDI-MS/MS analysis of some fucose-containing species out of the total pool of released KLH N-glycans. Proton adducts of the KLH AB-labelled N-glycans of composition H3N2F1 (m/z 1177; *A*), H4N2F1 (m/z 1339; *B*) and H5N2F1 (m/z 1501; *C*) were subjected to fragment ion analysis. Peaks are labelled with measured mass and deduced glycan composition. Fragment ions at m/z 650 or 812 indicative of unusual core-modifications are boxed. H: hexose; N: N-acetylhexosamine; F: fucose; AB: 2-aminobenzamide.

For the four major fucosylated species, fragment ion patterns were obtained from protonated precursors by nano-LC-ESI-IT-MS/MS and MALDI-MS/MS. This revealed for the $H_2N_2F_1$ precursor at *m/z* 1015 a fragmentation indicative of core-fucosylation (N_1F_1-2AB ion at *m/z* 488; confer Table 1, fraction 2). For the precursors at *m/z* 1177 ($H_3N_2F_1$), 1339 ($H_4N_2F_1$) and 1501 ($H_5N_2F_1$), unusual core modifications were indicated by ions at *m/z* 812 and/or 650, which could be interpreted as the protonated Y-type ions $H_2N_1F_1$-2AB and $H_1N_1F_1$ -2AB, respectively (Figure 2; fragment nomenclature according to (Domon & Costello, 1988)). Nano-LC-ESI-IT-MS/MS of the sodium adducts at *m/z* 1199 ($H_3N_2F_1$), 1361 ($H_4N_2F_1$) and 1523 ($H_5N_2F_1$) yielded similar fragment ions at *m/z* 672 and 834 (data not shown).

Treatment of KLH 2AB-labelled N-glycans with α-fucosidase from bovine kidney exhaustively removed the core-fucose from $H_2N_2F_1$ as monitored by MALDI-MS (Figure 1B) and led to the production of H_2N_2, as indicated by disappearance of the corresponding sodium adduct at *m/z* 1037. Signals ($[M+Na]^+$) at *m/z* 1199, 1361 and 1523, however, were largely resistant to enzymatic defucosylation (Figure 1B), which corroborated the assumption of an unusual core-modification as indicated by mass spectrometric fragmentation.

N-glycan fractionation and elucidation of unusual core-modifications

For a more detailed study of these unusual structures, the 2AB-labelled KLH N-glycan pool was fractionated by normal-phase HPLC (Figure 3). Glycans were structurally characterized by carbohydrate constituent analysis (data not shown), linkage analysis (Table 1), MALDI-MS/MS (Figure 4), and α-mannosidase treatment followed by analytical-scale normal-phase separation and nano-LC-ESI-IT-MS as well as -MS/MS of the individual fractions (Figure 3). MALDI-MS/MS of proton adducts revealed structures with unusual core-modifications in fractions 4, 7, 8 and 10, as indicated by fragment ions at *m/z* 812 and/or 650. Fraction 4 was shown to contain the two isobaric species $H_3N_2F_1$-a (60%) and $H_3N_2F_1$-b (40%). Using the two-dimensional separation system, the two species could be separated, and only the former compound was sensitive to α-fucosidase treatment. $H_3N_2F_1$-a was elucidated as Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-2AB consistent with (Kurokawa *et al.*, 2002).

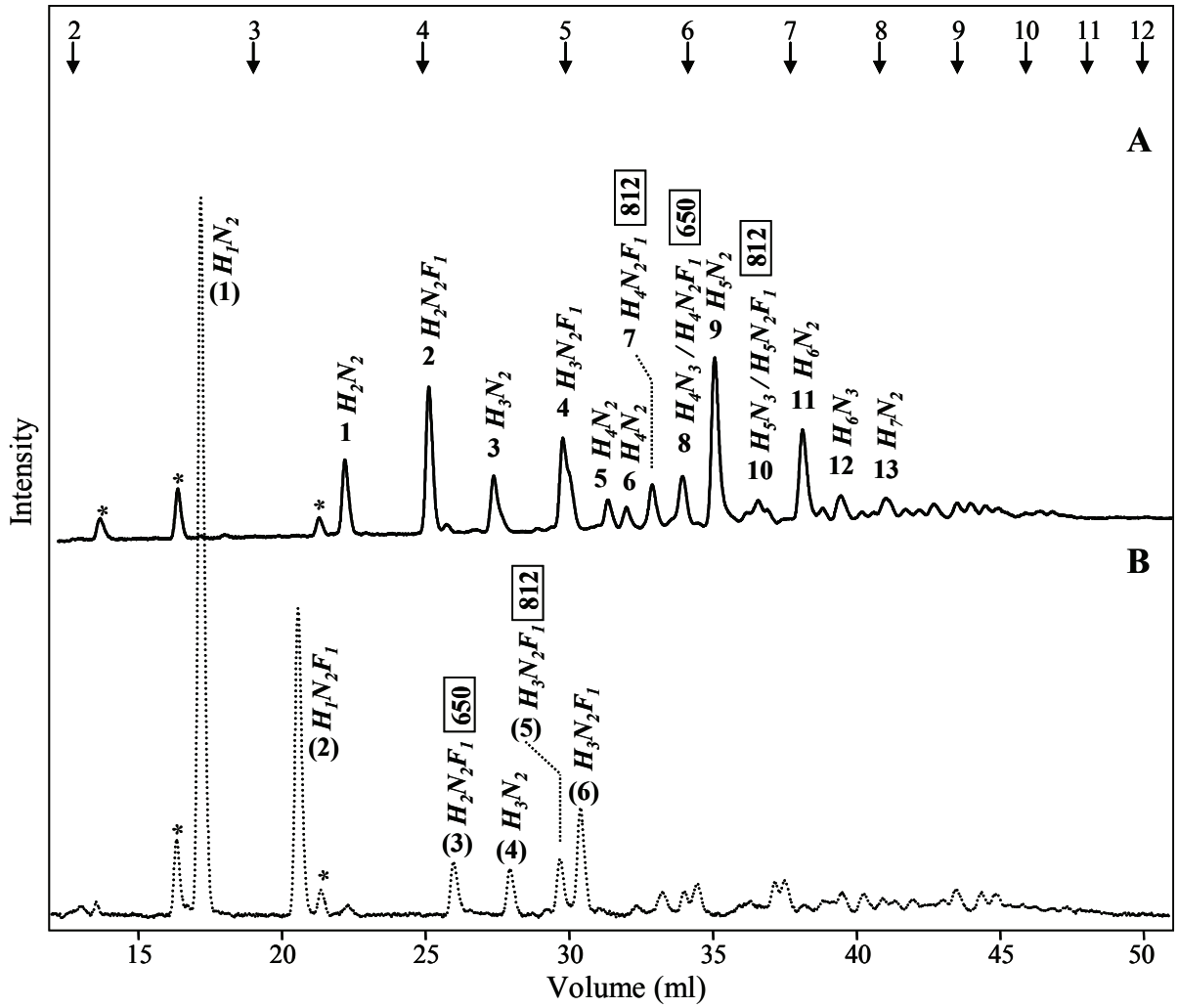

Figure 3. Normal phase HPLC fractionation of AB-labelled KLH N-glycans before and after α–mannosidase treatment. KLH AB-labelled N-glycans were fractionated by normal-phase chromatography before (*A*) and after (*B*) α-mannosidase treatment. Obtained glycans were subjected to MALDI-MS/MS (A) or nano-LC-ESI-IT-MS/MS (*B*) of their proton adducts, and fragment ions indicative of an unusual core modification are given in boxes. *: contaminant.

For $H_3N_2F_1$-b, MALDI-MS/MS (Figure 4A) revealed a hexose attached to the Fuc(α1-6)GlcNAc-2AB moiety (fragment at *m/z* 650). The compound was sensitive to α-fucosidase treatment only after removal of a galactose residue by β-galactosidase treatment, as analysed by normal-phase HPLC of the cleavage products using fluorescence detection (data not shown). This indicated a β-galactose capping of the core fucose. Together with the linkage analysis data (Table 1), the structure was deduced as Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Fuc(α1-6)]GlcNAc-2AB (schematically represented in Figure 4A).

Fraction 7 contained the species $H_4N_2F_1$-c for which MALDI-MS/MS revealed a major ion at *m/z* 812 indicating the attachment of 2 hexoses to the Fuc(α1-6)GlcNAc-2AB core region. This species was sensitive to Streptococcus 6646K β-galactosidase, which removed 2 galactose residues (Figure 5). Only after this β-galactosidase treatment, the core fucose could be enzymatically removed (data not shown). Together with linkage analysis data (Table 1), $H_4N_2F_1$-c glycans could be structurally characterized as Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Gal(β1-4)Fuc(α1-6)]GlcNAc-2AB (schematically represented in Figure 4D).

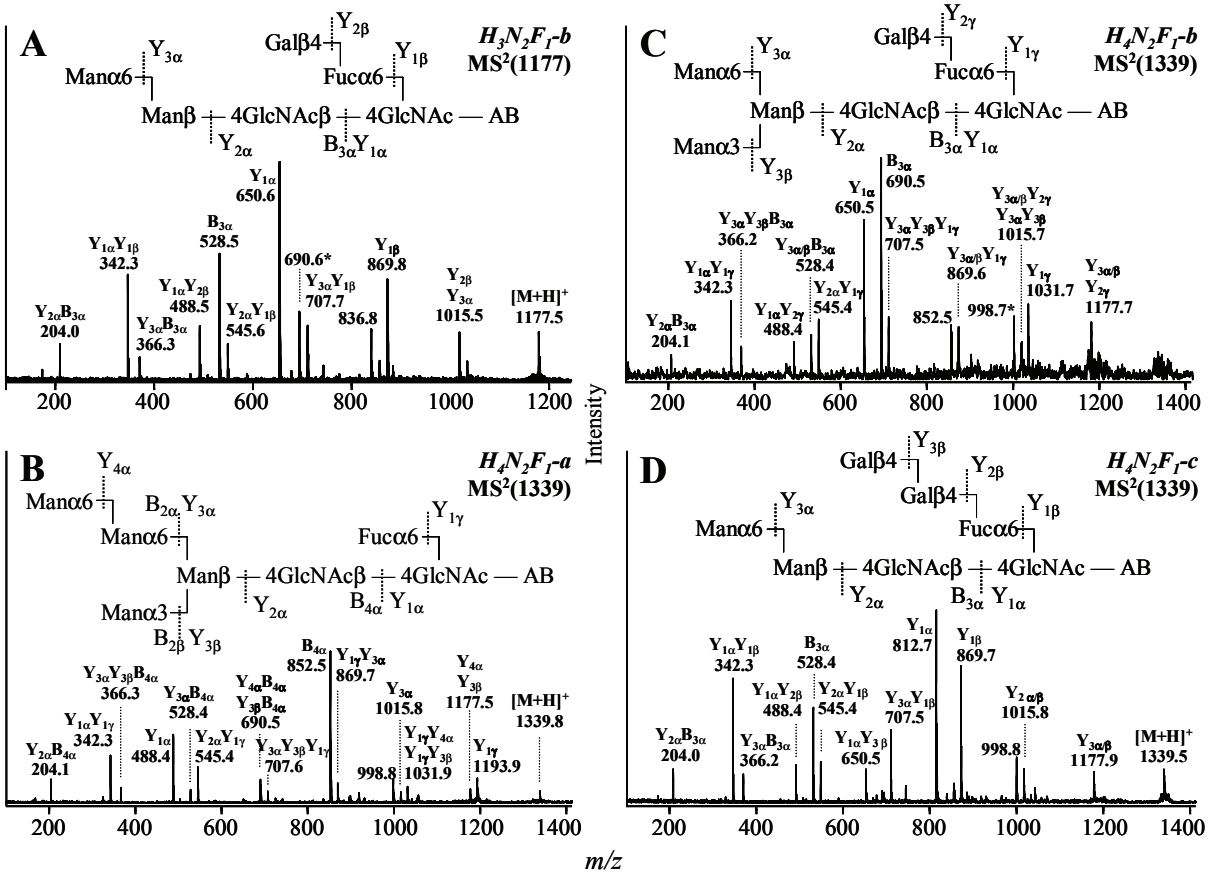

Figure 4. MALDI-MS/MS of individual, isobaric KLH N-glycans. By 2-dimensional HPLC fractionation, the individual N-glycan species H3N2F1-b (*A*), H4N2F1-a (*B*), H4N2F1-b (*C*) and H4N2F1-c (*D*) were obtained and were subjected as their proton adducts to MALDI-MS/MS. Possible fragmentation pathways are included in the structures. The assignment of fragments is in agreement with the nomenclature introduced by Domon and Costello (Domon & Costello, 1988).

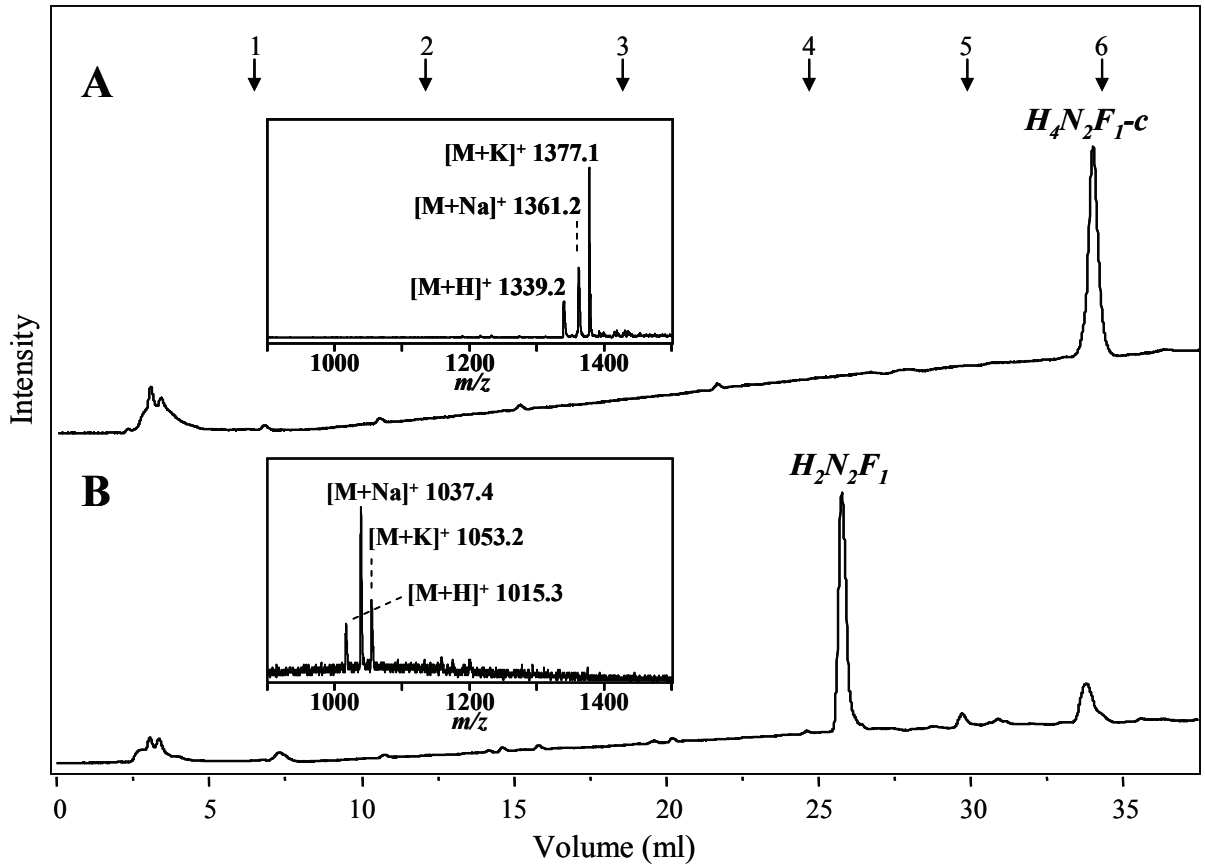

Figure 5. HPLC and mass spectrometry of H4N2F1-c before and after β-galactosidase treatment. H4N2F1-c was analysed by analytical normal phase HPLC with fluorescence detection before (A) and after (B) treatment with Streptococcus 6646 K β-galactosidase. Elution positions obtained for an AB-tagged dextran ladder are indicated by arrows labelled with the number of glucose residues. The insets show the corresponding mass spectra, which were obtained by nano-LC-ESI-IT-MS.

Fraction 8 comprised the two isobaric species, $H_4N_2F_1$-a and $H_4N_2F_1$-b, only the former of which was sensitive to α-fucosidase treatment. The two isobaric species $H_4N_2F_1$-a (60%) and $H_4N_2F_1$-b (40%) differed in retention properties in the two-dimensional separation system and were thus purified on an analytical scale for MALDI-MS/MS (Figure 4B and C). Together with linkage analysis and exoglycosidase treatment, $H_4N_2F_1$-a was elucidated as Man(α1-6)Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-2AB consistent with (Kurokawa *et al.*, 2002). For $H_4N_2F_1$-b, MALDI-MS/MS (Figure 4C) revealed structural similarities to $H_3N_2F_1$-b (Figure 4A), as both species exhibited major ions at *m/z* 650. Based on mass spectrometry, linkage analysis, β-galactosidase and α-fucosidase treatment, the structure was defined as Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Gal(β1-4)Fuc(α1-6)]GlcNAc-2AB (Figure 4C). MALDI-MS/MS of $H_5N_2F_1$ (Fraction 10) indicated the occurrence of galactosylated core structures (Table 1), yet full structural characterization was hindered by the relatively low abundance of these species.

Table 1. Structural data obtained for KLH 2AB-labelled N-glycans. For KLH 2AB-labelled N-glycans in fractions 1 to 11, the molecular masses were determined by MALDI-MS. Proton adducts were analysed by MALDI-MS/MS. In the case of non-resolved isobaric structures, MALDI-MS/MS was performed after isolation by 2-dimensional HPLC. Characteristic fragments observed are listed with the deduced composition given in parentheses. Bold type indicates Y-type ions indicative of unusual core-modification. 2AB, 2-aminobenzamide; F, fucose; H, hexose; N, N-acetylhexosamine. For linkage analysis (LA), partially methylated monosaccharide derivatives obtained after hydrolysis were analysed by GC-MS. t-Man, terminal mannose (2,3,4,6-tetra-O-methylmannitol as alditol acetate); 6-Man, 6-substituted mannose, etc. a, linkage analysis performed after enzymatic defucosylation and re-chromatography.

Fraction, Species	Determined mass	Key structural data
1, H_2N_2	$[M+H]^+$ 869.4; $[M+Na]^+$ 891.1	fragments: 342 (N_1-2AB); 528 (H_2N_1); 707 (H_1N_2 -2AB) LA: t-Man, 6-Man, 4-GlcNAc
2, $H_2N_2F_1$	$[M+H]^+$ 1015.4; $[M+Na]^+$ 1037.1	fragments: 342 (N_1-2AB); 488 (N_1F_1 -2AB); 528 (H_2N_1); 707 (H_1N_2 -2AB); 869 (H_2N_2-2AB) LA: t-Fuc, t-Man, 6-Man, 4-GlcNAc
3, H_3N_2	$[M+H]^+$ 1031.5; $[M+Na]^+$ 1053.1	fragments: 342 (N_1-2AB); 545 (N_2 -2AB); 690 (H_3N_1); 869 (H_2N_2 -2AB) LA: t-Man, 3,6-Man, 4-GlcNAc
4, $H_3N_2F_1$-a	$[M+H]^+$ 1177.2; $[M+Na]^+$ 1199.4	LA[a]: t-Man, 3,6-Man, 4-GlcNAc
4, $H_3N_2F_1$-b	$[M+H]^+$ 1177.2; $[M+Na]^+$ 1199.4	fragments: 342 (N_1-2AB); 528 (H_2N_1); **650 ($H_1N_1F_1$-2AB)**; 869 (H_2N_2-2AB); 1015 ($H_2N_1F_1$-2AB) LA: t-Man, t-Gal, 4-Fuc, 6-Man, 4-GlcNAc
5, H_4N_2-a	$[M+H]^+$ 1193.5; $[M+Na]^+$ 1215.4	fragments: 342 (N_1-2AB); 545 (N_2-2AB); 690 (H_3N_1); 707 (H_1N_2-2AB); 852 (H_4N_1); 1031 (H_3N_2-2AB) LA: t-Man, 6-Man, 3,6-Man, 4-GlcNAc
6, H_4N_2-b	$[M+H]^+$ 1193.5; $[M+Na]^+$ 1215.4	fragments: 342 (N_1-2AB); 545 (N_2-2AB); 690 (H_3N_1); 707 (H_1N_2-2AB); 852 (H_4N_1); 1031 (H_3N_2-2AB) LA: t-Man, t-Gal, 6-Man, 3,6-Man, 4-GlcNAc
7, $H_4N_2F_1$-c	$[M+H]^+$ 1339.6; $[M+Na]^+$ 1361.2	fragments: 342 (N_1-2AB); 528 (H_2N_1); 707 (H_1N_2-2AB); **812 ($H_2N_1F_1$-2AB)**; 869 (H_2N_2-2AB); 1015 ($H_2N_2F_1$-2AB); 1177 ($H_3N_1F_1$-2AB) LA: t-Man, t-Gal, 4-Fuc, 4-Gal, 6-Man, 4-GlcNAc
8, $H_4N_2F_1$-a	$[M+H]^+$ 1339.6; $[M+Na]^+$ 1361.2	fragments: 342 (N_1-2AB); 488 (N_1F_1-2AB); 545 (N_2-2AB); 690 (H_3N_1); 852 (H_4N_1); 1193 (H_4N_2-2AB) LA[a]: t-Man, 6-Man, 3,6-Man, 4-GlcNAc
8, $H_4N_2F_1$-b	$[M+H]^+$ 1339.5; $[M+Na]^+$ 1361.4	fragments: 342 (N_1-2AB); **650 ($H_1N_1F_1$-2AB)**; 690 (H_3N_1), 1031 (H_3N_2-2AB), 1177 ($H_3N_2F_1$-2AB) LA: t-Man, t-Gal, 4-Fuc, 3,6-Man, 4-GlcNAc
9, H_5N_2	$[M+Na]^+$ 1377.1;	LA: t-Man, 3,6-Man, 4-GlcNAc
10, H_5N_3	$[M+Na]^+$ 1580.1	-
10, $H_5N_2F_1$	$[M+H]^+$ 1501.4; $[M+Na]^+$ 1523.1	fragments: 342 (N_1-2AB); 366 (H_1N_1); 488 (N_1F_1-2AB); 528 (H_2N_1); 545 (N_2-2AB); **650 ($H_1N_1F_1$-2AB)**; 690 (H_3N_1); 707 (H_1N_2-2AB); **812 ($H_2N_1F_1$-2AB)**; 852 (H_4N_1); 869 (H_2N_2-2AB); 1014 (H_5N_1); 1031 (H_3N_2-2AB); 1177 ($H_3N_1F_1$-2AB); 1193 (H_4N_2-2AB); 1339 ($H_4N_1F_1$-2AB)
11, H_6N_2	$[M+Na]^+$ 1539.1	LA: t-Man, 2-Man, 3,6-Man, 4-GlcNAc

Taken together, the characterization of KLH 2AB-labelled N-glycans by mass spectrometry, linkage analysis, enzymatic cleavage and HPLC revealed two unusual types of core modification. While two glycans were shown to exhibit a Gal(β1-4)Fuc(α1-6)-modification of the innermost GlcNAc (Figure 4A and C), a further N-glycan exhibited an extension of this motif by a second galactose, namely Gal(β1-4)Gal(β1-4)Fuc(α1-6)- (Figure 4D).

$Man_{2-6}GlcNAc_2$ and Gal(βI-6)Man-exhibiting N-glycans

Using the set of methods mentioned above, the structures of H_2N_2 (Fraction 1), $H_2N_2F_1$ (Fraction 2) and H_3N_2 (Fraction 3) were deduced as Man(α1-6)Man(β1-4)GlcNAc(β1-4)GlcNAc-2AB, Man(α1-6)Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-2AB and Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-2AB, respectively, which is in agreement with former findings (Kurokawa *et al.*, 2002). H_4N_2-a (Fraction 5) was shown to represent the tetramannosidic structure Man(α1-6)Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-2AB. Linkage analysis of H_4N_2-b (Fraction 6) revealed equal amounts of terminal mannose and terminal galactose in addition to 6-substituted mannose, 3,6-disubstituted mannose and 4-substituted GlcNAc. These data are consistent with the N-glycan structure Gal(β1-6)Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)[Fuc(α1-6)]GlcNAc-2AB, which has been elucidated before (Kurokawa *et al.*, 2002).

For H_5N_2 (Fraction 9) and H_6N_2 (Fraction 11), linkage data (Table 1) were consistent with the oligomannosidic structures Man(α1-6)[Man(α1-3)]Man(α1-6)[Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-2AB and Man(α1-6)[Man(α1-3)]Man(α1-6)[Man(α1-2)Man(α1-3)]Man(β1-4)GlcNAc(β1-4)GlcNAc-2AB, respectively. Both structures have already been elucidated (Kurokawa *et al.*, 2002). Taken together, these data confirm the former finding of oligomannosidic structures as well as Gal(β1-6)Man-substituted N-glycans (Kurokawa *et al.*, 2002).

DISCUSSION

In this study, novel KLH N-glycans were characterized which exhibit an unusual core-modification containing a 4-substituted fucose residue. Interestingly, structures with 4-substituted fucose were very inefficiently released in former experiments, in which KLH was reduced, alkylated and trypsinised before PNGase F-treatment, as evidenced by the detection of only trace amounts of 4-substituted fucose in linkage analysis. Consequently, glycans containing internal fucose were not characterized in the former study (Kurokawa *et al.*, 2002). In the present investigation, PNGase F-release was performed on KLH denatured by

application of detergents and reducing agent as well as a heat step, which resulted in a more efficient release of the unconventionally core-modified N-glycans. Similarly, efficient release of certain glycans by PNGase F treatment of an insect-derived protein has been reported to depend on protein denaturation, resulting in very different glycan populations depending on variations in protein pre-treatment (Kim *et al.*, 2003).

The novel KLH N-glycans, which exhibit a core-fucosylation that resisted fucosidase treatment (Figure 1B), were isolated and structurally elucidated, which revealed two unusual types of core substitution. While two glycans were shown to carry a Gal(β1-4)Fuc(α1-6)-modification of the innermost GlcNAc (Figure 4A and C), one other N-glycan exhibited an extension of this motif by a second galactose, namely Gal(β1-4)Gal(β1-4)Fuc(α1-6)- (Figure 4D). The unconventional Gal(β1-4)Fuc(α1-6)- core modification has so far only been described for a monoantennary N-glycan derived from octopus rhodopsin (Zhang *et al.*, 1997) and a core difucosylated N-glycan from squid rhodopsin (Takahashi *et al.*, 2003). The extended structural motif comprising the digalactosyl unit attached to the fucose is described here for the first time. Although the substructure Gal(β1-4)Gal(β1- is found as terminal or internal element in various glycoconjugates of bacteria, plants and animals, with more than 100 entries in the carbohydrate database SweetDB (http://www.dkfz-heidelberg.de/spec2/sweetdb), its attachment to fucose as elucidated in this study has not been found before. In fish, the Gal(β1-4)Gal(β1- motif has been found as terminal element in the antennae of N-glycans (Taguchi *et al.*, 1993), while it has not yet been described for mammals.

It has been well documented that core modifications may confer immunogenic properties to N-glycans. Core (β1-2)-xylose and (α1-3)-fucose residues, relatively common in plant and insect glycoproteins (Kurosaka *et al.*, 1991; Wilson & Altmann, 1998; Staudacher *et al.*, 1999) have a strong contribution in IgE binding to plant glyco-allergens (van Ree *et al.*, 2000) and similarly, allergic reactions to bee venom may be induced by core (α1-3)-fucose carrying glycans (Dudler *et al.*, 1995). In addition, several helminth species, including schistosomes (Khoo *et al.*, 1997b), and *Haemonchus contortus* (Haslam *et al.*, 1996) produce unusual core-modified N-glycans that have been implicated in the glycan-induced immune responses particularly observed in parasitic helminth infections (van Die *et al.*, 1999; Faveeuw *et al.*, 2003).

To obtain a better molecular background for understanding the complex glycan-related inmunogenic properties of KLH, it is of importance to have detailed knowledge of the

structure of KLH glycans. The Fuc(α1-3)GalNAc element, previously identified on KLH N-glycans (Kantelhardt *et al.*, 2002), is the primary cross-reactive epitope with schistosome antigens, and recently, potentially immunogenic Gal(β1-6)Man-substituted N-glycans (Kurokawa *et al.*, 2002) of KLH were described. In view of the observed immunogenicity of N-glycans with unusual, non-mammalian core modifications it is likely that the novel Gal(β1-4)Gal(β1-4)Fuc(α1-6) substitution identified in this study also contributes to the immunostimulatory properties of KLH. Clearly, many interesting aspects of KLH glycosylation have now been revealed. In view of the reported binding of peanut agglutinin to KLH (Stoeva *et al.*, 1999), and induction of antibodies reactive with Gal(β1-3)GalNAc- (Thomsen-Friedenreich antigen) by KLH (Wirguin *et al.*, 1995), questions regarding the structure of KLH glycans remain. Future research applying alternative analytical approaches based on mass spectrometry of KLH-derived glycopeptides may help to answer these questions.

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7

General discussion

The main pathology of *Schistosomiasis mansoni* develops as the result of inflammatory reactions to eggs that get trapped in host tissues, mainly the liver or intestines, instead of leaving the body via the stool. Thus, the identification and characterisation of molecular components of the egg contributes to our understanding of schistosome egg biology, parasite-host interaction, and immunopathology. In addition, detection and analysis of egg-derived molecules in the blood or urine of the host may provide insight into the number of eggs present in the tissues and related morbidity. Previously, a quantitative assay for circulating soluble egg antigens (CSEA) has been developed in our laboratory. Using two anti-carbohydrate monoclonal antibodies (mAbs) 114-4D12 and 114-5B1, CSEA could sensitively be detected in serum or urine by sandwich ELISA (Nourel Din *et al.*, 1994b). The principal part of this thesis describes the detailed characterisation studies of the *S. mansoni* egg antigens that are recognised by mAb 114-4D12. Targeted-affinity purification and identification of "114-4D12 antigens" from SEA and egg secretions, and from urine of *S. mansoni*-infected individuals is described in chapters 3, 4 and 5. As a general basis for this work, the overall screening of fucosylated glycan motifs in different stages of the parasite was performed using a set of defined anti-schistosomal mAbs (chapter 2).

SURVEY OF DEVELOPMENTALLY EXPRESSED GLYCAN MOTIFS

The main binding characteristics of the mAbs used in chapter 2 had previously been defined on the basis of a panel of synthetic glycans (van Remoortere *et al.*, 2000). In the present study the mAbs were used in complementary techniques such as Western blotting, HP-TLC overlay and IFA. Results obtained with these different techniques were in line with each other and compared to MS rapidly indicated the presence of specific glycan motifs in the different stages and structures of the parasite. These expression patterns were largely in agreement with previous (Hokke *et al.*, 2001; Hokke *et al.*, 2007a) as well as subsequent (Hokke *et al.*, 2007b) MS-based structural studies of glycan subsets isolated from preparations of various life cycle stages. The observation of intense expression of fucosylated glycan motifs in eggs correlates with the finding that in eggs fucosyltransferases are present ~50 times the levels seen in other life cycle stages (Marques Jr *et al.*, 2001). Overall, this indicates that defined anti-carbohydrate mAbs are powerful tools to study the temporal and spatial expression of (fucosylated) glycan motifs.

MAB 114-4D12 & MAB 114-5B1: BROTHER AND SISTER

It has previously been shown that mAb 114-4D12 and mAb 114-5B1 recognise antigens that are at least 10,000 times more concentrated in eggs than in adult worms (Nourel Din *et al.*, 1994a). In addition, both mAbs react with components in other stages of the schistosome life

cycle (Bogers *et al.*, 1994; Nourel Din *et al.*, 1994b; Bogers *et al.*, 1995a; Bogers *et al.*, 1995b). Likewise, other mAbs recognising different fucosylated glycan motifs, showed most intense reactivity with the egg stage on Western blot (Chapter 2). The 114-4D12- and 114-5B1-staining patterns in IFA, Western blot (compare figure 6C of chapter 1 with figure 1D of chapter 2) and HP-TLC (compare figure 6B of chapter 1 with Figure 2D of chapter 2) proof that these two mAbs indeed bind partly overlapping sets of antigens, as was previously postulated on the basis of ELISA experiments by Nourel Din *at al.* (Nourel Din *et al.*, 1994b).

The 114-4D12 epitope was identified as Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (DF-LDN-DF; chapter 3). Glycans containing the DF-LDN-DF terminal motif were previously identified in *S. mansoni* cercarial and egg glycoproteins (Khoo *et al.*, 1995; Khoo *et al.*, 1997b) and egg glycolipids (Khoo *et al.*, 1997a). Despite indications for the existence of the DF-LDN-DF motif in adult worms (figure 6C, chapter 1 and (Bogers *et al.*, 1994; Nourel Din *et al.*, 1994b)) no glycans containing the DF-LDN-DF motif have yet been detected in worms by direct analytical methods such as MS (Nyame *et al.*, 1987; Nyame *et al.*, 1988a; Wuhrer *et al.*, 2006d). The discrepancy between the detection of glycan motifs by mAbs in a tissue section or on a blot and the failure to detect the corresponding motif by a direct MS method may be explained by the lower selectivity and in particular sensitivity of the latter method compared to immunochemical probing techniques.

With surface plasmon resonance (SPR) spectroscopy it has been shown that mAb 114-5B1 strongly binds LDN-DF (van Remoortere *et al.*, 2000), FFFGn, and with an insignificant but detectable affinity FFGn (van Roon *et al.*, 2005). In a separate set of SPR experiments to compare mAb 114-5B1 to 114-4D12 it was shown that mAb 114-4D12 strongly binds to FFGn, less to FFFGn, and hardly recognises LDN-DF and FGn (Figure 1). These results suggest that the minimal glycan structure recognised by mAb 114-4D12 is Fucα1-2Fucα1-3GlcNAcβ1-. Affinity chromatography of "authentic" egg-derived glycans however resulted in the selective purification of only those egg glycans that terminate with the Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc element (DF-LDN-DF) (Chapter 2, Figure 5). This indicates that the full epitope of 114-4D12 is DF-LDN-DF. Alternatively, it may be argued that the affinity-captured egg glycans always contain the DF-LDN-DF element simply because terminal Fucα1-2Fucα1-3GalNAcβ1- exclusively occurs in combination with the Fucα1-2Fucα1-3GlcNAcβ1- element, and because terminal Fucα1-2Fucα1-3GlcNAcβ1- might not occur in schistosome-derived material (Huang *et al.*, 2001; Khoo, Huang & Lee, 2001; Jang-Lee *et al.*, 2007). The absence of 3-mono-substituted GlcNAc in SEA-4D12-B (Table 2, chapter 3) in fact also excludes that a terminal Fucα1-2Fucα1-3GlcNAcβ1- motif

occurs on 114-4D12-bound egg glycans. How the affinities of mAb 114-4D12 for Fucα1-2Fucα1-3GlcNAcβ1- (FFGn), Fucα1-2Fucα1-3GalNAcβ1- and Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc (DF-LDN-DF) relate to each other could be further investigated by SPR studies using the individual synthetic glycans.

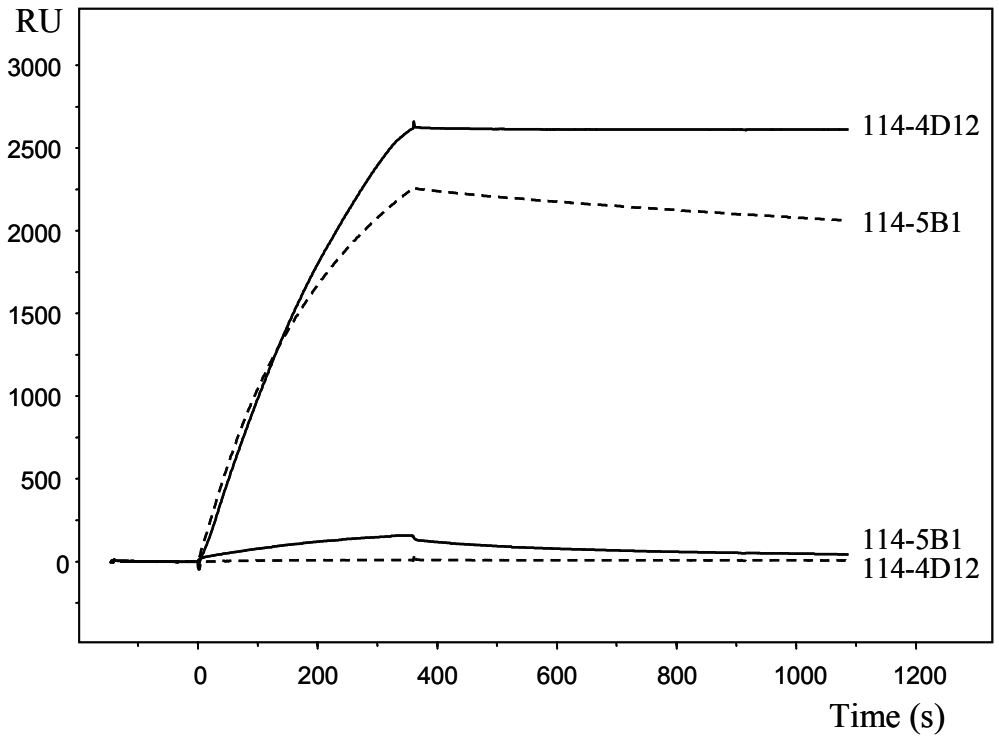

Figure 1. SPR sensorgrams obtained using a Biacore 3000 instrument (Biacore AB, Sweden). Interaction of MAb 114-4D12 (5 µg/ml) and 114-5B1 (5 µg/ml) with LDN-DF-BSA (dotted) and FFGn-BSA (line) (5000 RU immobilized).

To investigate the binding of 114-5B1 to released egg glycans, in a similar approach as for 114-4D12 (chapter 3) the 2AB-labelled hydrazinolysis-released glycan pool SEA-4D12-H was applied to a 114-5B1 affinity column (data not shown). In line with the SPR data (van Remoortere *et al.*, 2001) (Figure 1), the LDN-DF-containing glycan $H_1N_2F_3$ (see Figure 5, chapter 3) was eluted from the column in a delayed fraction, it was however not fully retained. Apparently, mAb 114-5B1 does not have the ability to strongly bind its epitope when monovalently presented, whereas mAb 114-4D12 does (chapter 3 - 5). MAb 114-4D12 seems to be unique in this aspect. In techniques, such as the diagnostic sandwich ELISA, IFA, immunoblotting and SPR, in which multivalent epitopes are available resulting in increased (combined) affinities both mAbs 114-4D12 and 114-5B1 function well.

SEA-4D12 IN COMPARISON TO OTHER EGG GLYCOPROTEIN PREPARATIONS

The glycosylation data presented in chapter 3 are in agreement with a recent structural study of (Jang-Lee *et al.*, 2007), who reported the presence of O-glycan-, but not N-glycan-associated DF-LDN-DF motifs on secreted egg proteins. In addition, Jang-Lee *et al.* detected a distinct H_2N_1 isomer in the O-glycans released by β-elimination from ESP. This isomer contained a novel core, formed by the Galβ1-4GlcNAc1-4Gal sequence in which the Gal residue is linked to the protein backbone (Jang-Lee *et al.*, 2007). At least two isomers of H_2N_1 have been detected in the hydrazinolysis-derived fraction SEA-4D12-H (see table 1, chapter 3). In the mass spectra of SEA-4D12-H the peak representing H_2N_1 was significant (data not shown) and preliminary ESI-quadrupole-TOF fragmentation data indicated the presence of both N-H-H-2AB and H-H-N-2AB sequences. Because of the massive degradation that occurred during the hydrazinolysis procedure (see chapter 3), it is not precisely known whether these short oligosaccharides were (partial) breakdown products, or whether they represented genuine intact O-glycans. It is clear, however, that glycans with the composition H_2N_1 are present also among the glycans released by β-elimination from SEA and SEA-4D12 (data not shown). Another isomer of the ESP released O-glycan H_2N_1 represented the *S. mansoni*-specific core sequence Galß1→3(Galß1→6)GalNAc (Jang-Lee *et al.*, 2007), which may also be present on SEA-4D12.

Jang-Lee *et al*. (2007) proposed that *S. mansoni* ESP only contains two major glycoproteins: Omega-1 and IPSE. IPSE does not contain the terminal DF-LDN-DF motif (Wuhrer *et al.*, 2006a), neither does Omega-1 (C.H. Hokke, personal communication). The presence of DF-LDN-DF on ESP-derived glycans as well as the 114-4D12-aided detection of egg-released compounds in IFA and in the host circulation by sandwich ELISA strongly suggests that so far unknown DF-LDN-DF-containing glycoproteins are secreted by the *S. mansoni* egg.

PROTEOMIC ANALYSIS

So far, we have not been able to identify schistosomal proteins that carry DF-LDN-DF-containing glycans. SEA-4D12 clearly consists of several glycoprotein antigens. Western blots (Figure 6D, chapter 1) and SDS-PAGE of SEA-4D12 showed several bands, predominantly at ~50 kD and above 100kD. The broadness of the bands indicates that these proteins are heterogeneously glycosylated. In addition, some bands may contain several glycoproteins.

For protein identification, bands were cut out, subjected to reduction/alkylation and trypsin digestion, and the extracted peptides were analyzed by LC-MS/MS. In SEA-4D12, α2-

macroglobulin, Sm-p40, tubulin, lactate dehydrogenase, HSP-70 and sequences with homology to α-galactosidases/N-acetylgalactosaminidases were identified. However, most of these proteins represent highly abundant egg proteins, some of which are most probably not glycosylated (Sm-p40, HSP-70), (Stadecker, Hernandez & Asahi, 2001; Curwen *et al.*, 2004) and therefore they are likely the result of non-specific binding during the immunopurification procedure. The unequivocal identification of DF-LDN-DF-containing glycoproteins through the direct analysis of glycopeptides in terms of glycan as well as peptide sequence appeared technically impossible at the moment my studies were performed. Due to the high variation in glycopeptide masses as a consequence of microheterogeneity the amount of each glycosylated isopeptide may fall below the detection limit, and the complexity of the samples may become enormous. In addition, the relatively high mass of glycopeptides, particularly if they contain more than one glycan, often renders them difficult to analyse by ion trap-MS. The continuing increase in sensitivity of MS instrumentation as well as the recent introduction of novel dissociation technologies such as ETD which is particularly suited for the analysis of post-translationally modified peptides is expected to contribute to identification of the DF-LDN-DF-containing glycoproteins in the near future.

Moreover, the schistosomal proteomics is currently probably limited by the incomplete assembly and annotation of the schistosomal genome (van Hellemond, van Balkom & Tielens, 2007). As a consequence the databases, to which the peptide identifications are submitted for a comparative search, contain either expressed sequence tags (EST), which do not contain the complete sequence of all transcribed genes and thus fail to identify some proteins, or contain genomic data, which contain high amounts of non-coding sequences (introns, transposons etc.) and thus increases the risk of false identifications. It is anticipated that the complete genomes of *S. mansoni* and *S. japonicum* will be reported in 2008. Nonetheless, despite the abundance of available sequence data, functional analysis of potential target genes will not be possible until reliable methods for reverse genetics in schistosomes become available (Brindley *et al.*, 2007).

EGG-DERIVED REDUCING OLIGOSACCHARIDES IN HOST CIRCULATION

When the structural information on the 114-4D12 epitopes was available, we analysed whether we could 114-4D12-affinity purify antigens from urine pools of *S. mansoni*-infected individuals and from egg incubate medium (egg excretory/secretory fraction). Surprisingly, we extracted unconjugated, but not peptide- or lipid-linked oligosaccharides with the terminal DF-LDN-DF motif from the urine pools as well as from the culture medium (chapter 3).

The MS-profiles generated from the 114-4D12-affinity purified oligosaccharides from infection urine (SmUrine-4D12) and egg incubate (EggESO-4D12) strongly resembled each other. This indicates that the oligosaccharides detected in the urine are of egg origin. Some differences between the EggESO-4D12 and SmUrine-4D12 fractions were observed however: in EggESO-4D12 a series of variably fucosylated linear HexNAc chains without the Hex residue is abundantly present, whereas in SmUrine-4D12 these structures are only detected as minor peaks. These differences could possibly be explained by the different developmental status of the eggs *in vivo* compared to *in vitro*. Alternatively, the observed differences between EggESO-4D12 and SmUrine-4D12 may result from specific clearance or processing of the egg-derived oligosaccharides by the host.

The free glycans we found in chapter 4 in SmUrine-4D12 are not the same antigens that are detected in the diagnostic CSEA ELISA, as the small, linear glycans only contain one epitope, whereas the presence of at least two glycan epitopes is required for detection by sandwich ELISA. The presence of the free glycans may however influence the outcome and sensitivity of the ELISA (Nibbeling *et al.*, 1997) by exhibiting inhibitory effects on binding of the mAbs to the larger, multivalent antigens. Which larger multivalent antigens are exactly detected in the sandwich ELISA thus still remains unknown. In theory, a glycan or glycopeptide containing a branched glycan thus expressing two epitopes (such as in the O-glycans represented in Figure 8, chapter 3) could possibly be bound by two mAbs at the same time. However, MALDI-TOF MS analysis of the 114-4D12 eluate from urine of infected individuals only revealed linear glycans and no indications of epitope-containing glycopeptides or branched glycans were observed. The SEA-4D12 glycoproteins as described in chapter 3 can be sandwiched in the diagnostic ELISA, but due to their size of more than 50kD these glycoproteins are not expected to be found as such in urine of infected individuals based on the classical concept of glomerular size selectivity. The intricate properties of the glomerular barrier are, however, still well hidden secrets (Haraldsson & Sorensson, 2004) and in addition in patients with heavy *S. mansoni* infection the glomeruli can be damaged resulting in proteinuria (Sobh *et al.*, 1987). More research would be needed to provide a conclusive answer to the question which schistosome egg-derived compounds are detected in the urine of *S. mansoni* infected individuals by sandwich ELISA.

IMMUNOLOGICAL INTERACTIONS WITH REDUCING OLIGOSACCHARIDES

In view of the reported glycan-dependent immunological activities of schistosome egg glycoproteins it can be speculated that unconjugated glycans may modulate those activities. In fact, it has been shown that SEA-induced IL-10 production by human peripheral blood

mononuclear cells (PBMC) is inhibited when the PBMC's were pre-treated with free LNFPIII (LeX –capped lactose) prior to SEA stimulation (Velupillai *et al.*, 2000). This suggests that immune responses to egg glycoproteins may be blocked by saturation of the host receptors such as C-type lectins or TLRs by free oligosaccharides containing similar glycan motifs as the active glycoproteins. Many studies have described the immune regulatory effects of SEA. Some of these studies observed an inhibitory effect of SEA on the activation of immune cells (Wright *et al.*, 1982; Kane *et al.*, 2004; van Liempt *et al.*, 2007) similar to Velupillai *et al*. (Velupillai *et al.*, 2000), but the existence and possible effect of free glycans in SEA was so far never taken into account. Therefore, it will be highly interesting to study the potential immunomodulatory effects of the free glycans described in this thesis.

In view of the above, it is also relevant to know whether other free glycans than those terminating with DF-LDN-DF (e.g. LeX-terminating) are being produced by schistosome eggs. In chapter 4 it was already suggested that the egg excreted/secreted reducing oligosaccharides were glycosphingolipid-derived. The smaller egg glycosphingolipid structures have been characterised by Wuhrer *et al*. (Wuhrer *et al.*, 2002), and none of the identified structures contained the terminal DF-LDN-DF motif. For example, one of the major glycans from SmUrine-4D12 is H1N4F4, with the proposed sequence Fucα1-2Fucα1-3GalNAc(Fucα1-3Fucα1-2)GlcNAc-HexNAc-HexNAc-Hex-. The structure of the glycan with the same composition H1N4F4 originating from egg glycolipids was Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-2Fucα1-3)GlcNAcβ1-3GlcNAcβ1-3GalNAcβ1-4Glc-. This suggests that variably fucosylated glycosphingolipid glycans exist, but obviously only those with a terminal DF-LDN-DF motif were purified from eggESO or SmUrine by 114-4D12 affinity purification.

To obtain an unbiased overview of the free glycans excreted/secreted by the egg more general purification techniques such as size-exclusion and solid phase extraction by graphitised carbon can be employed. An initial test (results not shown) revealed the presence of glycans with the general compositions N4Fx, H1N4Fx, H1N5Fx, H1N6Fx, and H2N6Fx (x = 3 - 7) Among these glycans, probably various non-DF-LDN-DF-containing compounds are present, due to the variable positions of the Fuc residues on the GalNAc/GlcNAc backbone.

THE POTENTIAL OF EGG EXCRETED GLYCANS AS DIAGNOSTIC MARKERS

Antigens excreted by the schistosome eggs (chapter 4 and 5) are expected to provide an estimate of the egg burden similar to the correlation observed between adult worm antigens and worm burden (Qian & Deelder, 1983). In addition, since eggs are primarily responsible

for the observed pathology in schistosomiasis, the quantitative detection of egg antigens could be expected to provide additional information on morbidity. Significant correlations have been described between the levels of CSEA (as detected by ELISA), egg counts, and ultrasound detectable pathology for *S. haematobium* (Kahama *et al.*, 1998) as well as *S. mansoni* (Hassan *et al.*, 1999) infections. Nibbeling *et al.* found that urine CSEA is still detectable six weeks after successful chemotherapy, while at that moment worm antigens had already disappeared and no viable eggs were detectable on parasitological examination, indicating that the measurement of egg antigen levels gives more insight into tissue egg load (Nibbeling, van Lieshout & Deelder, 1998). However, more research should be done to increase our knowledge about to what extent schistosome egg-derived molecular markers relate to the morbidity of the disease.

The egg-derived fuco-oligosaccharides are highly specific; so far the terminal DF-LDN-DF element has only been detected in *S. mansoni* derived molecules. Whereas in general glycosylation of different schistosome species is rather similar, the particular DF-LDN-DF terminal element is not expressed in *S. japonicum* (Khoo *et al.*, 1997a; Khoo *et al.*, 1997b). In addition, with the diagnostic 114-4D12/114-5B1 CSEA sandwich ELISA no antigens could be detected in urine or serum of *S. haematobium*- or *S. japonicum*-infected individuals (Nourel Din *et al.*, 1994b), which suggests that the DF-LDN-DF glycan epitope is also not expressed in *S. haematobium*. These observations imply that the DF-LDN-DF glycans are specific targets for the diagnosis of *S. mansoni* infections, but to offer final proof for this hypothesis urine of (heavily) *S. haematobium*- and *S. japonicum*-infected individuals would have to be tested.

Although egg antigen detection by MS is not suitable to meet the current need for more sensitive, field-applicable diagnostic tests for schistosomiasis, this novel approach might become valuable for diagnosis of travellers that return home with low intensity schistosome infections or e.g. to measure anti-fecundity effects of vaccine candidates. The fuco-oligosaccharides were detected in 9 out of 11 individual *S. mansoni* infection urines with variable infection intensities. The sample with the lowest infection intensity tested was a urine sample of an individual with 67 eggs per gram feces as mean of three consecutive duplicate 25-mg Kat Katz slides (chapter 5). Although a useful diagnostic test should positively demonstrate infection at much lower intensities, in our study we demonstrated the fuco-oligosaccharides captured from only 1 ml of this generally considered low infection intensity (<100 epg) urine sample. It is expected that detection limits will be significantly improved by increasing the urine sample volume, as was previously demonstrated (Nibbeling *et al.*, 1997).

As an additional advantage of the technique the mass-accuracy of MS guarantees that absolute specificity is retained.

FUTURE PROSPECTS

If, based on the current findings, a less labour-intensive technique could successfully be developed, larger sets of well-defined urine samples of schistosome-infected individuals should be screened for the concentration of fuco-oligosaccharides. This would allow to answer provocative questions such as whether this technique shows improved sensitivity compared to the current diagnostic methods and how these egg oligosaccharide markers relate to morbidity. The rapid improvements in automated injection and analysis techniques for off-line as well as on-line MALDI-TOF MS systems and the development of frontal affinity chromatography coupled to mass spectrometry (FAC-MS) (Ng *et al.*, 2007) should allow the high throughput analysis of large numbers of samples.

In order to facilitate further development of the technique a good internal standard would be highly valuable. Such an internal standard could be provided by synthetic DF-LDN-DF as this or other epitope-containing low molecular weight (glycan) structures are otherwise not readily available in sufficient amounts. The field of oligosaccharide synthesis has significantly improved in the last few years (Seeberger & Werz, 2007), which should allow synthesis of pure DF-LDN-DF in sufficient quantities. Fluorescent labelling of the internal standard (DF-LDN-DF) as well as of the oligosaccharide markers in urine of *S. mansoni* infected patients would allow specific and quantitative detection in chromatographic systems with fluorescence detection such as for example capillary electrophoresis (CE) directly coupled to MS.

Excretion of reducing oligosaccharides by parasite eggs has to our knowledge never been described before. It would be of interest to study whether other trematodes or nematodes also excrete reducing oligosaccharides. For these investigations a more general purification approach instead of affinity chromatography should be applied.

Analysis and characterisation of schistosome-specific glycan structures provides a basis for our understanding of the schistosome-host interactions. Basic molecular understanding of the schistosome biology, differentiation and immune regulation is of general scientific interest, but in addition provides essential information that may open –as shown in this thesis – new avenues to the development of novel diagnostics for this important disease.

List of abbreviations

LIST OF ABBREVIATIONS

2AB	2-aminobenzamide
APC	antigen presenting cell
Asn	asparagine
Asp	aspartate
ATT	6-aza-2-thiothymine
AWA	adult worm antigen
BCIP	X-phosphate/5-bromo-4-chloro-3'-indolyl-phosphate
BV	packed bead volume
CAA	circulating anodic antigen
CCA	circulating cathodic antigen
CE	capillary electrophoresis
Cer	ceramide
CFG	consortium for functional glycomics
ChS	chondroitin sulphate
CID	collision induced dissociation
CSEA	circulating soluble egg antigens
CV	column volumes
Cys	cysteine
Da	dalton
DC	dendritic cells
DeoxyHex	deoxyhexose
DHB	2,5-dihydroxybenzoic acid
DF	difucose, Fucα1-2Fucα1-3
DF-LDN-DF	Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc
DS	dermatan sulphate
ECD	electron capture dissociation
EggESO-4D12	114-4D12 affinity purified fraction of egg excretory/secretory oligosaccharides
ELISA	enzyme linked immuno sorbent assay
Epg	eggs per gram (feces)
ER	endoplasmatic reticulum
ES	excrery/secretory oligosaccharides from *S. mansoni* eggs
ESI	electrospray-ionisation
ETD	electron transfer dissociation
FAB-MS	Fast atom bombardment-mass spectrometry
FCS	fetal calf serum
FGn	Fucα1-3GlcNAc
FFGn	Fucα1-2Fucα1-3GlcNAc
FFFGn	Fucα1-2Fucα1-2Fucα1-3GlcNAc
F-LDN	Fucα1-3GalNAcβ1-4GlcNAc
F-LDN-F	Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAc
FT-ICR MS	Fourier transform ion cyclotron resonance MS
Fuc, F	*L*-fucose
GAG	glycosaminoglycan
GASP	gut-associated proteoglycan
GC-MS	gass chromatography-MS
Gal	*D*-galactose

GalNAc	*N*-acetyl-*D*-glucosamine
Glc	*D*-glucose
GlcA	glucaronic acid
GlcNAc	*N*-acetyl-*D*-glucosamine
GPI anchor	glycosylphosphatidylinositol-anchor
HA	hyaluronic acid
Hep	heparin
Hex, H	hexose
HexNAc, N	*N*-acetylhexosamine
HS	heparan sulphate
HSA	human serum albumin
IFA	immunofluorescence assay
IFN-γ	interferon-gamma
IRMPD	infrared multi photon dissociation
HPLC	high performance liquid chromatography
IE	immunoelectrophoresis
Ig	immunoglobulin
IL	interleukin
IT	ion trap
IRMPD	infrared multi photon dissociation
kD	kilo dalton
KLH	Keyhole limpet hemocyanin
KS	keratan sulphate
LC-MS	liquid chromatography-MS
LDN	LacdiNAc, GalNAcβ1-4GlcNAc
LDN-F	GalNAcβ1-4(Fucα1-3)GlcNAc
LDN-DF	GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAc
Lewis X, Le^X	Galβ1-4(Fucα1-3)GlcNAc
Lewis Y, Le^Y	Fucα1-2Galβ1-4(Fucα1-3)GlcNAc
LN	LacNAc, Galβ1-4GlcNAc
LNFPIII	lacto-N-fucopentaose
mAb	monoclonal antibody
MALDI	matrix-assisted laser desorption ionisation
MALDI-TOF MS	matrix-assisted laser desorption ionisation time-of-flight MS
Man	*D*-mannose
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MW	molecular weight
NBT	4-nitro blue tetrazolium chloride
NegUrine-4D12	114-4D12 affinity purified fraction of urine of endemic schistosome-negative individuals
NMR	nuclear magnetic resonance
NP	normal phase
PBMC	peripheral blood mononuclear cells
PCR	polymerase chain reaction
Pen, P	pentose
PGE_2	prostaglandin E_2

PNGase F	*N*-glycosidaseF,peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F from *Flavobacterium meningosepticum*
Pro	proline
RP	reverse phase
SEA	soluble egg antigen
SEA-B	glycans released from SEA by reductive β-elimination
SEA-4D12	mAb 114-4D12 affinity purified subset of SEA
SEA-4D12FT	SEA flow through fraction from mAb 114-4D12 affinity purification
SEA-4D12-H	glycans released from SEA-4D12 by hydrazinolysis
Ser	serine
SmUrine-4D12	mAb 114-4D12 affinity purified fraction of *S. mansoni* infection urine
TFA	trifluoroacetic acid
Th	T-helper
Thr	threonine
TLC	thin-layer chromatography
TLR	toll-like-receptor
WormESO-4D12	mAb 114-4D12 affinity purified fraction of worm-incubate supernatant
Xyl	D-xylose

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Summary

SUMMARY

Schistosomiasis, also known as bilharzia, is a disease that still occurs in many parts of Africa, the Middle East and Southern America, mainly Brasil and the Caribbean. Schistosomiasis is, after malaria, the second most common parasitic infection. Currently 200 million people are infected with the worms that cause the disease. *Schistosoma mansoni* is the most common schistosome species that infects humans.

S. mansoni uses freshwater snails of the species Biomphalaria as an intermediate host. In the snails asexual reproduction occurs and cercariae (larvae with a bifurcated tail) develop. When the cercariae emerge they propel through the water actively seeking for their final host. Upon contact with the human host the cercariae penetrate the skin. The parasite then migrates, via the circulation through the lungs and the hepatoportal circulation where they pair and develop into adult worms, before migrating to the mesenteric veins. The adult female worm, which is cylindrical, thinner and longer than the male, resides within the gynaecophoric canal of the adult male worm (measuring 1 to 2 cm long). The canal is a modification of the ventral surface of the male and forms a groove. The female worm lays hundreds of eggs (around 300) a day. It is assumed that approximately half of the eggs migrate through the wall of the intestine and are excreted in the faeces. Upon contact with fresh water, ciliated miracidia hatch from the excreted eggs and may then infect the intermediate snail host to complete the lifecyle. The other half of the eggs is taken by the blood flow and becomes lodged in the liver or intestines, where a plethora of excreted egg antigens induce strong granulomatous inflammatory responses. These inflammatory responses around the trapped eggs are the main causes of the chronic symptoms of the disease. Effective treatment exists, but in endemic areas people often get re-infected. No vaccine against schistosomiasis is currently available.

The schistosome produces many different sugar structures (glycans) that are not made by humans. Central to this thesis are sugar structures with a “double fucose” (fucose is a monosaccharide like glucose). Such fucosylated glycans are mainly produced by the eggs, which play a major role in the disease of schistosomiasis, mainly produce the fucosylated glycans. The human immune defence system responds to these parasite specific structures in several ways. High antibody responses have been measured against sugar structures with a “double fucose” moiety and moreover different types of immune cells are known to interact with these glycan structures. Despite these immunological responses the human host is not able to clear the parasitic infection. The adult worms survive for many years (up to 35 years) in the host’s circulation. The glycans are thought to play a role in the mechanisms that *S. mansoni* has developed to survive in the hostile environment of the human blood. In this

thesis the structures of several glycans containing the "double fucose" moiety have been characterised using different mass spectrometric techniques.

Chapter 1 is a general introduction on schistosomiasis and describes the different species of *Schistosoma*, the parasite's life cycle (figure 1), the symptoms of the disease, diagnostic methods and current and potential intervention methods. The second part of the introduction contains information on schistosome glycoconjugates and their interactions with the human host. In addition, techniques used to study glycans or glycoconjugates are discussed.

The research described in this thesis is based on the results of previous research performed in our group, in which a sandwich ELISA using two monoclonal antibodies (mAbs) was developed to measure specific *S. mansoni* egg glycoconjugates. The major part of this thesis concerns the identification and characterisation of the glycan structures that are recognised by one of these monoclonal antibodies, named mAb 114-4D12. In addition, the potential of these glycans for the development of new diagnostic methods is discussed.

Chapter 2 describes the mapping of fucosylated epitopes on glycoproteins and glycolipids of different life cycle stages of *S. mansoni*. Glycoprotein and glycolipid extracts from cercariae, adult worms and eggs were separated by SDS-PAGE and HP-TLC respectively, and were subsequently stained with a monoclonal antibody, which was known to recognise fucosylated glycans. Six different monoclonal antibodies, all recognising different fucosylated glycan epitopes were used in this study. Information was obtained about the type of carrier (protein or lipid) and in which life cycle stage these fucosylated glycan epitopes were expressed. The study showed that fucosylated glycans were most extensively expressed on egg glycoproteins.

In **chapter 3** the glycan epitope and the epitope containing glycan structures that are recognised by mAb 114-4D12, were identified. The 114-4D12 epitope containing glycans were isolated from *S. mansoni* SEA (soluble egg antigens) using an efficient 114-4D12 affinity purification method. The affinity purified fraction of SEA (SEA-4D12) contained predominantly O-glycans that were released from their protein backbones by two different chemical glycan-releasing methods namely hydrazinolysis and beta-elimination, to allow detailed analysis of the released glycans. Subsequently the acquired glycans were analysed using different mass spectrometric methods (MALDI-TOF MS and LC-MS) and additional glycan characterisation techniques such as 'linkage analysis'. The SEA-4D12-glycans were shown to be significantly richer in fucose compared to the glycans released from 'normal SEA'.

For reasons not yet understood a high degree of degradation occurred during the hydrazinolysis procedure, which resulted in the release of smaller, linear glycan structures with a galactose at the reducing end. These glycans released by hydrazinolysis were subsequently labelled with the fluorescent 2AB-label to facilitate efficient purification and sensitive detection. The 2AB-labelled glycans obtained by hydrazinolysis proved very useful for determining the exact mAb 114-4D12 glycan epitope. The pool of 2AB-labelled glycans was subjected to 114-4D12 affinity chromatography and only the epitope-containing glycan structures ($H_1N_2F_4$ and $H_1N_3F_6$) were captured on the 114-4D12 column and as a result were clearly separated from all other glycans. By doing this, the minimal glycan epitope of mAb 114-4D12 was identified as Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1-. This epitope occurs predominantly on branched O-glycans based on a GlcNAcβ1-6(Galβ1-3)GalNAc- type 2 'core-structure' with compositions $H_1N_{3-8}F_{4-12}$.

From the above described study it appeared that mAb 114-4D12 has unique abilities to bind a monovalently presented glycan epitope. We therefore decided to perform 114-4D12 affinity purification on a urine-pool from *S. mansoni* infected individuals and to study the elution fraction for epitope containing structures using MALDI-TOF MS. The result of this test was described in **chapter 4**. To our surprise a sharp and characteristic MALDI-TOF MS peak pattern was generated, of which the putative compositions suggested that a series of free glycans composed of one hexose (H), three to seven N-acetylhexosamines (N) and four to ten fucoses (F) ($H_1N_{3-7}F_{4-10}$) was captured. The reducing glycans were then 2AB-labelled, purified and further analysed by LC-MS. The question whether these glycans derived from the parasite could be answered by incubating *S. mansoni* worms and eggs in cell culture medium. The worm- and egg-supernatants were subsequently 114-4D12 affinity-purified in a similar way as the *S. mansoni* infection urine-pool. The 114-4D12-eluate of the *S. mansoni* egg-supernatant contained highly fucosylated free glycans while the 114-4D12 worm-eluate contained no peaks, and thus no free fucosylated glycans. The series of free glycans that were excreted by the eggs contained similar peaks to those visible in the 114-4D12 affinity-purified urine fraction and in addition contained a series of fucosylated glycans with composition: $N_{4-6}F_{4-10}$.

To our knowledge this is the first time that parasite eggs have been described to produce and excrete free glycans. At present, we neither know the synthesis route of these free glycans, nor whether in addition to the 114-4D12 recognised series of free glycans other glycans without or with less fucoses are produced.

In chapter 4 we described the technique for 114-4D12 affinity purification of *S. mansoni* egg-derived free glycans from a pool of urine samples from *S. mansoni* infected individuals. In **chapter 5** we investigated the possibilities of using these molecules as markers for infection for individual urine samples. An initial test was performed on 11 urine samples (1ml) from *S. mansoni* infected Senegalese individuals with variable intensity of infection. A specific MALDI-TOF MS peak pattern was obtained from nine out of the eleven infection urines. The described method was relatively labour intensive, which unfortunately did not allow us at that stage to test a much larger set of infection urines.

As the free glycans are excreted by the eggs, these structures could be seen as 'egg-load' related infection markers. As granuloma formation around the trapped eggs in liver or gut is the main cause of pathology the glycans are potential morbidity markers. To date there is still a need for a sensitive and highly specific method to diagnose people with light to moderate Schistosoma infections. Regularly travellers return home infected with schistosomiasis and infection intensity from the endemic population decreases due to expanded treatment programmes. For these reasons it would be worthwhile developing a (novel) sensitive and highly specific diagnostic method based on the detection of these free egg glycans. The method should also be less labour intensive in order to allow large sets of urines to be tested.

Keyhole limpet hemocyanin (KLH) is a glycoprotein from the mollusc *Megathura crenulata* that shares a glycan motif with *Schistosoma mansoni*. Based on this similarity KLH can be used for serodiagnosis purposes, by demonstrating the presence of cross-reactive antibodies in serum of *S. mansoni* infected patients. The immunogenic KLH glycans can, similarly to *S. mansoni* glycans induce granulomata. Moreover KLH has found several biomedical applications as immunogenic agent. In chapter 2 we demonstrated that the KLH / *S. mansoni* cross-reactive glycan epitope is terminal F-LDN-F, and that this glycan motif occurs on egg excreted glycoproteins and on glycolipids in parenchymal "spots or ducts" in adult worms. **Chapter 6** describes the characterisation of the KLH N-glycans and the identification of a new 'core'-modification: (Galβ1-4)$_{\pm}$Galβ1-4Fucα1- units (α1-6)-linked to the reducing end N-acetylglucosamine residue. This glycan modification does not occur on *S. mansoni* N-glycans, but is expected to be immunogenic, like other non-mammalian-type 'core'-modifications.

The closing chapter of this thesis contains a general discussion, **chapter 7**, which places the individual chapters into context and describes several aspects of our studies that were not yet described in one of the other chapters. The related diagnostic monoclonal antibodies 114-4D12 and 114-5B1 are mutually compared and the epitope characterisation method based on

natural glycans for 114-4D12 (as described in this thesis) is compared to the epitope characterisation methods based on synthetic glycan epitopes as previously employed for mAb 114-5B1. In addition the results of preliminary proteomics analysis of the 114-4D12 affinity purified glycoproteins are presented. Finally, future prospects are discussed.

Samenvatting

SAMENVATTING

De ziekte schistosomiasis, ook bekend als bilharzia, komt nog steeds in grote delen van de wereld voor. Na malaria is het de meest voorkomende parasitaire infectieziekte, momenteel zijn ruim 200 miljoen mensen besmet. Er zijn verschillende soorten *Schistosoma* die de mens als eindgastheer hebben, waarvan *Schistosoma mansoni* de meest voorkomende is. *S. mansoni* komt voor in grote delen van Afrika, het midden oosten en Zuid Amerika, met name in Brazilië en de Caraïben.

Slakken van het geslacht *Biomphalaria* die voorkomen in stilstaand zoet water worden door *S. mansoni* als tussengastheer gebruikt. Nadat de larven zich in de slak hebben vermenigvuldigd en ontwikkeld, komen vrijzwemmende cercariae met een gevorkte staart vrij. Mensen raken besmet wanneer zij in contact komen met water waarin cercariae leven. De cercariae dringen binnen een paar seconden de huid binnen en ontwikkelen zich in de aderen tot volwassen wormen. *S. mansoni* wormen leven in de poortaderen bij de lever. De mannetjes en vrouwtjes leven gepaard, het kortere (1 tot 2 cm) en plattere mannetje krult zich als het ware in de lengte om het langere, dunnere en ronde vrouwtje. Het vrouwtje legt honderden (circa 300) eitjes per dag. Ongeveer de helft van de eitjes verlaat het lichaam via de ontlasting. Als de eitjes in contact komen met zoet water barsten de eitjes open en komt het miracidium naar buiten dat zich met behulp van trilharen op het oppervlak voortbeweegt. Als het miracidium een slak infecteert is de levenscyclus volledig. De eitjes die het lichaam niet met de ontlasting verlaten, worden meegevoerd door de bloedstroom en komen terecht in lever of darmen waar zij ontstekingen veroorzaken. Deze ontstekingen rond de “verdwaalde” eitjes zijn de belangrijkste veroorzakers van de symptomen van de ziekte. Er is een effectief medicijn, maar in endemische gebieden raken mensen na behandeling meestal snel opnieuw besmet. Er is geen vaccin beschikbaar.

De parasiet produceert veel verschillende suikerstructuren (glycanen) die niet door mensen worden gemaakt. In dit proefschrift spelen suikerstructuren met een “dubbele fucose” (fucose is een monosacharide, net als glucose) een centrale rol. In het bijzonder de eieren, die bij de ziekte schistosomiasis een belangrijke rol spelen, produceren veel van deze gefucosyleerde suikerstructuren. Het menselijke afweersysteem reageert op deze vreemde structuren: er zijn hoge antilichaamresponsen gemeten tegen deze “dubbele fucose” en ook worden deze structuren herkend door verschillende typen afweercellen. Ondanks de immunologische reacties tegen deze en andere parasitaire componenten is het lichaam toch niet in staat de parasiet op te ruimen: de volwassen *Schistosoma*-wormen kunnen tot tientallen jaren in de bloedbaan van de mens overleven. Mogelijk spelen juist glycanen een rol bij de ontsnappingsmechanismen die *S. mansoni* heeft ontwikkeld om zo lang mogelijk in

“harmonie” met zijn gastheer te kunnen leven. In dit proefschrift is de structuur van verschillende glycanen met de “dubbele fucose” met behulp van massa-spectrometrie gekarakteriseerd.

In **hoofdstuk 1** wordt een algemene introductie gegeven over schistosomiasis: de verschillende soorten *Schistosoma*, de levenscyclus van de parasiet (figuur 1), de ziekteverschijnselen, methoden voor diagnostiek en de huidige en toekomstige bestrijdingsmethoden. Het tweede gedeelte van de inleiding behandelt de glycoconjugaten (glycanen zijn meestal gebonden aan een drager zoals eiwitten of lipiden) die schistosomen produceren en de mogelijke rol die zij spelen bij de interactie tussen de parasiet en zijn gastheer. Vervolgens worden de technieken besproken die kunnen worden gebruikt om glycanen of glycoconjugaten te analyseren.

Dit onderzoek is gebaseerd op de resultaten van eerder onderzoek in Leiden waarbij, met behulp van twee monoklonale antilichamen (mAbs), specifieke glycoconjugaten uit *S. mansoni* eieren met een sandwich ELISA in serum of urine van patiënten konden worden aangetoond. Het grootste deel van dit proefschrift beschrijft in detail welke glycanen een van deze twee antilichamen, mAb 114-4D12, herkent en op welke manier deze kunnen worden gebruikt voor het ontwikkelen van een nieuwe, potentieel gevoeliger diagnostische methode.

In **hoofdstuk 2** worden allereerst de antigenen in kaart gebracht die worden herkend door zes andere antilichamen waarvan we weten dat deze ook allemaal glycanen met een fucose herkennen. De glycoproteïnen en glycolipiden van drie verschillende stadia van de parasiet (cercariën, volwassen wormen en eieren) zijn gescheiden, met behulp van respectievelijk SDS-PAGE en HP-TLC en vervolgens aangekleurd met de monoklonale antilichamen. Op deze manier is informatie verkregen over in welk stadium en op welke drager (eiwit of lipide) de verschillende glycanen tot expressie komen. Met name de eiwitten van het ei bleken zeer rijk aan gefucosyleerde glycanen.

Hoofdstuk 3 beschrijft de identificatie van de glyco-epitoop die wordt herkend door mAb 114-4D12 en de onderliggende glycanen. Uit *S. mansoni* SEA (soluble egg antigens/oplosbare ei-antigenen) worden de 114-4D12-epitoop bevattende ei-antigenen (SEA-4D12) geïsoleerd met behulp van een efficiënte 114-4D12-affiniteitszuiveringmethode. SEA-4D12 bevat voornamelijk O-glycanen die zijn afgesplitst met behulp van twee verschillende chemische methoden: hydrazinolyse en bèta-eliminatie om een goede complete analyse mogelijk te maken. Vervolgens zijn de glycanen geanalyseerd met behulp van MALDI-TOF MS, LC-MS en aanvullende karakterisatietechnieken zoals ‘linkage analysis’.

De glycanen van SEA-4D12 zijn zeer rijk aan fucoses ten opzichte van de glycanen van 'gewoon SEA'. Tijdens de hydrazinolyse is door onbekende oorzaak in hoge mate degradatie opgetreden waardoor veel kleinere, lineaire structuren ontstonden met een galactose aan het reducerende eind, die ten behoeve van zowel zuivering als detectie met het fluorescente 2AB werden gelabeld. Deze 2AB-gelabelde kleine glycanen bleken uiteindelijk zeer waardevol voor het bepalen van de exacte glyco-epitoop van mAb 114-4D12. Op de pool van 2AB-gelabelde SEA-4D12 glycanen werd 114-4D12 affiniteitchromatografie toegepast. Alleen de epitoop-bevattende glycanen (H1N2F4 en H1N3F6) werden door het antilichaam gebonden en konden zo van de andere glycanen gescheiden worden. Op deze manier werd de glyco-epitoop van diagnostisch mAb 114-4D12 geïdentificeerd als Fucα1-2Fucα1-3GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAcβ1-. De oorspronkelijke O-glycanen die deze epitoop bevatten bleken voornamelijk vertakte structuren gebaseerd op een GlcNAcβ1-6(Galβ1-3)GalNAc-type 2 'core-structuur' met composities $H_1N_{3-8}F_{4-12}$.

Omdat uit de hierboven beschreven studie bleek dat mAb 114-4D12 de eigenschappen bezit om een monovalent gepresenteerde epitoop te binden, werd besloten om op een urinepool van mensen die met *S. mansoni*-geïnfecteerd waren, 114-4D12-affiniteitszuivering toe te passen en het eluaat te bestuderen op epitoop-bevattende structuren met behulp van MALDI-TOF MS. De resultaten hiervan worden beschreven in **hoofdstuk 4**. Tot onze verbazing werd een MALDI-TOF MS spectrum gegenereerd met een scherp piekenpatroon, waarvan de vermeende composities duidden op een serie vrije glycanen bestaande uit een hexose (H), drie tot zeven N-acetylhexosamines (N) en vier tot tien fucoses (F) ($H_1N_{3-7}F_{4-10}$). De reducerende glycanen werden 2AB-gelabeld, gezuiverd en verder geanalyseerd met behulp van LC-MS. De vraag of deze vrije glycanen afkomstig zijn van de parasiet werd beantwoord door zowel *S. mansoni* eieren als wormen te incuberen in medium en het supernatant ook met behulp van 114-4D12 affiniteitschromatografie te zuiveren. De *S. mansoni* eieren bleken de vrije glycanen uit te scheiden, de wormen niet. Naast de serie vrije glycanen die zichtbaar was in urine werd in het supernatant van eieren nog een serie gefucosyleerde glycanen gedetecteerd met compositie: $N_{4-6}F_{4-10}$.

Het is voor zover wij weten voor het eerst dat is beschreven dat een parasiet vrije glycanen produceert. Hoe de synthese hiervan verloopt is vooralsnog onduidelijk. Ook weten wij niet of naast deze gefucosyleerde structuren nog andere vrije glycanen met minder of zonder fucoses worden geproduceerd.

Mogelijk zouden de in dit proefschrift beschreven ei-gesecreteerde glycanen kunnen worden gebruikt als *S. mansoni* infectiemarkers. Om dat te onderzoeken is in **hoofdstuk 5** de 114-

4D12 affiniteitszuiveringmethode toegepast op 11 urines (1ml) van *S. mansoni* geïnfecteerde personen uit Senegal bij wie de intensiteit van infectie varieerde. Omdat de ontwikkelde methode zeer arbeidsintensief was, konden helaas geen grote aantallen urines op deze manier worden getest. In negen van de elf positieve urines werden de vrije glycanen met MALDI-TOF MS gedetecteerd. De negen geteste urines van niet geïnfecteerde personen uit Burundi vertoonden geen pieken in MALDI-TOF MS.

Omdat de vrije glycanen van eieren afkomstig zijn, zouden deze structuren kunnen worden gezien als 'ei-last' gerelateerde infectiemarkers terwijl ze tevens, omdat de granuloomvorming rond de achtergebleven eieren in de weefsels (met name lever en darm) de voornaamste oorzaak is van de pathologie van de ziekte, potentieel hebben als morbidity markers. Bovendien is er een steeds grotere behoefte aan een zeer gevoelige en specifieke methode om mensen met een lichte Schistosoma-infectie te kunnen diagnosticeren. Door het stijgend aantal reizigers naar verre bestemmingen komen steeds meer mensen thuis met een lichte Schistosoma-infectie en door succesvolle grootschalige behandelprogramma's daalt de intensiteit van infecties van de lokale bevolking. Het zou daarom zeer de moeite waard zijn om te kijken of het mogelijk is op basis van de vrije ei-glycanen een diagnostische methode te ontwikkelen die zeer gevoelig is en minder arbeidsintensief dan de methode zoals beschreven in hoofdstuk 5 zodat grote aantallen urines zouden kunnen worden getest.

Keyhole limpet hemocyanin (KLH) is een glycoproteïne van de mollusk *Megathura crenulata* dat een glycomotief deelt met *S. mansoni.* Op basis van deze kruisreactiviteit kan KLH worden gebruikt om de aanwezigheid van anti-*Schistosoma mansoni* antilichamen aan te tonen in serum van patiënten ten behoeve van diagnose. De immunogene KLH glycanen zijn net als glycanen van *S. mansoni* in staat granuloma te induceren en worden naast het gebruik voor diagnose van schistosomiasis voor verschillende medische toepassingen gebruikt. In hoofdstuk 2 hebben we laten zien dat de kruisreactieve glyco-epitoop terminaal F-LDN-F is, en dat dit motief voorkomt op ei-gesecreteerde glycoproteïnen en glycolipiden in en rond "kanalen" in het parenchym van volwassen wormen. **Hoofdstuk 6** beschrijft de karakterisatie van de N-glycanen van KLH en de identificatie van een nieuwe 'core'-modificatie: (Galβ1-4)$_{\pm}$Galβ1-4Fucα1-6 op de reducerende N-acetylglucosamine. Deze moficatie komt niet voor op *S. mansoni* N-glycanen.

Dit proefschrift wordt afgesloten met een algemene discussie, **hoofdstuk 7**. Daarin worden verbanden gelegd tussen de hoofdstukken en worden een aantal andere facetten van het onderzoek besproken waar in de individuele hoofdstukken geen ruimte voor was. De gerelateerde monoklonale antilichamen 114-4D12 en 114-5B1 worden onderling vergeleken

en de 114-4D12 epitoop karakterisatie op basis van natuurlijke glycanen wordt vergeleken met de 114-5B1 epitoop karakterisatie op basis van synthetische suikerstructuren. De resultaten en beperkingen van initiële proteomics analyses aan de 114-4D12 gezuiverde ei-antigenen worden gepresenteerd. Tot slot worden suggesties gegeven voor toekomstig onderzoek.

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CURRICULUM VITAE

Marjolein Louise Maria Robijn was born in Leidschendam on the 5th of August 1978. After she passed her V.W.O. exams at the Alfrink College in Zoetermeer in 1996, she commenced her academic studies in biomedical sciences at the Vrije Universiteit Amsterdam. From June to December 1999 she performed an undergraduate research project in medical microbiology at the Vrije Universiteit Amsterdam under the supervision of Dr B. J. Appelmelk. A second research internship was performed in the group of Professor I. R. Poxton in the field of microbial infection and immunity at the University of Edinburgh, for which she received support from an EU Erasmus 'free-movers' grant. Her stay in Edinburgh realised a long cherished wish to study abroad. Her masters thesis entitled "*Molecular aspects of schistosome-and other helminths - host modulatory compounds*" was written in preparation of her PhD at the department of Parasitology, Leiden University Medical Centre in Leiden. In December 2000 she obtained her doctorandus (Master of Science) degree.

The PhD project at the department of Parasitology, Centre for Infectious Diseases in Leiden started in January 2001 under the supervision of Professor A. M. Deelder and Dr C. H. Hokke. In 2002 she spent one month in the group of Professor P. M. Rudd at the Oxford Glycobiology Institute in Oxford, United Kingdom. Her study on the identification and interactions of glycosylated *S. mansoni* egg excreted antigens involved different glycosylation analysis-, proteomics- and mass spectrometric techniques and led to a European patent application. The thesis resulting from this work is entitled "*Targeted identification of* Schistosoma mansoni *egg glycans*".

Since November 2005 she works as a project officer for the European and Developing Countries Clinical Trial Partnership (EDCTP). This organisation aims through European research integration and in partnership with African countries to develop new clinical interventions to fight HIV/AIDS, malaria and tuberculosis.

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The greatest obstacle to discovery is not ignorance - it is the illusion of knowledge.

Daniel J. Boorstin