Parasite glycans and antibody-mediated immune responses in *Schistosoma* infection

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SUMMARY

Schistosome infections in humans are characterized by the development of chronic disease and high re-infection rates after treatment due to the slow development of immunity. It appears that anti-schistosome antibodies are at least partially mediating protective mechanisms. Efforts to develop a vaccine based on immunization with surface-exposed or secreted larval or worm proteins are ongoing. Schistosomes also express a large number of glycans as part of their glycoprotein and glycolipid repertoire, and antibody responses to those glycans are mounted by the infected host. This observation raises the question if glycans might also form novel vaccine targets for immune intervention in schistosomiasis. This review summarizes current knowledge of antibody responses and immunity in experimental and natural infections with *Schistosoma*, the expression profiles of schistosome glycans (the glycome), and antibody responses to individual antigenic glycan motifs. Future directions to study anti-glycan responses in schistosomiasis in more detail in order to address more precisely the possible role of glycans in antibody-mediated immunity are discussed.

Key words: *Schistosoma*, antibodies, glycans, immune response.

SCHISTOSOMIASIS

Schistosomiasis is a chronic and potentially deadly parasitic disease caused by members of the helminth genus *Schistosoma* of which *S. haematobium*, *S. mansoni* and *S. japonicum* are the most widespread in humans. Approximately 207 million people in tropical and sub-tropical areas are infected with schistosomes and 779 million people are at risk of being infected (Gryseels *et al.* 2006; Steinmann *et al.* 2006). Schistosomes have a complex life-cycle in which larval, adult worm and egg stages interact with the human host, each playing a role in immunology, immunopathology and maintenance of infection. Eggs deposited in the organs of the host cause a strong immune response leading to the formation of periovular granulomas which eventually may give rise to fibrosis and organ failure.

Two immunologically distinct phases develop after a schistosome infection, the acute and the chronic phase. Acute schistosomiasis occurs during a primary infection upon first contact with the parasite while chronic schistosomiasis develops when immune responses become modulated, as observed in individuals living in endemic areas with continuous exposure to the parasites. Development of pathology depends on individual immune responses and infection intensity, but may lead to life-threatening inflammatory and obstructive disease (Gryseels *et al.* 2006).

Despite the strong immune response mounted, schistosomes can survive for years in the human host (Caldas *et al.* 2008). Effective treatment of schistosomiasis, at the individual level or in mass treatment programmes, is mainly by use of the chemical agent Praziquantel (PZQ) (Fenwick and Webster, 2006; Gray *et al.* 2011), and alternative anti-schistosomal drugs are currently also being investigated (Utzinger *et al.* 2010). However, drug treatment does not prevent reinfection and therefore a more definitive solution may be the development of a prophylactic vaccine inducing protection against schistosomiasis. Detailed epidemiological and immuno-epidemiological studies in endemic areas have shown that natural human immunity to *Schistosoma* does occur, although it takes many years of infection to develop (Wilkins *et al.* 1987; Butterworth *et al.* 1988*b*; Fulford *et al.* 1992; Ouma *et al.* 1998; Vereecken *et al.* 2007). Young children are far more susceptible to re-infection than older children and adults even in communities where adults are more exposed than children (Kabatereine *et al.* 1999). Other studies have also shown a peak shift in prevalence and intensity of infection with age (Woolhouse *et al.* 1991; Fulford *et al.* 1992; Muller-Graf *et al.* 1997; Mutapi *et al.* 1997) which provides

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evidence for a gradually acquired protective immunity in *Schistosoma* infection (Woolhouse, 1998).

PROTECTIVE ROLE OF ANTIBODIES IN *SCHISTOSOMA* INFECTION

Several immunological parameters, including specific antibody and T cell responses, are predictive of the age-dependent immunity or susceptibility to re-infection after treatment (Butterworth *et al*. 1988*a*; Khalife *et al*. 1989; Leenstra *et al*. 2006; Vereecken *et al*. 2007). In the mouse model, it has been shown that B cell-deficient mice singly vaccinated with radiation-attenuated *S. mansoni* cercariae are significantly less protected against challenge infection than vaccinated wildtype mice (Jankovic *et al*. 1999), suggesting a role for antibodies in immunity. Furthermore, repeated vaccination increased protection in wildtype mice but not in B cell-deficient mice (Anderson *et al*. 1999; Jankovic *et al*. 1999). Serum transfer studies with rabbits, rats and mice showed that serum from vaccinated animals can protect against infection with *S. mansoni* when transferred to non-vaccinated animals (Ford *et al*. 1984; Bickle *et al*. 1985; Mangold and Dean, 1986). Also in baboons and vervet monkeys vaccination with irradiated *S. haematobium* and *S. mansoni* cercariae, respectively, results in high antibody titers correlating with protection (Harrison *et al*. 1990; Yole *et al*. 1996).

In natural human infection, protective IgE, IgG and IgA-mediated immune responses have been reported (Black *et al*. 2010). IgA and IgE are isotypes that can mediate antibody-dependent cellular cytotoxicity of schistosomula *in vitro* (Butterworth *et al*. 1975, 1977; Capron *et al*. 1978; Dunne *et al*. 1993) and high levels of IgE and IgA against adult worm antigens have been associated with increased resistance to re-infection after treatment (Dunne *et al*. 1992; Hagan *et al*. 1991; Naus *et al*. 1998; Walter *et al*. 2006; Vereecken *et al*. 2007; Jiz *et al*. 2009; Black *et al*. 2010). One of the most predominant antigens for protective IgE antibody responses is SmTAL1 (formerly known as Sm22·6) (Dunne *et al*. 1992, 1997; Fitzsimmons *et al*. 2007). IgE response patterns against SmTAL1 resemble those against adult worm antigen (Walter *et al*. 2006) and are associated with reduced odds of re-infection (Dunne *et al*. 1992, 1997; Webster *et al*. 1996; Pinot de Moira *et al*. 2010), indicating that anti-SmTAL1 IgE could be a factor in host defence or a marker for resistance against infection. Although mice also produce large amounts of IgE upon *Schistosoma* infection (De Oliveira Fraga *et al*. 2010) the role of this IgE is not clear. Some reports have shown a protective role in primary infection, while others show a detrimental or even the absence of a functional role for IgE (Amiri *et al*. 1994; King *et al*. 1997; El *et al*. 1998), which may be partly explained by the lack of expression of the high affinity receptor FcεR1 on mouse eosinophils (De Andres *et al*. 1997).

With respect to IgG antibodies, immuno-epidemiological studies have suggested that IgG1 and IgG3 are correlated with protection to reinfection, while IgG2 has a dual function. In the presence of activated eosinophils IgG2 has an effector function, but in the presence of normal eosinophils it acts as a blocking antibody with detrimental consequences for the expression of protective immunity (Butterworth *et al*. 1988*a*; Khalife *et al*. 1989). The latter observations have been further supported by *in vitro* studies of antibody-dependent cell-mediated cytotoxicity to schistosomula in which killing was mediated by IgG1 and IgG3, but not by IgG2 (Khalife *et al*. 1989). IgG4 can block protective IgE activity (Wachholz and Durham, 2004), and a high level of IgG4 antibodies, or rather an increased IgG4/IgE ratio, is associated with susceptibility to re-infection (Hagan *et al*. 1991; Dunne *et al*. 1992; Viana *et al*. 1995; Satti *et al*. 1996; Zhang *et al*. 1997; Li *et al*. 2001; Jiz *et al*. 2009; Pinot de Moira *et al*. 2010). Elevated IL-10 levels are also considered a major risk factor for re-infection (Van den Biggelaar *et al*. 2002; Leenstra *et al*. 2006; Mutapi *et al*. 2007; Caldas *et al*. 2008), possibly by stimulating B cells to switch to IgG4 production (Wachholz and Durham, 2004).

The individual target antigens of these antibodies, protective or not, are largely unknown. Relatively few protein antigens are exposed to the host in the schistosome tegument, but more antigens become exposed when schistosomes die and fall apart, possibly giving rise to more effective immune responses and the development of immunity against *Schistosoma* infection. Humans treated with PZQ, which disrupts the schistosome tegument thereby exposing underlying antigens to the host, develop a serological profile similar to that of resistant individuals (Mutapi *et al*. 1998; Correa-Oliveira *et al*. 2000). IgE, IgG3 and IgA levels to adult worm antigens and SEA become higher after treatment with PZQ whereas IgG4 levels decrease (Webster *et al*. 1997*a*, *b*; Mutapi *et al*. 2003).

SCHISTOSOMA GLYCAN ANTIGENS

While major interest is focused on surface-exposed and/or secretory proteins of schistosome larval and adult worm stages to discover novel targets for immune intervention by vaccination, the antigenic carbohydrate chains (glycans) expressed on schistosome proteins have been receiving far less attention. Specific repertoires of glycans with numerous different structural characteristics are abundantly expressed on secreted and membrane-bound proteins of each schistosome life stage (Nyame *et al*. 2004; Hokke and Yazdanbakhsh, 2005; Hokke *et al*. 2007*a*) and these glycans are capable of, or involved in the activation and modulation of the host immune

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response (Velupillai *et al*. 2000; Okano *et al*. 2001; Van der Kleij *et al*. 2002; Hokke and Yazdanbakhsh, 2005; Van Die and Cummings, 2010). Furthermore, high levels of antibodies against glycan antigens are generated during natural and experimental *Schistosoma* infection (Eberl *et al*. 2001; Hokke and Deelder, 2001; Nyame *et al*. 2003; Hokke *et al*. 2007*a*, *b*; Kariuki *et al*. 2008), indicating that it would be worth examining the impact of these antibody responses on the development of immunity to schistosome infection.

Elaborate structural and biochemical studies on schistosome glycans expressed throughout the different life stages of the parasite have indicated that hundreds of different glycan structures are present within the N- and O-linked glycans and the glycolipids (Hokke *et al*. 2007*a*). Most of these glycomics data have been generated by various mass spectrometric techniques from whole parasite extracts, although some studies have been performed on more restricted groups of proteins (e.g. secretions, or single purified glycoproteins). The occurrence of the different glycans and glycan motifs throughout the schistosome life stages within the mammalian host are summarized below.

Glycans of cercariae and schistosomula

A clear feature of both protein- and lipid-linked glycans of cercariae is the abundance the immunogenic glycan motif Galβ1-4(Fucα1-3)GlcNAc (Lewis X, LeX, see Table 1 for definition of the glycan elements). The majority of cercarial N-glycans are modified with a core β2-xylose as well as an α6-fucose, while no core α3-fucosylated glycans are detected in this life stage (Khoo *et al*. 2001; Hokke *et al*. 2007*a*). Furthermore, LDN-based structures are present as part of the cercarial glycocalyx O-glycans, but in glycolipids and N-glycans they occur only in minor amounts (Wuhrer *et al*. 2000; Huang *et al*. 2001; Khoo *et al*. 2001; Hokke *et al*. 2007*a*; Jang-Lee *et al*. 2007). Interestingly, the cercarial glycocalyx has been reported to carry complex O-glycans with repeating units of unique multi-fucosylated (Fucα1-2Fucα1-3, DF) LDN motifs (Khoo *et al*. 1995; Huang *et al*. 2001). The multi-fucosylated LDN-motifs were found however in only low abundance on the cercarial excretory/secretory proteins (Jang-Lee *et al*. 2007). In addition to LeX, the cercarial glycolipids express the Fucα1-3Galβ1-4(Fucα1-3)GlcNAc (pseudo-LeY) motif (Wuhrer *et al*. 2000), which to date has not been observed in other *S. mansoni* life stages.

The glycosylation of the schistosomula which develop after transformation of the penetrating cercariae is less thoroughly studied and limited data on glycan structures are available. While O-linked and lipid glycosylation have never been analysed, one mass spectrometric analysis of N-glycosylation of *in vitro* transformed 3-day old schistosomula exists (Hokke *et al*. 2007*a*). In comparison to cercariae, the expression of LeX-containing glycans is reduced, and truncated N-glycans are more prevalent. Xylosylation of complex glycans is nearly absent, but a major fraction of truncated glycans still carries this motif. Monoclonal antibody (mAb) studies have indicated the presence of LeX, LDN and GalNAcβ1-4(Fucα1-3)GlcNAc (LDN-F) on the surface of schistosomulae (Koster and Strand, 1994; Nyame *et al*. 2003). As LDN and LDN-F motifs are not clearly detectable on N-glycans of schistosomula, these might be expressed by O-glycans and/or glycolipids.

Glycans of adult worms

Upon maturation of the larvae into adult worms, xylosylation and α3-core fucosylation of N-glycans decreases further, and also N-glycans with LeX motifs become less abundant. Instead, N-glycosylation of adult worms is mainly characterized by α6-core fucosylated, mono- and di-antennary glycans terminating with LDN (Wuhrer *et al*. 2006*b*; Hokke *et al*. 2007*a*). Minor subsets include diantennary glycans with mixed LDN, *N*-acetyllactosamine (LN) and LeX termini as well as linear repeats of these structures (Wuhrer *et al*. 2006*b*). Although male and female glycans in general display a similar N-glycosylation profile, subtle differences in the minor glycan subsets are observed, with females expressing more LN/LeX-type glycans, whereas LDN/LDN-F-type glycans are more prevalent in males (Wuhrer *et al*. 2006*c*). Immunofluorescence studies using mAbs revealed that these gender-specific glycans were at least in part found on the tegument, which might have consequences for immune responses elicited by the two sexes. O-glycans could not be directly detected within the adult worm extract also used to characterize the N-glycans (Wuhrer *et al*. 2006*b*), but previously worms were shown to excrete the highly antigenic circulating cathodic antigen (CCA) and circulating anodic antigen (CAA) from the gut that carry long O-linked carbohydrate chains containing repeats of LeX units and a unique GlcA-substituted GalNAc polymer, respectively (Bergwerff *et al*. 1994; Van Dam *et al*. 1994). Worm glycolipids have to date been poorly defined in terms of glycosylation. However, using defined anti-glycan antibodies, TLC overlays of worm glycolipids indicated the presence of (multi-)fucosylated LDN structures including LDN-F and LDN-DF (Robijn *et al*. 2005), as well as the presence of LeX (Van Stijn *et al*. 2010).

Glycans of eggs and miracidia

The glycan profile of eggs evidently differs from that of adult worms. Within the N-glycan pool, β2-core

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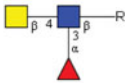
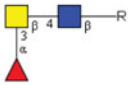
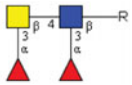
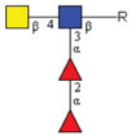
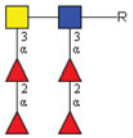
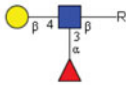
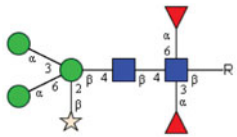
Table 1. Antibody responses to defined antigenic glycan elements in natural and experimental schistosome infections

Element	Glycan structure	Structure in symbols	Main life stage expression[b]	Antibody isotype[c]	Reference(s)
LDN	GalNAcβ1-4GlcNAcβ1-		cer (P) som (P) worms (P) eggs (P)	IgA^{H}, $IgG^{P,H,M}$, $IgM^{P,H,M}$	Eberl *et al.* 2001; Nyame *et al.* 1999, 2003; Van Remoortere *et al.* 2000, 2001
LDN-F	GalNAcβ1-4(Fucα1-3)GlcNAcβ1-		cer (P) som (P) worms (P+L) eggs (P+L)	$IgA^{H,M}$, IgE^{M}, $IgG^{P,H}$, $IgM^{P,H,M}$	Eberl *et al.* 2001; Nyame *et al.* 2000, 2003; Van Remoortere *et al.* 2000; Naus *et al.* 2003
F-LDN	Fucα1-3GalNAcβ1-4GlcNAcβ1-		cer (P+L) worms (L) eggs (P+L)	IgG^{H},IgM^{H}	Kantelhardt *et al.* 2002; Naus *et al.* 2003
F-LDN-F	Fucα1-3GalNAcβ1-4(Fucα1-3) GlcNAcβ1-		cer (P+L) worms (L) eggs (P+L)	IgG^{P}, IgM^{M}	Van Remoortere *et al.* 2003*b*; Robijn *et al.* 2005
LDN-DF	GalNAcβ1-4(Fucα1-2Fucα1-3)GlcNAβ1-		cer (P+L) worms (L) eggs (P+L)	$IgG^{H,M}$, $IgM^{H,M}$	Van Remoortere *et al.* 2000; Naus *et al.* 2003
DF-LDN-DF	Fucα1-2Fucα1-3GalNAcβ1-4 (Fucα1-2Fucα1-3)GlcNAβ1-		cer (P+L) eggs (P+L)	IgG^{M}	Robijn *et al.* 2007
LeX	Galβ1-4(Fucα1-3)GlcNAcβ1-		cer (P+L) som (P) worms (P+L) eggs (P)	$IgA^{H,M}$, $IgG^{P,H,M}$, $IgM^{P,H,M}$	Richter *et al.*1996; Van Remoortere *et al.* 2000, 2001; Eberl *et al.* 2001; Nyame *et al.* 2003, 1997, 1996; Van Roon *et al.* 2004
[a]Core α3-Fuc [a]Core α6-Fuc [a]Core β2-Xyl	Manα1-3(Manα1-6)(Xylβ1-2) Manβ1-4GlcNAcβ1-4GlcNAcβ1-		<u>B2-Xyl (P)</u> cer, som, eggs <u>α3-Fuc (P)</u> eggs <u>α6-Fuc (P)</u> cer, som, worms, eggs	$IgE^{M,H}$	Van Die *et al.* 1999; Nyame *et al.* 2003; Meevissen *et al.* 2011*a*

CAA	[-6(GlcAβ1-3)GalNAcβ1-]$_n$	worms (P)	IgG1^M, IgG3^M, IgM^M	Deelder *et al.* 1996
Poly LeX (CCA)	[-3Galβ1-4(Fuc α 1-3) GlcNAcβ1-]$_n$	worms (P)	IgG1$_M$, IgG3$_M$, IgM$_M$	Van Roon *et al.* 2004
Mannosidic	Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAcβ1-	cer (P) som (P) worms (P) eggs (P)	N.D[d]	Van Remoortere *et al.* 2003*a*

blue square, *N*-acetylglucosamine; yellow square, *N*-acetylgalactosamine; green circle, mannose; yellow circle, galactose; red triangle, fuccose; open star, xylose; blue/white diamond, glucuronic acid.

[a] N-glycan core modifications which can be present separately or combined.

[b] Major expression of glycan elements on glycoproteins and/or glycolipids in cercariae (cer), schistosomula (som), adult worms (worms) and eggs. P, present as a protein conjugate; L, present as a lipid conjugate. Mainly based on Hokke *et al.* 2007*a*, 2007*b*.

[c] Only antibody responses in infected mice (M), primates (P) and humans (H) are noted.

[d] Monoclonal antibodies against the glycan were obtained from *S. mansoni* infected mice. No elevated antibody levels were found in sera of infected humans.

xylosylation as observed in the cercarial stage re-appears, and a set of α3-core fucosylated glycans can be detected (Khoo *et al.* 1997; Hokke *et al.* 2007*a*). As in most other life stages, antenna structures on N-, O- glycans for a large part consist of fucosylated LN and LDN motifs, including LeX and LDN-F structures (Khoo *et al.* 1997; Wuhrer *et al.* 2002). This is in line with the observations for major secretory egg-glycoproteins omega-1, IPSE/α1 and κ5 which carry these types of terminal motifs on a α3/α6-difucosylated N-glycan core, in the case of κ5 in a unique combination with core-linked xylose (Wuhrer *et al.* 2006*a*; Jang-Lee *et al.* 2007; Meevissen *et al.* 2010, 2011*a*). Another characteristic feature of egg glycans is the occurrence of multi-fucosylated antenna structures containing the Fucα1-2Fucα1-3 (DF) motif (Bergwerff *et al.* 1992; Khoo *et al.* 1997; Jang-Lee *et al.* 2007). On glycolipids, these motifs are expressed in the form of repeating -4(Fucα1-2Fucα1-3)GlcNAcβ1-units terminating with (Fucα1-2)$_{0/1}$Fucα1-3GalNAcβ1- at the non-reducing end. Notably, egg glycolipids do not seem to express the LeX element (Robijn *et al.* 2005). The egg shell surface most likely contains both N- and O-glycans and the presence of fucosylated LDN and LeX was shown by monoclonal antibodies (Dewalick *et al.* 2011), but the precise composition of these glycans remains unknown.

The N-glycans of miracidia, which constitute the major part of mature eggs are indeed very similar to the ones found in egg extracts (Hokke *et al.* 2007*a*). However, eggs contain additional glycan structures such as those found on omega-1 and IPSE/α1, which are expressed in the sub-shell area within the egg (Hokke *et al.* 2007*a*). The glycan structures of miracidial O-glycans and glycolipids have not been analysed yet, but, as observed for N-glycans, are expected to be largely similar to the respective egg glycans.

ANTIBODY RESPONSES AGAINST *SCHISTOSOMA* GLYCAN ELEMENTS

It has been shown that high levels of antibodies directed against glycan epitopes are present in sera from *Schistosoma* infected individuals, and an unidentified subset of these antibodies may be protective (Richter *et al.* 1996; Nyame *et al.* 2000; Van Remoortere *et al.* 2000; Eberl *et al.* 2001; Naus *et al.* 2003; Kariuki *et al.* 2008). These observations led to the hypothesis that glycans may also form the basis for a vaccine that induces antibody-mediated protection against schistosomes. The molecular nature of the glycan epitopes recognized by antibodies in natural schistosomiasis infection serum is however still largely unknown. Below we summarize current knowledge of specific glycan targets of antibodies in schistosomiasis.

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The Lewis X motif

Antibodies against the LeX element have been identified in sera from naturally infected humans (Nyame *et al*. 1996, 2003; Van Remoortere *et al*. 2001), experimentally infected primates (Nyame *et al*. 1996; Eberl *et al*. 2001) and rodents (Richter *et al*. 1996; Nyame *et al*. 1997; Van Remoortere *et al*. 2000; Van Roon *et al*. 2004). All species investigated generated mainly IgM antibodies against the LeX element although low levels of IgG and IgA were observed as well (Richter *et al*. 1996; Nyame *et al*. 1997; Van Roon *et al*. 2004). LeX is expressed not only in monomeric but also in linear oligomeric and polymeric forms, the latter as the major immunogenic part of CCA (Van Dam *et al*. 1994). Interestingly, the response against monomeric LeX was much lower than the response against di- and trimeric LeX with a different temporal response pattern in mice. Both antibody responses peaked at 8 weeks after infection, but the anti-di- and tri-meric LeX response rapidly declined while the response against the monomeric LeX showed a gradual decline (Van Roon *et al*. 2004). Furthermore, the response pattern for the polymeric LeX containing CCA was different from the anti-mono-, di- and trimeric responses as it appeared later and is more prolonged (Van Roon *et al*. 2004). Together these observations indicate that specific and different antibody responses are generated against structurally related but non-identical presentations of the LeX antigen.

The LeX element is also present on a limited set of cell surface expressed and/or secretory glycolipids and glycoproteins of the human host, and it has been shown that anti-LeX antibodies from sera of infected humans are able to mediate complement-dependent cytolysis (Nyame *et al*. 1996, 1997). Whether this also leads to significant pathology *in vivo* is unknown but it is an important reason why LeX is not a straightforward potential glycan-vaccine candidate. Since schistosomes express different structural forms of LeX associated with different antibody responses, specific LeX conjugates or multimers may still be explored as vaccine candidates (Van Dam *et al*. 1994; Van Roon *et al*. 2004). It is worth noting that in particular the LeX motif, as part of synthetic or natural glycoconjugates, has also been shown to harbour immunomodulatory properties via interactions with antigen presenting cells such as DC and macrophages (Harn *et al*. 2009). Several other schistosome glycans, including LDN and LDN-F, may also be involved in inducing innate and modulatory immune mechanisms in the host, in addition to being a direct target of the antibody response (Van Die and Cummings, 2010; Meevissen *et al*. 2011*b*).

LDN and its fucosylated derivatives

Antibody responses against LDN and fucosylated variants have been shown in humans (Van Remoortere *et al*. 2001; Naus *et al*. 2003; Nyame, 2003), primates and mice (Nyame *et al*. 1999, 2000; Van Remoortere *et al*. 2000; Eberl *et al*. 2001). Schistosomes express the more exceptional glycan motifs F-LDN (Kantelhardt *et al*. 2002) and multi-fucosylated LDN (Khoo *et al*. 1995, 1997; Wuhrer *et al*. 2002) as well as the more widely expressed glycans LDN and LDN-F which are shared between schistosomes and mammalian hosts including humans (Hakomori *et al*. 1981; Fox *et al*. 1983; Spooncer *et al*. 1984; Fukuda *et al*. 1985; Van Kuik *et al*. 1991; Yan *et al*. 1993; Bergwerff *et al*. 1995; Dell *et al*. 1995; Khoo *et al*. 1995; Van den Eijnden *et al*. 1995). Antibody responses to these less specific LDN and LDN-F motifs are generally low and predominantly of the IgM type, while antibody responses against the more exceptional elements LDN-DF and F-LDN are more pronounced and are predominantly of the IgG isotype in infected humans and chimpanzees (Khoo *et al*. 1997; Van Remoortere *et al*. 2001; Kantelhardt *et al*. 2002; Naus *et al*. 2003). For LDN-DF it has been shown that isotypes can differ between age groups and groups infected with different *Schistosoma* species. Sera of *S. japonicum* individuals contained mainly IgG to LDN-DF, while *S. mansoni*-infected individuals show IgM antibody responses. Both isotypes were observed in high amounts in *S. haematobium* infected individuals (Van Remoortere *et al*. 2001). For all *Schistosoma* species, the antibody response against LDN-DF is higher in children than in adults, which may or may not be the result of the higher infection intensities generally observed in children. While *S. mansoni* infection is dominated by IgM in residents of an endemic area, an immigration study showed that young children upon their first year of exposure induce high IgG1 mediated responses against LDN-DF that decline within 2 years to levels comparable with children are exposed and infected more frequently (Naus *et al*. 2003). This indicates that antibody levels as well as isotypes/subclasses to specific glycan antigens are correlated to specific infection characteristics.

Core-α3-Fuc and core-β2-Xyl

The core-α3-Fuc and core-β2-Xyl modifications occurring in schistosomes also occur in other invertebrates and in plants, but not in mammalian species. Sera from *S. mansoni*-infected mice contain antibodies that specifically reacted with complex type glycans containing these modifications since the antibodies bound to proteins of *S. mansoni* and *Arabidopsis thaliana* but not to proteins from mutant *A. thaliana* lacking the core antigens (Van Die *et al*. 1999). Interestingly, these antibodies were of the IgE isotype and not IgM or IgG which is mainly the case for all other glycan antigens described. The generation of IgE against core-α3-Fuc and/or

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core-β2-Xyl suggests that antibodies against these elements are generated later in infection during the development of a Th2 response (Faveeuw *et al*. 2003). This is consistent with the presence of core-α3-Fuc in schistosomes on egg and miracidia glycans only (Khoo *et al*. 1997; Hokke *et al*. 2007*a*). In natural human infection, the major anti-glycan IgE response appears to be directed against the N-glycan core structure of the major egg glycoprotein κ5, which uniquely carries both β2-Xyl and α3/α6-core-difucosylation (Meevissen *et al*. 2011*a*). The core-β2-Xyl modification, often in combination with the commonly occurring α6-core Fuc is also present on cercariae and schistosomula glycans and is therefore exposed to the host immune system at an earlier stage after infection (Hokke *et al*. 2007*a*, *b*). It would be interesting to explore further IgE and other types of antibodies against core-α3-Fuc and core-β2-Xyl in infection in humans and determine if they play a role in immunity.

Antibody responses to Schistosoma *glycans: protective or a smoke screen?*

In immunization studies with radiation-attenuated cercariae which induce protection in animal models, strong antibody responses against glycans are observed (Richter *et al*. 1996; Eberl *et al*. 2001; Kariuki *et al*. 2008). It has been argued that in general these responses are merely a smoke screen rather than involved in the protective response, but it could also be hypothesised that responses to specific subset of glycan elements may be be linked to protective immunity. Cytolytic capacity and protective properties have been described for glycan-specific antibodies (Nyame *et al*. 1996, 1997, 2003). IgE directed against glycolipids has been shown to be negatively correlated with egg output at 2 years post-treatment, indicating that it could play a role in resistance to reinfection (Van der Kleij *et al*. 1999). However, IgM and IgG2 antibodies that reacted with carbohydrate epitopes expressed on the surface of schistosomula and eggs were negatively associated with resistance to reinfection which is probably due to blocking activity of the antibody isotypes (Butterworth *et al*. 1988*a*). These observations for anti-glycan antibodies are in line with the protective IgE and blocking IgM/IgG2 responses described above. In vaccination/infection studies in chimpanzees (Eberl *et al*. 2001) and baboons (Kariuki *et al*. 2008) it was noticed that anti-glycan antibodies were predominantly produced against cercarial and egg secretions of *Schistosoma* during the early phases of infection but that antibodies to peptide epitopes become more prominent during the chronic phase of infection when protective immune responses are generated (Eberl *et al*. 2001). These observations support the smoke screen theory which reasons that high antibody responses towards glycans are beneficial for the parasite rather than the host by subverting the immune system away from epitopes that could provoke protective immune responses. This hypothesis was further supported by the perception that immunization with eggs resulted in high antibody titers in mice, which were cross-reactive with cercarial and egg secretions, but did not result in increased protection (Kariuki *et al*. 2008).

It will be necessary to study in more detail responses to individual glycan antigens rather than to crude glycoprotein mixtures, in particular to those that are expressed by larval and worm stages but not by eggs, before conclusions can be drawn about their detrimental or beneficial effects to host immunity.

SHOTGUN GLYCAN MICROARRAYS: EFFECTIVE TOOLS FOR STUDYING ANTIBODY-GLYCAN INTERACTIONS

The notion that high antibody titers against glycan elements do not correlate with protection *per se* makes the identification of potential vaccine candidates more complex and asks for more advanced strategies than simply identifying glycan elements towards which antibodies are generated. So far research has been focused on antibody responses against a limited set of synthetic glycoconjugates representing antigenic schistosome glycan elements such as listed in Table 1. It is very likely that in a *Schistosoma* infection antibodies are generated against a much wider range of glycan elements not tested so far, and that the larger underlying glycan structure as produced by the schistosome itself contributes to antibody specificity and affinity, and thereby also immunological functionality. Additionally, a protective immune response may be formed by the combined action of multiple antibodies against various glycans and glycoproteins and rather than against a single antigen. With the recent development of glycan microarrays it is possible to study antibody responses against multiple glycans simultaneously. Glycan microarrays contain small amounts of a large number of glycans presented on a surface to quantitatively measure their interaction with complementary molecules, analogous to arrays developed for gene transcription analysis or the study of protein-protein interactions (Bergwerff *et al*. 1994; Blixt *et al*. 2004; Bochner *et al*. 2005; Gryseels *et al*. 2006; De Boer *et al*. 2007; Lonardi *et al*. 2010; Smith *et al*. 2010). So far, glycan microarrays have been explored in particular for studying glycan ligands of mammalian lectins, for example in the framework of the Consortium of Functional Glycomics in the USA (www.functionalglycomics.org). In different versions, these glycan microarrays contain up to about 600 synthesized glycan structures and motifs mainly related to the mammalian glycome (Blixt *et al*. 2004; Bochner *et al*. 2005; Smith *et al*. 2010). While efficiently generating high quality data and new

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hypotheses, drawbacks of these arrays are the dependence on laborious and time-consuming synthesis of glycans to be printed on the arrays and the relatively limited glycan repertoire covered.

These limitations can be overcome with the development of shotgun glycan microarray approaches. Shotgun glycan microarrays have the potential to study protein-glycan interactions at the whole natural glycome level (De Boer *et al*. 2007; Song *et al*. 2011). These arrays consist of glycans isolated directly from relevant cells or organisms thereby completely avoiding the need for synthetic glycan structures. Also a major advantage of the shotgun glycan microarray approach versus the conventional synthetic array approach is the inclusion of unique and unusual (e.g. pathogen specific) glycans that would not be available through chemical synthesis because these glycans have simply never been structurally identified. The natural glycans are obtained via routine analytical procedures and, after chromatographic separation, individual glycans from complex glycomes can be printed in an array format. When constructed of pathogen-derived glycans, such arrays can for instance be applied to determine specific anti-glycan antibodies by incubating the arrays with infection sera and subsequent detection with fluorescently labeled secondary antibodies Glycan microarrays have already been applied to examine anti-glycan antibodies in sera of healthy individuals (Oyelaran *et al*. 2009), of *S mansoni*-infected subjects (De Boer *et al*. 2008), and of patients with Lyme's disease (Song *et al*. 2011), indicating the potential of glycan arrays as a tool for anti-glycan antibody profiling. Comparing antibody response profiles in sera of infected, non-infected and resistant individuals and cohorts using these shotgun glycan microarrays could be a promising strategy to discover glycan antigens and vaccine candidates for various infectious diseases (Oyelaran and Gildersleeve, 2007).

SCHISTOSOMA GLYCANS AS VACCINE CANDIDATES

Despite the fact that antibody-mediated immune responses can confer resistance against re-infection with *Schistosoma* there is no effective vaccine available yet (Hotez *et al*. 2010; McManus and Loukas, 2008). Initial research with the radiation-attenuated schistosome vaccine showed high levels (up to 90%) of immunity in animal models against a challenge infection (Bickle, 2009) but with the current protein vaccine candidates such effective levels have not yet been reached (McManus and Loukas, 2008; Hotez *et al*. 2010). Although so far largely unexplored, glycans may also be considered as targets to develop vaccines to schistosome infections, and in fact other helminth infections in which antigenic glycans play equally prominent roles. Glycans are extensively surface exposed, they are present at a higher density than proteins, often in a multivalent pattern, and one single glycan can be expressed by multiple proteins thereby allowing the targeting of more than one protein by the same antibody.

Immunization with glycans generally induces poor antibody responses due to T-cell independent mechanisms that result in the production of IgM (Astronomo and Burton, 2010). IgM is associated with blocking of immunity in *Schistosoma* infection (Butterworth *et al*. 1988*a*) and not the isotype of choice in vaccine development. However, conjugation of glycans to a protein carrier results in T cell-dependent immune responses with subsequent production of IgG antibodies against the carbohydrate antigen. Such vaccines have been shown to confer protection against a variety of microbes including, *Haemophilus influenza type b, Neisseria meningitidis, Salmonella typhi* and *Streptococcus pneumonia* (reviewed in Astronomo and Burton, 2010). By generating an anti-glycan antibody response of high class isotype that activates a protective mechanism via an appropriate specific glycan target a *Schistosoma* glycan-based vaccine may be feasible.

Although a significant amount of structural information on schistosome glycans is available, the identity of the proteins on which these glycans are expressed is known in only a few cases. Protein-specific information may in many cases easily be obtained by Western blot using monoclonal antibodies against glycan epitopes (Meevissen *et al*. 2010, 2011*a*). *Vice versa*, proteomic studies identifying promising vaccine targets often contain information on putative N- and O-glycosylation sites but lack identification of glycans that are expressed by the protein (Ribeiro de *et al*. 2000; Siddiqui *et al*. 2003; Al-Sherbiny *et al*. 2003; Shalaby *et al*. 2003; Tran *et al*. 2006; Farias *et al*. 2010). In particular, in studies on glycoprotein antigens it will be of importance to identify glycan structures on individual proteins. Antibodies to native glycoproteins may actually bind to the glycan structures rather than the protein. In addition, it is becoming clear that proper glycosylation of protein vaccines may be of utmost importance via its capacity to modulate and activate the anti-protein response induced by immunization (Okano *et al*. 2001; Singh *et al*. 2009, 2011; Van Montfort *et al*. 2011).

CONCLUDING REMARKS

In parallel with proteomics technologies, sensitive glycomic techniques are generating a wealth of structural information on glycans of schistosomes. Novel technologies such as glycan arrays now allow the screening of antibody responses to individual components of a pathogen glycome. Further research on schistosome glycan antigens will contribute to a better understanding of glycan-induced immune

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responses, and may contribute to the development of an effective vaccine against schistosomes and other infectious helminths.

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