Carbohydrate profiling and protein identification of tegumental and excreted/secreted glycoproteins of adult *Schistosoma bovis* worms

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Abstract

Schistosoma bovis is a parasite of wild and domestic ruminants that is broadly distributed throughout many tropical and temperate regions of the old world. *S. bovis* causes severe health problems and significant economic losses in livestock, but in contrast to human schistosomes, *S. bovis* has been little investigated at a molecular level. Since schistosome glycans and glycoproteins can play important roles in the host-parasite interplay, the aims of the present work were: (i) to characterize the glycans expressed by adult *S. bovis* worms on their excreted/secreted (ES) and tegumental (TG) glycoproteins, and (ii) to identify their carrier protein backbones by mass spectrometry. Using a panel of lectins and monoclonal and polyclonal anti-glycan antibodies we observed: (i) the absence of sialic acid in *S. bovis*; (ii) the presence of complex-type N-glycans and LDN antennae on ES glycoproteins; (iii) the presence of glycans containing the Fucα1-2Galβ motif in many TG glycoproteins, and (iv) the presence of glycans containing the Fucα1-3GlcNAc motif on many ES and TG glycoproteins but, simultaneously, the absence of the F-LDN(-F) glycans from both the ES and TG glycoproteins. Interestingly, we also found the Lewis^X and Lewis^Y antigens co-expressed on several TG isoforms of ATP:guanidino kinase and glyceraldehyde-3-phosphate dehydrogenase. Finally, by ELISA we observed the presence of antibodies against Lewis^X, Lewis^Y and F-LDN(-F) in the sera of sheep experimentally infected with *S. bovis*.

Keywords: *Schistosoma bovis*; glycans; glycoproteins; Lewis antigens; mass spectrometry

1. Introduction

Blood flukes of the genus *Schistosoma* are one of the major causes of human and domestic animal disease in many tropical and subtropical countries. There are three major species that infect humans, namely *S. mansoni*, *S. japonicum* and S. *haematobium*, and at least ten species have been reported to infect domestic ruminants naturally. Among these second species, only *S. mattheei* and *S. bovis* have received some attention, mainly because of their recognized veterinary significance (De Bont and Vercruysse, 1998; Vercruysse and Gabriel, 2005).

S. bovis lives in the portal mesenteric system of its ruminant hosts and shows a wide geographic distribution that includes practically the whole of Africa, the Middle East (Israel, Iran, Iraq), and the Mediterranean region (Corsica, Sardinia, Sicily, Italy and Spain) (De Bont and Vercruysse, 1998; Moné et al., 1999). In addition, *S. bovis* belongs to the same species complex as the human pathogen *S. haematobium* and indeed is an immunological analogue of *S. haematobium* (Agnew et al., 1989), making studies on *S. bovis* an attractive goal from the perspectives of both veterinary and comparative medicine.

Nonetheless, in spite of the veterinary importance of this parasite, studies on *S. bovis* have been scant in comparison with as those carried out on human schistosomes and have mainly focused on the life cycle and epidemiology of the worm. The pathology and treatment of the infection and certain features of the host immune response have also been studied, but little is known of the immunological mechanisms involved in ruminant schistosomiasis (Johansen et al., 1996; De Bont and Vercruysse, 1998; Viana da Costa et al., 1998; Rodriguez-Osorio et al., 1999; Vercruysse and Gabriel, 2005). Similarly, work aimed at the molecular characterization of *S. bovis* has been scarce and has focused on only a few molecules, such as glutathione S-transferase, which has been addressed in several studies owing to its potential as a vaccine (Trottein et al., 1992; Boulanger et al., 1999; De Bont et al., 2003).

The need for a better understanding on how *S. bovis* interacts with its host at molecular level, as well as the search for diagnostic antigens and targets for new vaccines and drugs

encouraged us to study the parasite molecules involved in the host-parasite interplay. Since such molecules should be among those that the parasite exposes to the host along the period of infection, we began our study by carrying out a proteomic analysis of the tegument and the excreted/secreted products of the adult worms (Pérez-Sánchez et al., 2006). This study allowed us to identify 18 parasite proteins located at the host-parasite interface. At least 4 of these proteins (enolase, glyceraldehyde-3-phosphate dehydrogenase, serine protease inhibitor and superoxide dismutase) could counteract the host defence mechanisms. The remaining proteins identified are also likely to play some kind of role in the host-*S. bovis* relationships not only because they form part of the host-parasite interface but also because, for many of them, their molecular functions suggest that this would indeed be the case.

While very important, proteins are not the only kind of parasite molecules involved in the schistosome-host interactions. It is currently recognized that schistosomes produce a variety of complex glycans, which are expressed on glycolipids and glycoproteins, and that these glycans can play important roles in the induction and modulation of the host immune response as well as in the immunopathology of schistosomiasis (Hokke and Deelder, 2001; Nyame et al. 2003, 2004; Hokke and Yazdanbakhsh, 2005). For this reason, schistosome glycans and glycoconjugates are now regarded as good immunodiagnostic targets and potential targets for vaccine development (Nyame et al., 2003, 2004). In fact, schistosome glycobiology has recently become an area of intensive research and a significant amount of structural and functional information on schistosome glycans is available, at least for human schistosomes. What is still almost completely lacking, however, is information about which glycoproteins these glycans are expressed on (Hokke and Yazdanbakshs, 2005).

Regarding *S. bovis*, almost nothing is known about its glycans and the glycoproteins on which are they expressed. It is necessary to determine which proteins of *S. bovis* are glycoproteins and how these glycoproteins are glycosylated. In this work, we initiated such a determination by studying the glycans expressed on the excreted/secreted and tegumental glycoproteins of adult *S. bovis* worms. Since these two glycoprotein fractions are exposed to the

105 host during infection, it is likely that their glycans would be involved in the host-parasite
106 interplay as immunogens or immunomodulators.

107 We paid particular attention to some of the carbohydrate motifs that are known to occur
108 in human schistosomes and that have immunogenic and immunomodulatory properties, such as
109 GalNAcβ1-4GlcNAcβ1 (LDN), GalNAcβ1-4(Fucα1-3)GlcNAcβ1 (LDN-F), Fucα1-
110 3GalNAcβ1-4GlcNAcβ1 (F-LDN), Fucα1-3GalNAcβ1-4(Fucα1-3)GlcNAcβ1 (F-LDN-F) and
111 Galβ1-4(Fucα1-3)GlcNAcβ1 (Lewis^X, Le^X) (Van Liempt et al., 2004; Hokke and
112 Yazdanbakshs, 2005; Meyer et al., 2005; Robijn et al., 2005; van de Vijver et al., 2006; Wuhrer
113 et al., 2006a, 2006b). Additionally, although the tetrasaccharide Fucα1-2Galβ1-4(Fucα1-
114 3)GlcNAcβ1 (Lewis^Y, Le^Y) has so far not been found in human schistosomes (Nyame et al.,
115 1998), here we also investigated the presence of Le^Y on the glycoproteins of adult *S. bovis*
116 worms.

117 With these aims, two extracts containing the tegumental and excreted/secreted proteins
118 of *S. bovis* adult worms were probed with eight lectins, two monoclonal antibodies (anti-Le^X
119 and anti-Le^Y), and a polyclonal antibody against Keyhole limpet haemocyanin (KLH). KLH and
120 human schistosomes share the carbohydrate epitopes F-LDN and F-LDN-F (Geyer et al., 2005;
121 Robijn et al., 2005), allowing the anti-KLH antibody to recognize those epitopes, if present, in
122 *S. bovis*. Both protein extracts were analysed by combining lectin blotting, Western blotting and
123 immunoprecipitation with 1-D and 2-D electrophoresis. The glycoproteins thus detected were
124 identified by mass spectrometry analysis and, finally, by ELISA the presence of antibodies
125 against Le^X, Le^Y and F-LDN(-F) in the sera of sheep experimentally infected with *S. bovis* was
126 demonstrated.

127

128 **2. Material and Methods.**

129

130 *2.2. Maintenance of Schistosoma bovis life-cycle*

131

A strain of *S. bovis* from Salamanca (Spain) was maintained at the laboratory in its natural hosts: *Planorbarius metidjensis* snails and sheep. Each snail was infected with five miracidia from eggs obtained from the faeces of experimentally infected sheep. The infected snails were maintained at the laboratory under controlled conditions until the emission of cercariae. Then, the sheep were infected percutaneously with 2,000 *S. bovis* cercariae by submerging a fore-limb for 30 min in 150 ml of cercarial suspension. At 6 months post-infection the sheep were sedated with ketamine (10 mg/kg) and then sacrificed by bleeding after sectioning the jugular vein. *S. bovis* adult worms were recovered by dissection of mesenteric vessels from the entire gut and washed thoroughly in PBS at 37ºC. The viability of the worms was examined microscopically, after which intact parasites were processed immediately to obtain the two protein extracts to be studied (see below). The sheep were bled before and along the infection to obtain sera. As a rule, bleeding was performed once a week during the first four weeks post-infection and then once a month until the time of sacrifice.

2.2. Preparation of extracts of excretory-secretory and tegumental proteins

Extracts of excretory-secretory and tegumental proteins were prepared from live adult worms as follows. The parasites were washed twice in RPMI 1640 medium supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml) and then cultured for 6 h in the same medium at 37ºC and 5% CO_2. This culture medium was recovered and designated ES. Following this, to obtain the tegumental extract the parasites were washed twice in TBS and incubated in 1% Triton X-100 in TBS at 4ºC with gentle shaking for 30 min, after which the medium from this incubation, designated TG, was recovered. Both extracts -ES and TG- were clarified by centrifugation at 10,000 *g*, dialyzed against water for 24 h, and concentrated by vacuum centrifugation to a concentration of > 3 µg/µl. A cocktail of protease inhibitors (1mM EDTA, 1mM N-Ethylmaleimide, 0.1 µM Pepstatin A, 1 mM PMSF and 0.1 mM N-Tosylamide-L-phenylalanine chloromethyl ketone) was added to each extract (Maizels et al., 1991). The

protein concentration of the ES extract was measured with the Bradford assay (Bio-Rad, Hercules, CA, USA) and that of the TG by the method of Markwell et al. (1978).

2.3. SDS-PAGE

Protein samples were subjected to SDS-PAGE in a Laemmli (1970) discontinuous gel system using the Mini-Protean Cell system (Bio-Rad). The stacking gel was 3% and the resolving gel was a 5-20% gradient. Usually, the gels were loaded with 10 µg of protein per lane. After running, the gels were either electrotransferred onto nitrocellulose membranes or stained with silver nitrate. The electrotransfer step was carried out in 2 mM Tris, 192 mM glycine buffer, pH 8, at 400 mA for 90 min.

2.4. Two-dimensional electrophoresis (2-DE)

2-DE was performed as described in Perez-Sánchez et al. (2006). The isoelectric focusing (IEF) was run in 7-cm IPG strips (Bio-Rad) with linear pH ranges of 5-8 and 7-10 using a Protean IEF Cell (Bio-Rad). Protein samples (25 µg of ES or TG, as well as immunoprecipitation pellets -see below-) were diluted in 125 µl of rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 50 mM DTT, 0.2% IPG buffer, bromophenol blue traces) and were allowed to mix gently for 1 h at room temperature before centrifugation at 18,000 *g* over 30 min to remove all particulate material. The supernatants were applied to the IPG strips by in-gel rehydration at 20ºC for at least 12 h. IEF was run at 50 µA/strip for a total of 20,000 Vh. Next, the strips were reduced in equilibration buffer (6 M urea, 0.05 M Tris pH 8.8, 2% SDS, 20% glycerol) containing 2% DTT over 15 min and then alkylated in equilibration buffer containing 2.5% iodoacetamide for 10 min. The second dimension was performed on 12% polyacrylamide gels and the resulting 2-D gels were either stained with a mass spectrometry-compatible silver stain (Stochaj et al., 2003), or electrotransferred onto nitrocellulose membranes in a similar way to that of the 1-D gels. The stained 2-D gels were scanned using an ImageScanner (Amersham

Biosciences, Uppsala, Sweden) and their images were analysed using the ImageMaster 2D Platinum Software v5.0 (Amersham Biosciences).

2.5. 1-D and 2-D lectin blotting

Samples of ES and TG extracts, previously resolved by SDS-PAGE and electrotransferred to nitrocellulose membranes, were probed with the eight lectins listed in Table 1. The digoxigenin-labelled lectins GNA, SNA, MAA, DSA and PNA, all of them included in the DIG Glycan Differentiation Kit (Roche, Mannheim, Germany), were used following the instructions of the kit manufacturer. Briefly, the nitrocellulose membranes were blocked and subsequently incubated with the corresponding lectin (see Table 1 for lectin concentration). Then, they were incubated with an alkaline phosphatase-conjugated anti-digoxigenin antibody (1:1000) and the reactive bands were revealed with a substrate solution containing NBT and X-phosphate.

The biotinylated lectins WFA, LTA and UEA-I (Vector Laboratories, Peterborough, UK) were used as follows. Nitrocellulose membranes were blocked with 3% BSA in TBS (50 mM Tris-HCl, 150 mM NaCl, pH 7.5), washed 3 times with TBS containing 0.3% Tween-20, and incubated with the respective lectin diluted in dilution buffer (TBS, 0.05% Tween-20, 1% BSA). After 3 new washes, the membranes were incubated with 1:1000 avidin-peroxidase (Sigma, Saint Louis, MO, USA) in dilution buffer and washed again 3 times. Reactive bands were revealed with 4-Cl-1-naphthol. Incubations were carried out at room temperature for 1 h, and the washes were done for 10 min per each wash.

The lectins GNA, WFA, LTA and UEA-I were those that recognized bands on one or both extracts. Consequently, a second 1-D blotting with these lectins was performed, but this time including the appropriate monosaccharide as a lectin binding inhibitor; respectively, α-methyl-D-mannoside (0.5 M), N-acetyl-D-galactosamine (0.2 M) and L-fucose (1 and 3 M). All three monosaccharides were purchased from Sigma.

After the inhibition experiments had been completed, these four lectins were used again as probes in a subsequent 2-D lectin blotting. The ES extract was resolved in 2-D gels in the 5-8 and 7-10 pH ranges, transferred to nitrocellulose membranes, and re-screened with GNA, WFA and LTA. The TG extract was re-screened in a similar way, but in this case with LTA and UEA-I. Blotting conditions were similar to that used in the 1-D lectin analysis. The resulting 2-D blots were scanned and their images were analysed using the ImageMaster software. These blots and their homologous silver-stained gels were aligned to isoelectric point (pI) and molecular weight (MW) and then matched by ImageMaster software in order to identify the lectin-binding spots in the gels. Then, the spots of interest were excised from the gels and analysed by mass spectrometry for protein identification.

2.6. 1-D Western blotting

ES and TG samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 2% BSA in PBS and washed 3 times with PBS containing 0.05% Tween 20. Following this, they were incubated with 2 µg/ml of primary antibody in dilution buffer (PBS, 0.05% Tween 20, 1% BSA): anti-Le^X and anti-Le^Y monoclonal antibodies (Calbiochem, San Diego, CA, USA) and rabbit anti-KLH policlonal antibody (Sigma). After 3 new washes, the membranes were incubated with the respective secondary antibody: peroxidase-conjugated goat anti-mouse IgM (1:1000) (Stressgen Bioreagents, Victoria, Canada) and peroxidase-labelled goat anti-rabbit IgG (1:5000) (Sigma). The membranes were washed again and the reactive bands were visualised with either 4-Cl-1-naphthol or with the Immun-Star HRP chemiluminescent substrate (Bio-Rad) followed by digitalization using the Fluor-S Multimager system (Bio-Rad). Incubations were performed at 37 ºC for 1 h, and the washes were carried out at room temperature for 10 min per each wash.

2.7. Immunoprecipitation (IP)

242 The glycoproteins of the TG extract that were recognized in the Western blots by the
243 monoclonal antibodies anti-Le^X and anti-Le^Y were subsequently separated by IP. The IP
244 protocol was an adaptation of that described by Harlow and Lane (1988) and proceeded as
245 follows.

246 Samples of 100 µg of protein G-Sepharose 4B (Sigma) were sensitised with 10 µg of
247 each monoclonal antibody in 100 µl of PBS. The reactions were carried out in 1.5 ml test tubes
248 at 4ºC for 12-16 h with gentle shaking. They were then centrifuged for 2 min at 3000 rpm and
249 the supernatants were removed (non-bound material). The pellets (bound antibody) were
250 washed 3 times with 1 ml of PBS and resuspended in 100 µl of PBS. The binding of the
251 antibodies to protein G was checked by running 10 µl of each supernatant and pellet in SDS-
252 PAGE. In parallel, to discard direct binding of TG components to protein G, 100 µg of TG were
253 incubated with 100 µg of protein G-Sepharose 4B.

254 Both monoclonal-sensitised protein G-Sepharose pellets (containing close to 9 µg of
255 bound antibody each) were then incubated with 100 µg of TG in a total volume of 100 µl of
256 PBS at 37 ºC for 1 h with gentle shaking. Following this, they were centrifuged as above and the
257 supernatants were removed and preserved. The pellets were washed and resuspended in 100 µl
258 of PBS. The IP results were checked by running 20 µl aliquots of both supernatants and pellets
259 in SDS-PAGE. The remaining 80 µl of each pellet was then electrophoresed in 2-D gels in the
260 5-8 and 7-10 pH ranges. The gels were stained with a mass spectrometry-compatible silver
261 stain, and the major spots corresponding to the immunocaptured proteins were excised from the
262 gels and analysed by mass spectrometry.

263

264 *2.8. Mass spectrometry analysis and protein identification*

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266 These analyses were carried out as described in Pérez-Sánchez et al. (2006). Protein
267 spots were excised manually and then digested automatically using a Proteineer DP protein
268 digestion station (Bruker-Daltonics). The digestion protocol used was that of Schevchenko et al.
269 (1996). For peptide mass fingerprinting and the acquisition of LIFT TOF/TOF spectra, an

aliquot of α-cyano-4-hydroxycinnamic acid in 33% aqueous acetonitrile and 0.1% trifluoroacetic acid was mixed with an aliquot of the above digestion solution and the mixture was deposited onto an AnchorChip MALDI probe (Bruker-Daltonics).

Peptide mass fingerprint spectra were measured on a Bruker Ultraflex TOF/TOF MALDI mass spectrometer (Bruker-Daltonics) (Suckau et al., 2003) in positive ion reflector mode. Mass measurements were performed automatically using fuzzy logic-based software or manually. Each spectrum was calibrated internally with the mass signals of trypsin autolysis ions to reach a typical mass measurement accuracy of ± 25 ppm. The measured tryptic peptide masses were transferred by means of the MS BioTools program (Bruker-Daltonics) as inputs to search the NCBInr database using Mascot software (Matrix Science; London, UK). When necessary, MS/MS data from LIFT TOF/TOF spectra were combined with PMF data for database searches with a parent ion mass tolerance of 20 – 40 ppm and a fragment ion mass accuracy of 0.25 – 0.50 Da.

2.9. ELISA

ELISA was performed to investigate the presence of antibodies against Le^X, Le^Y and F-LDN(-F) in the sera of sheep experimentally infected with *S. bovis*. To do so, the sera of 3 sheep were analysed by ELISA against the following antigens: ES and TG extracts, KLH (Sigma) and the neoglycoproteins LNFPIII-BSA (Dextra Laboratories, Reading, UK) and Le^Y hexasaccharide-BSA (Le^Y-BSA). Le^Y-BSA was synthesized by the reductive amidation method of Gray (1974), following a procedure similar to that described by Nyame et al. (1996). Briefly, 200 µg of Le^Y-hexasaccharide (Dextra Laboratories) was mixed with 67 µg of BSA and 52 µg of $NaBH_3CN$ in 50 µl 0.2 mM KH_2PO_4 pH 7.0 and left at room temperature for 14 days to allow covalent binding of the sugar to BSA. The neoglycoprotein synthesized was purified from uncoupled sugar and salts by dialysis using Slide-A-Lyzer Mini Dialysis Units (Pierce Biotechnology, Rockford, IL, USA). Analysis by Western blot with the anti-Le^Y monoclonal antibody revealed that the oligosaccharide was coupled to BSA.

ELISA plates were coated overnight at room temperature with 0.2 µg of antigen/well in 100 µl of carbonate buffer, pH 9.6, and subsequently blocked with 5% BSA in PBS at 37ºC for 2 h. The wells were washed 4 times with PBS containing 0.05% Tween 20 and incubated at 37 ºC for 1 h with 100 µl of the sera diluted 1:50 in dilution buffer (PBS, 0.3% Tween 20, 1% BSA). After 4 new washes, the wells were incubated at 37 ºC for 1 h with 100 µl of peroxidase-labelled donkey anti-sheep IgG (1:8000) (Sigma) or peroxidase-labelled rabbit anti-sheep IgM (1:6000) (Bethyl Laboratories, Montgomery, TX, USA) and washed again 4 times. Finally, o*rtho*-phenylene-diamine was used as chromogen substrate for peroxidase.

3. Results

3.1. 1-D lectin blots of ES and TG extracts

As can be seen in Fig. 1(A), both extracts were resolved by SDS-PAGE in approximately 30 bands, whose MWs ranged between 7 and 200 kDa. Fig. 1(C) shows that lectins SNA, MAA, DSA and PNA did not recognize any band on any extract apart from some unspecific binding of lectin MAA on TG. This lack of reactivity indicates that the ES and TG glycoproteins do not contain sialic acid (NeuNAc), lactosamine (LacNAc) and Galβ1-3GalNAc.

Lectins GNA and WFA recognized a group of bands of high MW on the ES -although not on the TG-, which is indicative of the presence, respectively, of terminal mannosyl residues and GalNAcβ1-4GlcNAcβ1 (LDN) on the ES glycoproteins. By contrast, lectin UEA-I recognized numerous bands on the TG -but not on the ES-, signalling the presence of the Fucα1-2Galβ terminal sequence on numerous TG glycoproteins. Finally, lectin LTA recognized numerous bands on both the ES and TG, showing that the Fucα1-3GlcNAc motif is abundantly expressed on the glycoproteins of both extracts. The binding of GNA, WFA, LTA and UEA-I to those glycoproteins was inhibited by the corresponding mononosaccharide, except in the case of a broad band of 55 kDa revealed by GNA on TG, which did not disappear in the inhibition

experiment (not shown); furthermore, no reactive bands were observed when the ES and TG were probed directly with avidin-peroxidase alone.

3.2. 2-D lectin blots and identification of lectin-binding proteins of the ES and TG extracts

Both extracts were re-screened by 2-D lectin blotting but using only the lectins that gave positive reactions in the 1-D blotting, i.e., GNA, WFA, LTA and UEA-I.

The ES extract resolved into approximately 400 spots, whose pIs and MWs ranged between pH 5-9 and 15-190 kDa (Fig. 2(A), 2(B)). LTA bound to 176 of these spots, which were distributed between pH 5-9 and 32-190 kDa (Fig. 2(C), 2(D)). By contrast, GNA (Fig. 2(E), 2(F)) and WFA (Fig. 2(G), 2(H)) bound to only 20-25 spots, most of which were located between pH 5-5.7 and 46-190 kDa. Matching of the lectin blots with their homologous silver-stained gels allowed us to localize in the 2-D gels a total of 55 of those spots recognized by the lectins (Fig. 2). These 55 spots were cut from the gels and subjected to MS analysis. Table 2 shows the MS analysis results. Thirty-four spots out of 55 were identified and these 34 spots corresponded to 8 different glycoproteins, one from the host (pre-pro serum albumin) and 7 from the parasite: surface protein-fluke (SPF), ATP:guanidino kinase (ATPGK), serine protease inhibitor (serpin), enolase, fructose 1,6 bisphosphate aldolase (FbisPA), glyceraldehyde-3-phophate dehydrogenase (GAPDH) and cathepsin B endopeptidase (CBendo). For each protein we identified between 2 and 9 isoforms. Lectin LTA bound to all these ES glycoproteins except serpin and low MW SPF isoforms. On the other hand, lectin GNA bound to serpin and all SPF isoforms and lectin WFA bound to serpin and high MW SPF isoforms.

The TG extract resolved in more than 600 spots, whose pIs and MWs ranged between pH 5-9.5 and 15-160 kDa (Fig. 3(A), 3(B)). LTA bound to approximately 167 of these spots, which were distributed between pH 5.9-9.5 and 44-98 kDa (Fig 3(C), 3(D)). Lectin UEA-I bound to almost the same spots as LTA (Fig 3(E), 3(F)). Matching of the LTA and UEA-I blots with the homologous silver-stained gels allowed us to localize 38 lectin-binding spots in the 2-D gels (Fig. 3). These spots were cut from the gels and subjected to mass spectrometry analysis.

As can be seen in Table 3, 20 spots out of 38 were identified that corresponded to 5 different parasite glycoproteins: ATPGK, enolase, actin, FbisPA and GAPDH. For each glycoprotein we identified between 2 and 7 isoforms. All these isoforms, except those of actin, which only fixed LTA, were bound by both lectins: LTA and UEA-I.

Overall, we identified 8 parasite glycoproteins: 4 of them (ATPGK, enolase, FbisPA and GAPDH) were found in both extracts; another 3 (SPF, serpin and CBendo) were found only in the ES extract, and the remaining one, actin, was found only in the TG extract. All these glycoproteins except serpin fixed LTA, which indicates that they all carry the Fucα1-3GlcNAc motif. In contrast, only serpin and the high MW isoforms of SFP fixed GNA and WFA, indicating that these proteins are the ones that carry glycans containing terminal mannosyl residues and LDN. Finally, only the TG isoforms of ATPGK, enolase, FbisPA and GAPDH fixed UEA-I, showing that only these isoforms contain the Fucα1-2Galβ terminal sequence on their glycans.

3.3. Western blots on ES and TG extracts with anti-KLH, anti-Le^X and anti-Le^Y.

Fig. 1(B) shows that the anti-KLH antibody did not recognize any band on the ES or TG, which indicates that the F-LDN(-F) epitopes are not expressed on ES and TG glycoproteins of adult worms.

On the other hand, the anti-Le^X and anti-Le^Y monoclonal antibodies did not recognize any band on the ES extract, but they both recognized bands on the TG extract and, in fact, they recognized essentially the same ones: a band of 44 kDa and, less intensely, a band of nearly 92 kDa (Fig. 1(B)).

3.4. Immunoprecipitation and identification of the Le^X- and Le^Y-bearing glycoproteins from the TG

Fig. 4(A) shows that both monoclonal antibodies were efficiently coupled to protein G-Sepharose 4B, and that no protein of TG bound directly to protein G-Sepharose 4B. Consequently, we incubated the TG with the anti-Le^X and anti-Le^Y antibodies coupled to protein G-Sepharose 4B and examined the results of the immunoprecipitation by SDS-PAGE. Fig. 4(B) shows that both antibodies captured the two bands of 44 and 92 kDa previously revealed by Western blotting. Then, both IP pellets were subjected to 2-D electrophoresis and in both cases we observed the same result (Fig. 4(C)). Both bands of 92 kDa separated into 3 tiny spots each (spots no. 1-3 and 9-11) and both bands of 44 kDa separated into 5 spots each (spots no. 4-8 and 12-16). All these spots were cut from the gels and identified by MS analysis. Table 4 shows that spots no. 1-3 and 9-11 were identified as ATPGK and that spots 4-8 and 12-16 were identified as GAPDH.

3.5. Presence of antibodies against Le^X, Le^Y and F-LDN(-F) in the sera of S. bovis-infected sheep.

The 3 *S. bovis*-infected sheep analysed developed very similar antibody responses. They all showed high levels of IgG and IgM anti-ES (Fig. 5(A), 5(B)) and moderate levels of IgG and IgM anti-TG (Fig. 5(C), 5(D)). The IgG anti-ES had reached maximum levels by the 8th week post-infection (w.p.i.) whereas the IgM anti-ES reached maximum levels early on, by the 4th w.p.i.; after that, the levels of both isotypes remained relatively constant until the sacrifice of sheep, in the 24th w.p.i. By contrast, the levels of both IgG and IgM anti-TG antibodies underwent a slow but constant increase from week 0 to week 24 p.i.

High levels of anti-KLH antibodies were also detected in the infected sheep (Fig. 5(E), 5(F)). Anti-KLH IgG levels peaked by week 4-8 and remained high up to week 24. Anti-KLH IgM levels peaked by week 4 and decreased to a minimum by week 8-12; after that time, they increased slightly up to week 24.

Moderate to high levels of anti-Le^X antibodies were present in all the infected sheep (Fig. 5(G), 5(H)). Anti-Le^X IgG levels peaked between weeks 4 to 8 and then decreased but

408 remained detectable until week 24. Anti-Le^X IgM levels peaked by week 4 and decreased
409 slightly, although within a moderate range, until week 24.
410 Moderate levels of anti-Le^Y antibodies were also present in all the sheep analyzed (Fig.
411 5(I), 5(J)). The anti-Le^Y response was very similar to the anti-TG response; i.e., the levels of
412 both anti-Le^Y antibody isotypes, IgG and IgM, increased slowly but constantly from week 0 to
413 week 24 p.i. At the time of sacrifice, the levels of anti-Le^Y antibodies reached values 2- to 3-
414 fold higher than the pre-infection level.
415
416 **4. Discussion**
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418 As pointed out in the Introduction, the aim of this work was dual: on one hand to
419 characterise the glycans expressed by adult *S. bovis* worms on their excreted/secreted and
420 tegumental glycoproteins, and -on the other- to identify the glycoproteins bearing those glycans.
421 We chose these two protein fractions, ES and TG, because they contain the glycoproteins that
422 are exposed to the host during infection and this affords their glycans the opportunity to
423 participate in the host-parasite interplay. Such involvement would make these glycans -and their
424 carrier glycoproteins- potential targets for immunodiagnostics and vaccines (Nyame et al., 2003,
425 2004).
426 We started the characterization of the glycans of the ES and TG with a simple 1-D lectin
427 blotting in which we used as many as 8 lectins that covered a broad range of binding
428 specificities for terminal carbohydrate sequences (Fig. 1(C)). This lectin binding assay provided
429 us with information about the type of terminal sugar sequence present -or absent- on the ES and
430 TG glycoproteins, guiding us in later analyses.
431 Not surprisingly, the 1-D lectin blot confirmed the absence of sialic acid residues on the
432 ES and TG glycoproteins of *S. bovis*. This finding is consistent with the fact that the sialic acid
433 is lacking in schistosome glycans in general (Nyame et al. 1998).
434 The 1-D lectin blot indicated that the ES and TG glycoproteins also lacked the
435 carbohydrate motifs recognized by lectins PNA (Galβ1-3GalNAc-Ser/Thr) and DSA (LacNAc,

436 GlcNAc-Ser/Thr). The absence of these motifs from adult *S. bovis* ES and TG glycoproteins,
437 however, does not rule out their presence on proteins from other parts of adult worms or even
438 other developmental stages of *S. bovis*. In fact, Galβ1-3GalNAc-Ser/Thr and GlcNAc-Ser/Thr
439 have been found in *S. mansoni* on glycoproteins from homogenates of whole adult worms and
440 schistosomula (Nyame et al., 1987, 1988); in addition, unusual O-glycans that terminate with
441 GlcNAc or LacNAc have been described to occur on the *S. mansoni* cercarial glycocalyx
442 (Huang et al., 2001).

443 Lectin GNA did not recognize any glycoprotein on the TG extract, but it recognized two
444 glycoproteins on the ES extract that were later identified as surface protein-fluke (SPF) and
445 serine protease inhibitor (serpin) (Fig. 2(E) and Table 2). Since GNA indicates the presence of
446 terminal mannose residues on "high mannose" and "complex"-type N-glycans, it is reasonable
447 to speculate that the *S. bovis* SPF and serpin would carry this kind of N-glycans. Complex N-
448 glycans contain the so-called chitobiose core ($Man_3GlcNAc_2$), which is common to all
449 eukaryotes. Recently, complex N-glycans in which the chitobiose core was partially substituted
450 by the so-called core β2-xylose (Man_3(Xyl)-R) and core α3-fucose (Fucα1-3GlcNAc) have been
451 detected in *S. mansoni* excreted/secreted products of schistosomula, adults, and eggs (Faveeuw
452 et al., 2003). Moreover, these N-glycans are very antigenic and induce strong Th2 responses
453 (Faveeuw et al., loc. cit.). Since the *S. bovis* SPF and serpin glycoproteins are also strongly
454 antigenic and very abundant in the ES extract (Pérez-Sánchez et al., 2006), it is tempting to
455 surmise that they could carry complex N-glycans similar to those described in *S. mansoni*.
456 Evidently, however, demonstration of this hypothesis would require detailed carbohydrate
457 structural analyses.

458 Regarding the SPF isoforms (Table 2), the broad range of MWs (47-191 kDa) exhibited
459 by them is noteworthy. Although some degree of proteolysis cannot be ruled out, a better
460 explanation for this phenomenon could be differences in the degree and type of glycosylation
461 among isoforms. In fact, while all the SPF isoforms were recognized by GNA, those with the
462 highest MW were also recognized by lectins WFA and LTA (Table 2), indicating the presence

463 of additional glycan decorations that, in turn, may account for the increase in the MW of those
464 isoforms.

465 The positive reaction with lectin WFA suggest that LDN (GalNAcβ1-4GlcNAcβ1) is
466 expressed by *S. bovis* (Fig. 2(G)). LDN is an antigenic motif abundantly expressed by
467 schistosomes, but also by humans and probably by many other mammals (Hokke and
468 Yazdanbakhsh, 2005). In *S. mansoni*, LDN is expressed by cercariae, schistosomula, adults, and
469 eggs, and it is localized on the surface of all vertebrate stages analyzed (Nyame at al., 2003).
470 We found two WFA-binding glycoproteins -SFP and serpin (Table 2)- that were both present in
471 the ES extract but not in the TG extract. Since these two glycoproteins have been reported to be
472 tegument-associated proteins (Abath and Werkhauser, 1996), their presence in the ES extract
473 could be explained by the shedding of tegument components to the incubation medium during
474 preparation of the ES extract.

475 Lectin LTA binds to the Fucα1-3GlcNAc glycan motif. This motif can be found
476 forming part of several glycans that are abundantly expressed on the glycoproteins of
477 schistosomes, such as LDN-F, F-LDN-F and Le^X (Nyame et al., 2003; Robijn et al., 2005).
478 Other glycans that also contain this motif are Le^Y and pseudo-Le^Y (Fucα1-3Galβ1-4(Fucα1-
479 3)GlcNAcβ1). Le^Y has never been found in schistosomes (Nyame et al., 1998) but pseudo-Le^Y
480 has been found in schistosome cercarial glycolipids (Wuhrer et al., 2000; Meyer et al., 2005).

481 Thus, given the ample range of glycans that could be bound by the lectin LTA and the
482 fact that many of these glycans are present in schistosome glycoproteins, the high number of
483 bands that were recognized by LTA in the adult *S. bovis* ES and TG extracts (Fig. 1(C)) was not
484 unexpected. Very similar band profiles have been revealed by lectin LTA on Triton X-100
485 extracts from whole *S. haematobium* and *S. japonicum* adult worms (Nyame et al., 1998), which
486 suggests strong similarities among the fucosylated glycoproteins of adult *S. bovis*, *S.*
487 *haematobium* and *S. japonicum* worms.

488 Regarding the identification of the LTA-binding fucosylated glycoproteins of adult *S.*
489 *bovis* worms, the analysis by mass spectrometry of a fraction of the LTA-binding spots allowed
490 us to identify 7 glycoproteins: SPF, ATPGK, enolase, FbisPA, GAPDH, CBendo and actin. All

491 of them except SPF, CBendo and actin were present in both extracts. This is consistent with the
492 findings of previous works (Pérez-Sanchez et al., 2006), except for GAPDH and FbisPA, which
493 were only found in the TG extract.

494 Moreover, not all these LTA-binding glycoproteins were antigenic. Only the high-MW
495 SPF isoforms, enolase, GAPDH and CBendo were antigenic (Perez-Sánchez et al., loc. cit.),
496 suggesting that there is no direct relationship between the presence of the Fucα1-3GlcNAc motif
497 in *S. bovis* glycoproteins and the antigenicity of these glycoproteins. In fact, the glycans that
498 contain only this kind of fucosylated motif, such as LDN-F and Le^X, generally induce weak
499 antibody responses, although they may have immunomodulatory effects (Robijn et al., 2005).
500 By contrast, glycans containing the Fucα1-3GalNAcβ1- or Fucα1-2Fucα1- motifs, such as F-
501 LDN(-F) and (DF-)LDN-DF, induce strong antibody responses in human and primate
502 schistosomiasis (van Remoortere et al., 2003; Robijn et al., 2005; Wuhrer et al., 2006b). Bearing
503 this in mind, we analysed whether the ES and TG glycoproteins of adult *S. bovis* worms carried
504 F-LDN(-F) and Le^X. Additionally, since Le^Y also contains the LTA-binding motif Fucα1-
505 3GlcNAc we also checked the presence of Le^Y in those glycoproteins.

506 It is well established that KLH and schistosomes share the carbohydrate epitopes F-
507 LDN(-F) (Geyer et al., 2005); this is indeed why we used an anti-KLH polyclonal antibody as a
508 probe in the search for F-LDN(-F)-containing glycans in *S. bovis*. The 1-D western blots with
509 anti-KLH clearly showed that the F-LDN(-F) epitopes were absent from the adult ES and TG
510 glycoproteins (Fig. 1 (B)). This antibody, however, recognized numerous bands on extracts of
511 glycoproteins from the cercariae and eggs of *S. bovis* (personal observation, data not shown).
512 These results suggest that *S. bovis* expresses F-LDN(-F) on cercarial and egg glycoproteins but
513 not on adult worm glycoproteins, in agreement with the results described for *S. mansoni* by
514 Robijn et al. (2005). These authors also reported that *S. mansoni* expresses F-LDN(-F) on
515 glycolipids of cercariae, adults and eggs. Accordingly, it is possible that *S. bovis* might also
516 express F-LDN(-F) on its glycolipids, although here this point was not assessed. In any case, the
517 fact that *S. bovis* expresses F-LDN(-F), at least in its cercarial and egg glycoproteins, is also

supported by the strong IgG and IgM antibody responses against KLH that we observed in all the sheep infected with *S. bovis* (Fig. 5(E), 5(F)).

In the search for Le^X- and Le^Y-bearing glycoproteins within the adult worm ES and TG extracts we obtained some remarkable results (Fig. 4(B), 4(C) and Table 4). First, we found Le^X in *S. bovis*, as was expected in view of its abundant expression in the *Schistosoma* genus (Nyame et al., 1998; Hooke and Yazdanbakshs, 2005). However, we also found Le^Y and this observation was somewhat surprising because this glycan has never been recorded in the genus *Schistosoma*. In this sense, Nyame et al. (1998) elegantly demonstrated the absence of Le^Y, Le^b (Fucα1-2Galβ1-3(Fucα1-4)GlcNAcβ1) and Le^a (Galβ1-3(Fucα1-4)GlcNAcβ1) in the *Schistosoma* species that infect humans.

Second, we found both the Le^X and Le^Y antigens in the TG but not in the ES extract. This is in agreement with the observation that Le^X is expressed by other schistosomes on their tegument surface (Nyame et al., 2003). Nevertheless, we found both Lewis antigens in two glycoproteins, ATPGK and GAPDH, that were present in both the ES and TG extracts, suggesting that the ATPGK and GAPDH isoforms present in the ES extract do not express Lewis groups.

Third, in the TG extract the two anti-Lewis monoclonal antibodies recognised exactly the same isoforms of ATPGK and GAPDH. This finding, together with the well established absence of Le^Y in the human-infecting schistosomes, suggested that we might be facing a problem of cross-reactivity between both monoclonal antibodies, such that the anti-Le^Y antibody would be recognising the Le^X antigen. However, we ruled out this possibility because both antigens (Le^X and Le^Y) are quite different and, above all, because we observed by means of an inhibition ELISA -using the neoglycoproteins BSA-LNFPIII and BSA-Le^Y- that there was no cross-reactivity at all between both monoclonal antibodies (data not shown). Consequently, the presence of Le^Y on these two *S. bovis* glycoproteins appeared to be real. In addition, we observed antibody responses against both the Le^X and Le^Y epitopes in all the sheep infected with *S. bovis* (Fig. 5(G)-5(J)). The existence of these humoral responses and the different kinetics of the anti-Le^X and anti-Le^Y antibodies reinforced the idea that *S. bovis* actually

546 expresses these two different Lewis antigens. Finally, the spot pattern recognized by lectin
547 UEA-I on the TG extract also supported this idea. As can be seen in Fig. 3 (F), the ATPGK and
548 GAPDH isoforms that were recognized by the anti-Le^Y monoclonal antibody were also bound
549 by lectin UEA-I, which confirms that they carry the Fucα1-2Galβ1- motif, which, in turn, forms
550 part of the Le^Y antigen.

551 Overall, these results indicate that both the Le^X and Le^Y antigens are co-expressed on
552 the same isoforms of ATPGK and GAPDH by adult S. *bovis* worms, although definitive
553 demonstration of this point requires detailed structural analyses.

554 Regarding the possible biological significance of this fact, some data recently published
555 in relation to the Lewis antigens could provide some clues. It has been demonstrated that the
556 Lewis antigens Le^X, Le^a, Le^b and Le^Y bind to the host lectin DC-SIGN through the amino acid
557 Val^{351} (see Meyer et al., 2005). It has also been shown that DC-SIGN also binds to pseudo-Le^Y,
558 a glycan antigen that so far only has been found within schistosomes, in particular on cercarial
559 glycolipids from *S. mansoni* (see Meyer et al., 2005). DC-SIGN is a C-type lectin expressed on
560 the surface of dendritic cells, which in a concerted action with Toll-like receptors determine the
561 balance in dendritic cells between the induction of immunity and tolerance against a particular
562 antigen. It has been hypothesized that the binding of parasite Le^X and pseudo-Le^Y to host DC-
563 SIGN may allow schistosomes to mislead the host immune system by down-regulating dendritic
564 cell function in all stages of infection (Meyer et al., loc. cit). Perhaps this hypothesis could also
565 be applied to the Le^X and Le^Y expressed by *S. bovis*.

566 In conclusion, the identification in *S. bovis* of all these glycans and carrier proteins
567 provides new and exciting perspectives for future studies on the biological roles of these
568 molecules in host-*S. bovis* relationships, and, perhaps, for their potential use as diagnostic or
569 vaccine antigens.

570

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572

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695
696 Table 1. Lectins used as probes in the screening of the ES and TG extracts.
697

Lectin	Source	Carbohydrate motif binding specificity	Label	Working concentration
GNA	*Galantus nivalis*	Manα1-3Man Manα1-6Man Manα1-2Man	digoxigenin	1 µg/ml
SNA	*Sambucus nigra*	NeuNAcα2-6Galβ	digoxigenin	1 µg/ml
MAA	*Maackia amurensis*	NeuNAcα2-3Galβ	digoxigenin	5 µg/ml
DSA	*Datura stramonium*	Galβ1-4GlcNAc (LacNAc) GlcNAc-Ser/Thr	digoxigenin	1 µg/ml
PNA	*Arachis hypogea*	Galβ1-3GalNAc-Ser/Thr	digoxigenin	10 µg/ml
WFA	*Wisteria floribunda*	GalNAcβ1-4GlcNAc (LDN)	biotin	1 µg/ml
LTA	*Lotus tetragonolobus*	Fucα1-3GlcNAc	biotin	1 µg/ml
UEA.I	*Ulex europaeus*	Fucα1-2Galβ	biotin	1 µg/ml

698

699 Table 2. Identification by MALDI-TOF-TOF MS and NCBInr database searching of the ES
700 proteins revealed by lectins LTA, GNA and WFA (from Fig. 2). SPF, surface protein-fluke;
701 Pre-proSA, pre-pro serum albumin; ATPGK, ATP:guanidino kinase; Serpin, serine protease
702 inhibitor; FbisPA, fructose 1,6 bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate
703 dehydrogenase; CBendo, cathepsin B endopeptidase.
704

Spot no.	Lectin bound	Protein	Species	Accession No. (NCBI)	MW (kDa) Theo/exp	pI Theo/exp	Cover. %	MASCOT score
1	LTA,GNA,WFA	SPF	*S. mansoni*	T30271	191/191	4.7/5.1	6	94
2	LTA,GNA,WFA	SPF	*S. mansoni*	T30271	191/153	4.7/5.2	5	84
3	LTA,GNA,WFA	SPF	*S. mansoni*	T30271	191/123	4.7/5.1	6	90
32	GNA	SPF	*S. mansoni*	T30271	191/51	4.7/5.5	7	101
33	GNA	SPF	*S. mansoni*	T30271	191/52	4.7/5.6	6	83
34	GNA	SPF	*S. mansoni*	T30271	191/52	4.7/5.7	5	83
47	GNA	SPF	*S. mansoni*	T30271	191/46	4.7/5.4	6	86
48	GNA	SPF	*S. mansoni*	T30271	191/46	4.7/5.5	6	90
49	GNA	SPF	*S. mansoni*	T30271	191/47	4.7/5.6	7	101
16	LTA	Pre-proSA	*Ovis aries*	NP_001009376	71/89	5.8/6.0	19	120
17	LTA	Pre-proSA	*Ovis aries*	NP_001009376	71/89	5.8/6.1	13	84
18	LTA	Pre-proSA	*Ovis aries*	NP_001009376	71/89	5.8/6.2	14	96
19	LTA	Pre-proSA	*Ovis aries*	NP_001009376	71/88	5.8/6.62	16	105
20	LTA	Pre-proSA	*Ovis aries*	NP_001009376	71/88	5.8/6.3	19	120
25	LTA	ATPGK	*S. mansoni*	P16641	77/93	8.1/7.3	19	120
26	LTA	ATPGK	*S. mansoni*	P16641	77/93	8.1/7.4	34	208
27	LTA	ATPGK	*S. mansoni*	P16641	77/92	8.1/7.6	12	112
28	LTA	ATPGK	*S. mansoni*	P16641	77/92	8.1/7.8	19	120
29	GNA,WFA	serpin[b]	*S. haematobium*	AAA19730	44/66	4.9/5.4	-	47
30	GNA,WFA	serpin[b]	*S. haematobium*	AAA19730	44/65	4.9/5.5	-	49
31	GNA,WFA	serpin[a]	*S. haematobium*	AAA19730	44/66	4.9/5.6	19	88
35	LTA	Enolase[a]	*S. mansoni*	AAC46886	47/57	6.2/6.9	10	111
36	LTA	Enolase	*S. mansoni*	AAC46886	47/57	6.2/7.1	17	113
38	LTA	FbisPA	*S. mansoni*	AAA57567	40/46	7.4/6.8	49	225
39	LTA	FbisPA	*S. mansoni*	AAA57567	40/46	7.4/7.0	17	99
40	LTA	FbisPA	*S. mansoni*	AAA57567	40/46	7.4/7.1	49	225
41	LTA	FbisPA	*S. mansoni*	AAA57567	40/46	7.4/7.4	51	228
42	LTA	FbisPA	*S. mansoni*	AAA57567	40/46	7.4/7.7	51	230
43	LTA	GAPDH	*S. mansoni*	JL0121	37/44	7.7/7.8	21	90
44	LTA	GAPDH [a]	*S. mansoni*	JL0121	37/44	7.7/8.4	21	115
45	LTA	GAPDH	*S. mansoni*	JL0121	37/44	7.7/8.5	17	81
46	LTA	GAPDH [a]	*S. mansoni*	JL0121	37/44	7.7/8.6	17	134
51	LTA	CBendo	*S. mansoni*	CAC85211	40/41	5.7/6.2	21	88
52	LTA	CBendo	*S. mansoni*	CAC85211	40/41	5.7/6.4	19	86

705

706 [a] Spots identified using PMF data in combination with MS/MS data.
707 [b] Spots unidentified using PMF data but identified using MS/MS data.
708

Table 3. Identification by MALDI-TOF-TOF MS and NCBInr database searching of the TG proteins revealed by the lectins LTA and UEA-I (from Fig. 3). ATPGK, ATP:guanidino kinase; FbisPA, fructose 1,6 bisphosphate aldolase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Spot no.	Lectin bound	Protein	Species	Accession No. (NCBI)	MW (kDa) Theo/exp	pI Theo/exp	Cover. %	MASCOT score
2	LTA, UEA-I	ATPGK	*S. mansoni*	P16641	77/90	8.1/7.7	19	120
3	LTA, UEA-I	ATPGK	*S. mansoni*	P16641	77/90	8.1/7.8	34	208
4	LTA, UEA-I	ATPGK	*S. mansoni*	P16641	77/89	8.1/8.0	12	112
22	LTA, UEA-I	Enolase[a]	*S. mansoni*	AAC46886	47/58	6.2/6.7	22	90
23	LTA, UEA-I	Enolase[a]	*S. mansoni*	AAC46886	47/58	6.2/6.7	10	111
24	LTA, UEA-I	Enolase	*S. mansoni*	AAC46886	47/58	6.2/6.8	14	97
25	LTA, UEA-I	Enolase	*S. mansoni*	AAC46886	47/58	6.2/7.0	16	86
26	LTA, UEA-I	Enolase	*S. japonicum*	P33676	48/58	6.2/7.2	24	125
29	LTA, UEA-I	Enolase [b]	*S. mansoni*	AAC46886	47/48	6.2/6.6	-	61
30	LTA, UEA-I	Enolase [b]	*S. mansoni*	AAC46886	47/48	6.2/6.8	-	82
27	LTA	Actin	*S. mansoni*	AAA62377	42/48	5.5/6.2	29	152
28	LTA	Actin	*S. mansoni*	AAA62377	42/46	5.5/6.4	18	113
33	LTA, UEA-I	FbisPA	*S. mansoni*	AAA57567	40/48	7.4/7.4	51	228
35	LTA, UEA-I	FbisPA	*S. mansoni*	AAA57567	40/48	7.4/7.6	17	99
36	LTA, UEA-I	FbisPA	*S. mansoni*	AAA57567	40/48	7.4/7.7	51	230
31	LTA, UEA-I	GAPDH [b]	*S. mansoni*	JL0121	37/42	7.7/7.1	-	107
32	LTA, UEA-I	GAPDH	*S. mansoni*	JL0121	37/44	7.7/7.2	22	119
34	LTA, UEA-I	GAPDH	*S. mansoni*	JL0121	37/44	7.7/7.6	17	81
37	LTA, UEA-I	GAPDH	*S. mansoni*	JL0121	37/44	7.7/7.8	21	90
38	LTA, UEA-I	GAPDH[a]	*S. mansoni*	JL0121	37/44	7.7/8.4	21	115

[a] Spots identified using PMF data in combination with MS/MS data.
[b] Spots unidentified using PMF data but identified using MS/MS data.

720 Table 4. Identification by MALDI-TOF-TOF MS and NCBInr database searching of the TG
721 proteins that contain Le^X and Le^Y groups (from Fig. 4). ATPGK, ATP:guanidino kinase;
722 GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
723

Spot no.	Protein	Species	Accession No. (NCBI)	MW (kDa) Theo/exp	pI Theo/exp	Cover. %	MASCOT score
1, 9	ATPGK	*S. mansoni*	P16641	77/90	8.1/7.7	19, 21	120, 123
2, 10	ATPGK	*S. mansoni*	P16641	77/99	8.1/7.8	30, 34	201, 208
3, 11	ATPGK	*S. mansoni*	P16641	77/89	8.1/8.0	12,16	112, 118
4, 12	GAPDH	*S. mansoni*	JL0121	37/44	7.7/7.6	22, 27	100, 115
5, 13	GAPDH	*S. mansoni*	JL0121	37/44	7.7/7.9	21, 26	90, 115
6, 14	GAPDH	*S. mansoni*	JL0121	37/44	7.7/8.3	23, 25	115, 115
7, 15	GAPDH	*S. mansoni*	JL0121	37/44	7.7/8.4	15, 17	78, 81
8, 16	GAPDH	*S. mansoni*	JL0121	37/44	7.7/8.5	28, 29	134, 139

724
725
726

Figure captions

Figure 1. (A) SDS-PAGE: the ES and TG extracts were resolved in 5-20% polyacrylamide gels and stained with silver nitrate. (B) 1-D Western blots of the ES and TG immunostained with the polyclonal anti-KLH antibody (developed with 4-Cl-1-naphthol) or the anti-Le^X and anti-Le^Y monoclonal antibodies (developed with luminol). (C) 1-D lectin blots of the ES and TG extracts: lectins GNA, SNA, MAA, DSA, PNA, WFA, LTA and UEA-I were used as probes. The arrow indicates the band not abolished in the inhibitory assays.

Figure 2. Two-dimensional images of the ES extract. (A) and (B) silver-stained 12% polyacrylamide gels with pH ranges of 5-8 and 7-10. Two-dimensional lectin blots in the pH 5-8 and 7-10 ranges with LTA (C) and (D), GNA (E) and (F) and WFA (G) and (H). Numbered circles indicate the lectin-binding spots, which were analyzed by mass spectrometry.

Figure 3. Two-dimensional images of the TG extract. (A) and (B) silver-stained 12% polyacrylamide gels with pH ranges of 5-8 and 7-10. Two-dimensional lectin blots in the pH 5-8 and 7-10 ranges with LTA (C) and (D), and UEA-I (E) and (F). Numbered circles indicate the LTA- and UEA-I-binding spots, which were analyzed by mass spectrometry.

Figure 4. Immunoprecipitation and identification of the Le^X- and Le^Y-bearing glycoproteins from the TG. (A) Silver-stained SDS-PAGE of the supernatants (1) and pellets (2) of the coupling reaction of the anti-Le^X and anti-Le^Y mAb, and TG to protein G-Sepharose 4B. HC, mAb heavy chains; LC, mAb light chains. Arrows indicate contaminants also bound by protein G. (B) Silver-stained SDS-PAGE of the supernatants (1) and pellets (2) from the immunoprecipitation of TG with the mAb coupled to protein G-Sepharose 4B. The inmunocaptured bands are circled and annotated with their apparent MW. (C) Images of the immunoprecipitation pellets after 2-D electrophoresis in gels of 12% polyacrylamide and pH

755 ranges of 5-8 and 7-10. Numbered circles indicate the spots corresponding to the
756 immunocaptured proteins, which were analyzed by mass spectrometry
757
758 Figure 5. ELISA. IgG and IgM antibody responses to ES, TG, KLH and the neoglycoproteins
759 BSA-LNFPIII (Le^X) and BSA-Le^Y in three sheep infected with *S. bovis*. Each series of points
760 represents an individual sheep. The plots are typical of three experiments.

Figure 1

Figure 1

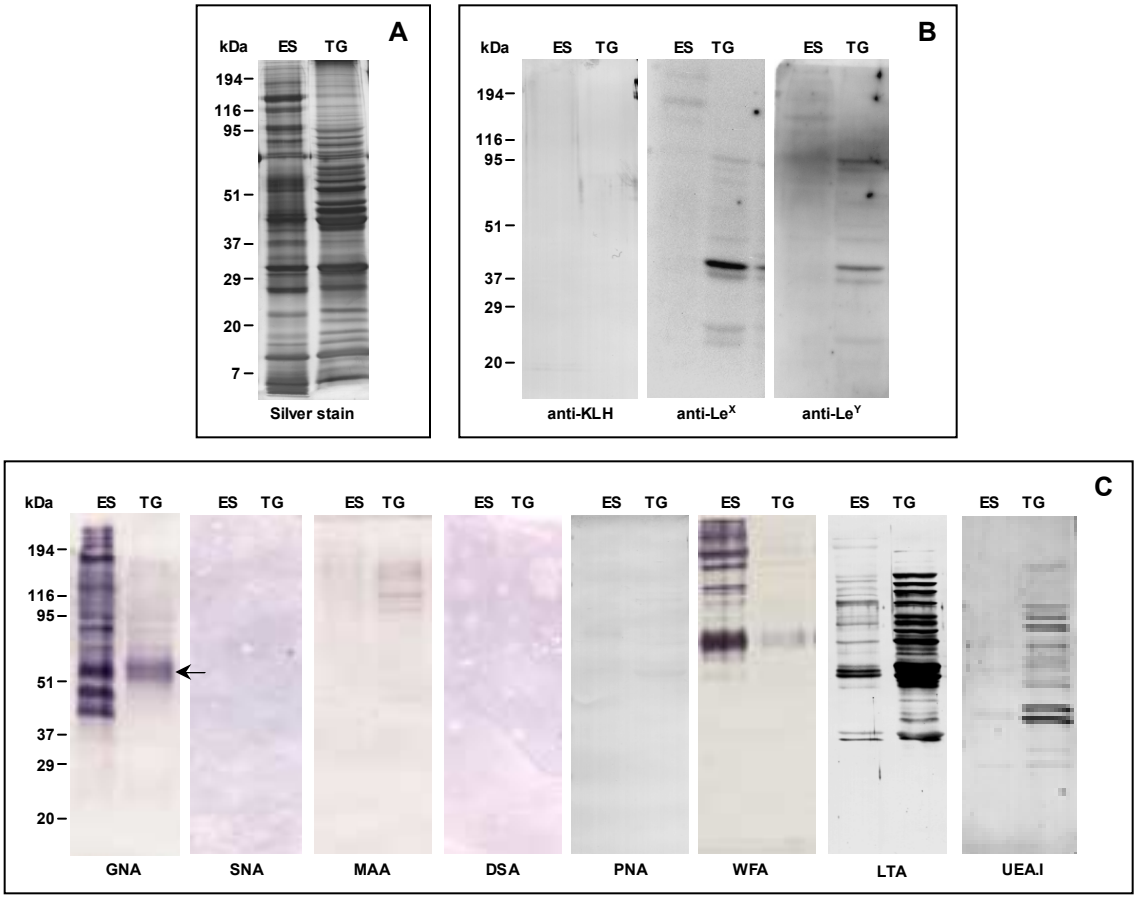

Figure 2

Figure 2

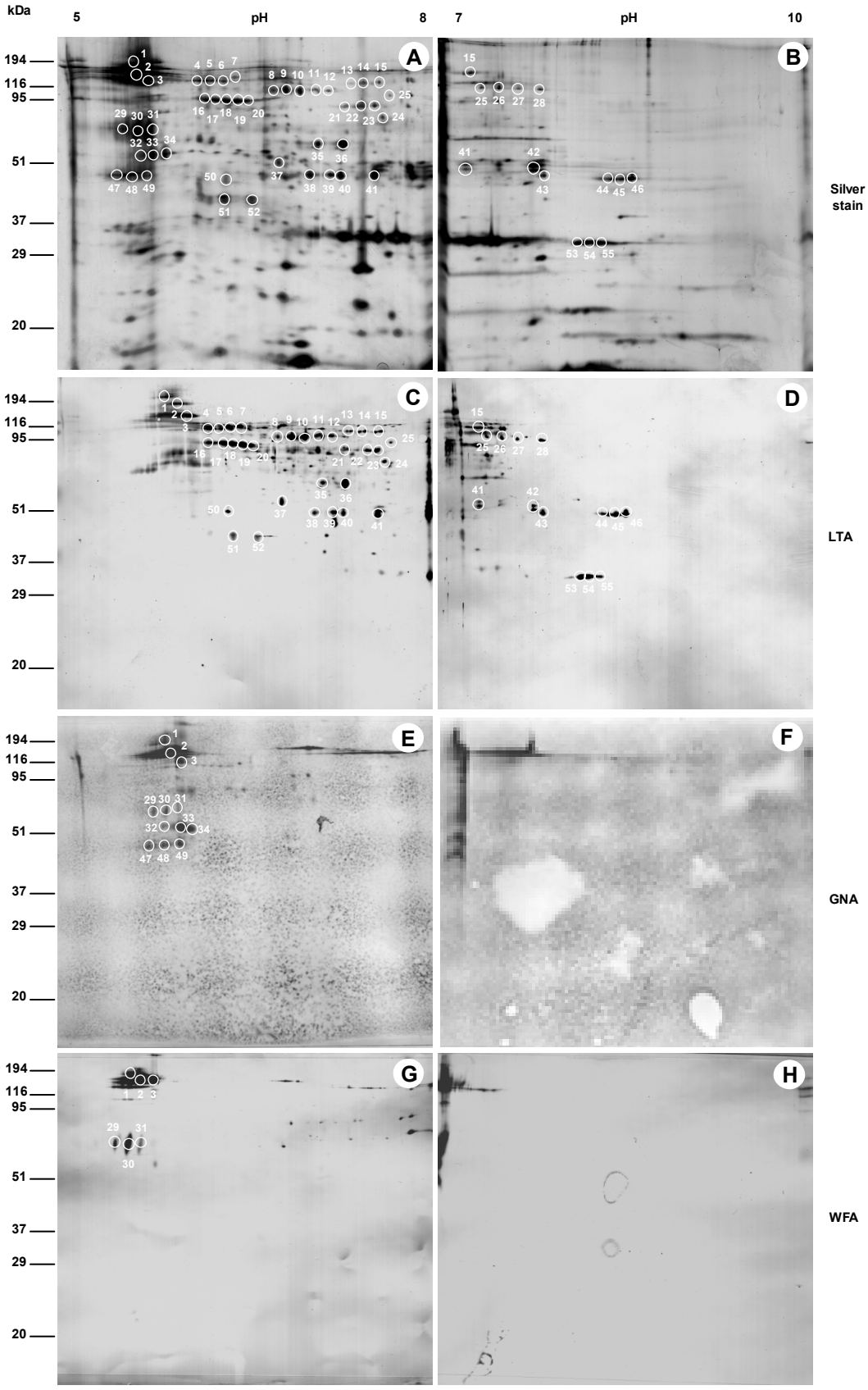

Figure 3

Figure 3

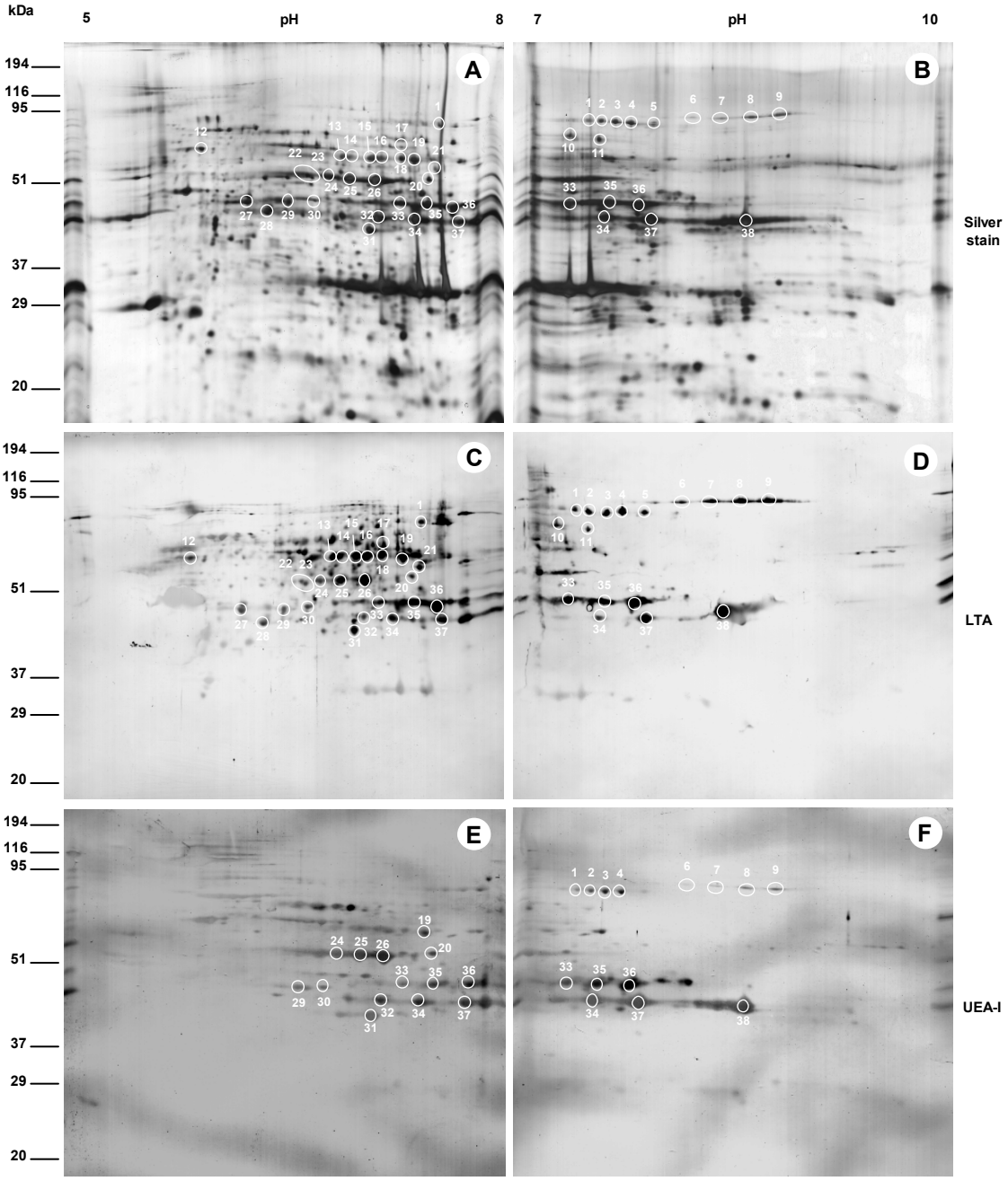

Figure 4

Figure 4

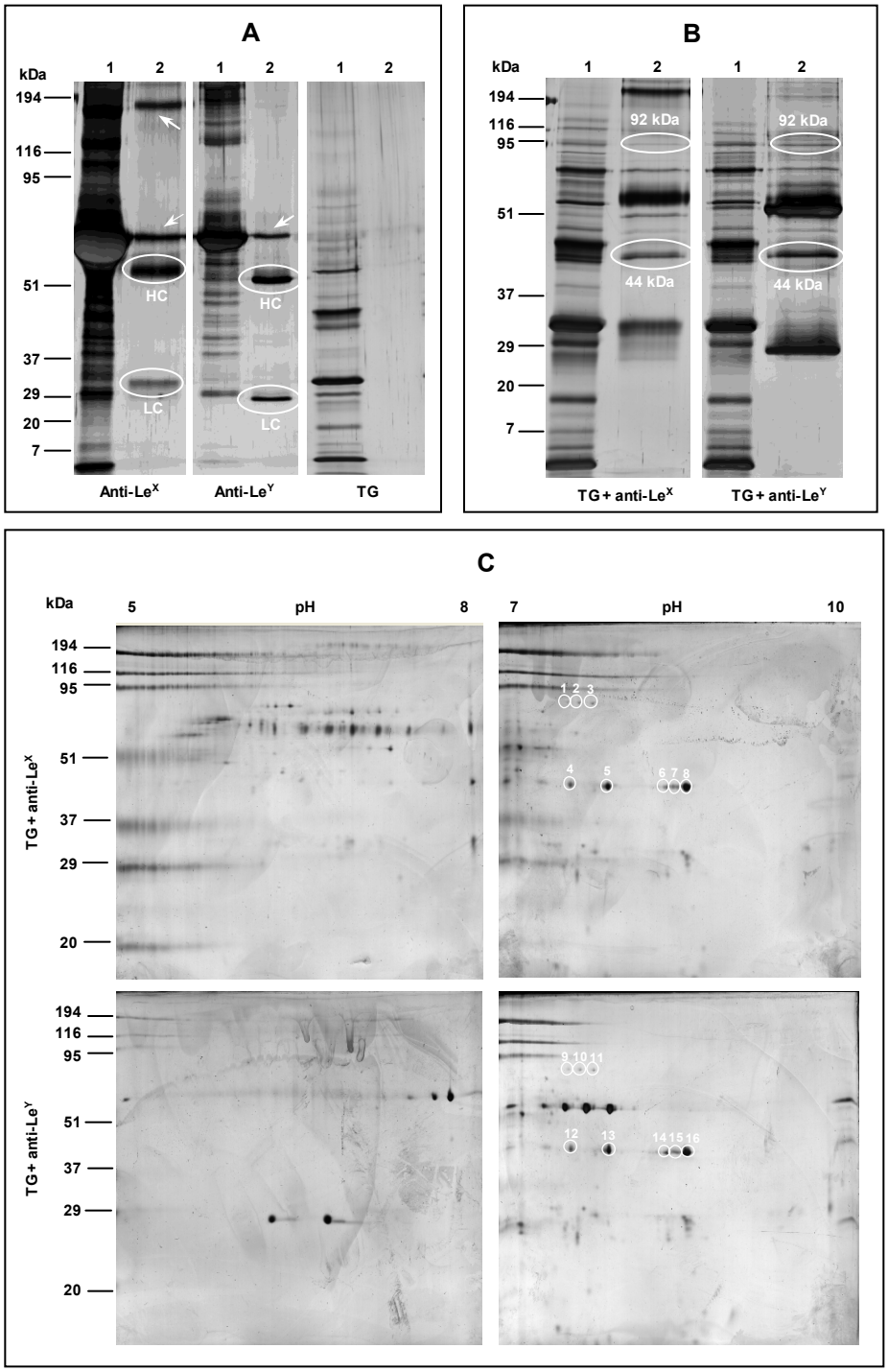

Figure 5

Figure 5