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Detection of O-glycosylated proteins from different *Trichinella* species muscle larvae total extracts

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Summary

The aim of the work was to analyze oligosaccharide composition with the focus on O-linked glycoproteins presence in the total extract obtained from different *Trichinella* species muscle larvae by means of lectin affino-blot with lectins selected for their sugar specificity. The absence of reactivity with two lectins, *Tritrichomonas mobilensis* lectin and *Maackia amurensis* agglutinin, indicated that the species of the *Trichinella* genus do not synthesize sialic acid. Reactivity with *Helix pomatia* lectin, *Vicia villosa* lectin-B4, peanut agglutinin and *Ulex europeus* lectin-I identified the presence of O-linked glycans identical to carcinoma-associated Tn-antigen (GalNAc-α-Ser/Thr) and T-antigen (Gal-β1,3-GalNAc-α-Ser/Thr) and also structures analogous to ABH-blood group antigens. The results obtained may contribute to a better understanding of the glycobiology of this parasitic nematode in relation to occupation of its intracellular niches.

Keywords: *Trichinella*, O-glycans, lectin-blot

Introduction

Glycoconjugates play a significant role in the life cycle and pathology of most major parasites. It is becoming clear that many of the protozoal and helminthic parasites rely on carbohydrate-binding proteins in the host to promote their parasitism, and they have elaborated intriguing strategies to defeat the anticarbohydrate immunity of the host (Schmidt & Roberts, 1996).

The mechanisms by which infectious *Trichinella* larvae recognise, invade, and migrate within the intestinal epithelium are still unknown. However, it is likely that carbohydrate structures on the nematode contact surface and/or in its excretory/secretory products (ESP) are important in these events (Butcher *et al*., 2000). *Trichinella* glycosylated ESP had been found inside of the hypertrophic nuclei of nurse cells (Vassilatis *et al*., 1996; Yao & Jasmer, 1998), raising the assumption that they could be involved in the induction of the aberrant skeletal muscle phenotype. It was shown however, that recombinant 49 kDa ES protein of *T. nativa* does not lose its antigenicity, although the recombinant form was deglycosylated. Moreover, it was reactive with five sera from mice infected with different *Trichinella* strains (Zheng *et al.,* 2007). The glycobiology of *Trichinella* is becoming a subject of rising interest. N-glycosylation has been characterized in detail in adult *T. spiralis* total extracts (Morelle *et al*., 2000) and also in ESP from *T. spiralis* muscle larvae (Gruden-Movsesijan *et al*., 2002), in which the presence of the unusual sugar tyvelose has been discovered (Wisnewski *et al*., 1993; Reason *et al*., 1994). Except for one report (Gruden-Movesijan *et al*., 2002), there is virtually no information available on the structure and the synthesis of O-glycans in *Trichinella*. This work is focused on the demonstration of the mucin-type O-glycosylated proteins in muscle larvae crude extract from five different *Trichinella* isolates by a set of lectins selected according to their sugar specificity.

Material and methods

Parasites and crude extract preparation

The reference strains *T. spiralis* (code ISS03), *T. pseudospiralis* (code ISS13), *T. britovi* (code ISS02), *T. murrelli* (code ISS035) and *T. nelsoni* (code ISS029) used in this study were kindly provided by Prof. Eduardo Pozio (Instituto Superiore di Sanita, Italy). Briefly, BALB/C mice were inoculated with 400 infectious larvae of each isolate. *Trichinella* spp. muscle larvae were recovered 45 days post infestation (d.p.i.) by artificial digestion of muscle tissue of the carcasses according to a standard protocol, as referenced in Pozio *et al.* (1992) and Zarlenga *et al.* (1999). Muscle larvae of each isolate were washed three times with physiological saline solution, then homogenized in glass-Teflon homogenizer in phosphate buffered saline, pH 7.2

Table 1. Carbohydrate binding specificity of the lectins applied in this study

Lectin	Abbreviation	Carbohydrate binding specificity	Concentration
Helix pomatia agglutinin	HPA	GalNAc-α-	20µg/ml
Vicia villosa lectin-isoform B4	VVL-B4	GalNAc-α-	20µg/ml
Arachis hypogea agglutinin	PNA	Galβ3GalNAc	50µg/ml
Ulex europaeus agglutinin-I	UEA-I	Fucα2Gal	50µg/ml
Maackia amurensis lectin-II	MAL	Neu5Acα3Galβ4GlcNAc	6.6µg/ml
Tritrichomonas mobilensis lectin	TML	Neu5Ac	2.5µg/ml

(PBS), on an ice bath and centrifuged at 10 000g/min for 30 min. The supernatant was used as a crude extract and stored at -80˚C. The protein content was determined by the method of Bradford (1976).

SDS-PAGE and Western blot

Components of the *Trichinella* crude extract products of all five isolates were separated on 10 % SDS-polyacrylamide mini-gel electrophoresis under reducing conditions as described previously (Laemli, 1970) and then subjected to Western blotting on nitrocellulose membranes (Sigma) according to Towbin *et al.* (1979). Membranes were blocked with 5 % non-fat dry milk in tris-buffered saline (TBS), pH 7.2 for 30 min.

Blots were incubated with biotinylated lectins- *Arachis hypogea* agglutinin (PNA), *Vicia villosa* lectin, B_4-isoform ($VVL-B_4$) and *Maackia amurensis* lectin, type II (MAL-II, Vector Laboratories), *Helix pomatia* agglutinin (HPA) and *Ulex europaeus* agglutinin, type I (UEA-I, Sigma-Aldrich), and *Tritrichomonas mobilensis* lectin (TML, Calbiochem-

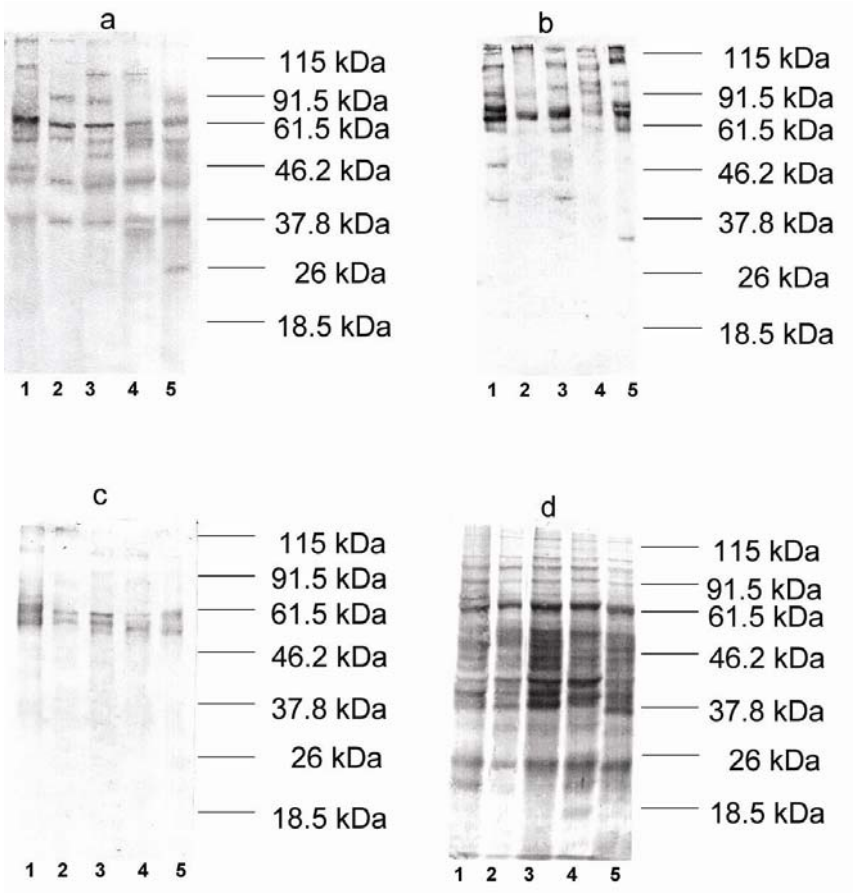

Fig. 1. Affino-blot analyses of *Trichinella* spp glycoproteins from muscle larvae total extracts, 45 d.p.i. The oligosaccharide sequence of the glycoproteins was probed with lectins of different carbohydrate specificity: a-VVL-B4; b-PNA; c-HPA; d-UEA-I. Specificity of binding was controlled by inhibition with appropriate sugars (data are not presented here).
Line 1 *T. nelsoni*; 2 *T. murrelli*; 3 *T. britovi*; 4 *T. pseudospiralis*; 5 *T. spiralis*.

Novabiochem). The sugar specificities and the concentrations used are listed in Table 1. The specificity of the binding was checked by inhibition of the particular lectin with 0.1 M solution of the target monosaccharide: galactose for PNA, N-acetylgalactosamine for VVL-B_4 and HPA, fucose for UEA-I and sialic acid for TML (Sigma-Aldrich). The membranes were incubated with the lectins for 1 hour at room temperature, washed three times with TBS for 5 min, then incubated 30 min with streptavidin-HRP (Sigma-Aldrich) and washed three times with TBS. The color reaction was developed by 10 min incubation with 0.18% diamino benzidine solution (Dako Cytomation) (TBS pH 7.2, 0.05% H_2O_2). The approximate molecular masses of the proteins identified by lectin-blot were estimated versus protein standards (Beijing Genetech Co., Ltd) ranging from 115 to 18.5 kDa.

Results

The proteins of the muscle larvae total extracts from different *Trichinella* isolates were separated in gels, which were stained with Coomassie brilliant blue. The pattern of the bands did not show remarkable differences between the particular strains. The pattern of crude extract glycoproteins, visualized by binding with different lectins, is shown in Fig. 1. All applied lectins, except for MAL-II and TML, showed sugar-specific reactions with *Trichinella* total extract products. The five *Trichinella* isolates share several bands of glycoproteins identified by HPA, VVL-B_4, PNA and UEA-I, summarized in Table 2. Among the bands common for all strains, there was a glycoprotein with molecular mass of 58 kDa that simultaneously reacted to HPA, VVL-B_4 and PNA, and another one of 111 kDa, which interacted with both PNA and UEA-I. The most abundant number of glycoproteins common to all strains was reactive with UEA-I. The greatest interspecies variety was displayed by the products identified with PNA.

Discussion

O-linked glycans are involved in cell-adhesion events during sperm-egg fertilization, host-microbial interactions and viral infections (Yang et *al*., 2000; Hooper & Gordon, 2001; Primakoff & Myles, 2002). A variety of tumor-associated antigens are O-linked glycans (Brockhausen, 1999). Recently, evidence was provided that Tn-antigen is widely distributed among the parasites belonging to both major phyla, the Plathelminthes and the Nemathelminthes (Casaravilla *et al*., 2003), and sialyl-Tn-antigen was particularly found in *Echinococcus granulosus* and *Fasciola hepatica* (Alvarez Errico *et al*., 2001; Freire *et al*., 2003).

Our study was focused on identifying the O-glycosylation in total extract proteins from members of the *Trichinella* genus. For this purpose we used the lectins VVL-B_4 and HPA to demonstrate Tn-structures (Tollefsen & Kornfeld, 1983; Roth, 1984), and PNA because of its specificity to T-antigen (Lotan, 1975). To determine whether these structures had been sialylated, we used also MAL-II and TML. The first lectin recognizes α-2,3-linked sialic acid, and the second one identifies sialic acid residues without any linkage preference (Knibs *et al.*, 1991; Babál *et al*., 1994). Finally, UEA-I was applied to indicate α2-bound Fuc (Sugii & Kabat, 1982).

The results showed that glycoproteins bearing both Tn- and T-antigens are abundantly present in somatic extracts of *Trichinella* spp (Fig. 1-a, b). T-antigen (Galβ3GalNAc-αSer/Thr) derives after β3-galactosylation of Tn-structure (GalNAc-αSer/Thr) (Brockhausen, 1999). One glycoprotein with molecular mass of 58 kDa was common to all isolates (Table 2) and simultaneously interacted with HPA, VVL-B_4 and PNA. It seems that not all Tn- structures are good substrates for the β3-galactosyltransferase in *Trichinella* because this 58 kDa glycoprotein exposes T-antigens, illustrated by PNA reactivity, together with Tn-structures, which was shown through the interaction with HPA and

Table 2. Interaction of *Trichinella* spp. total extract products with lectins

Molecular masses of proteins in the total extract of *T. spiralis, T. pseudospiralis, T. britovi, T. murrelli* and *T. nelsoni* (kDa) which reacted in lectin-blot						
	Common bands	*T. spiralis*	*T. pseudospiralis*	*T. britovi*	*T. murrelli*	*T. nelsoni*
HPA	105, 60, 58, 54	-	-	-	-	-
VVL-B_4	67, 58, 43, 36	91, 51, 25	103, 33	103, 51	91	109, 48
PNA	111, 58	105, 85, 62, 52, 28	98, 85, 79	98, 52, 38	-	98, 73, 62, 44, 38
UEA-I	111, 108, 102, 98, 85, 73, 62, 41, 40, 35, 23	52, 46, 45, 43	52, 20, <18	44, 43	-	20
MAL-II	-	-	-	-	-	-
TML	-	-	-	-	-	-

VVL-B_4. This observation could be explained by the fact that the specificity of this enzyme is controlled by the amino acid sequence of the peptide substrates (Granovsky *et al*., 1994).

Obviously, sialyl-Tn and sialyl-T do not exist in *Trichinella* spp. since their somatic glycoproteins reacted neither with TML, nor with MAL-II. This was in accordance with the previous report on the absence of acidic sugars in adult *T. spiralis* (Morelle *et al*., 2000). Moreover, alcian-blue staining of *T. spiralis* muscle larvae tissue sections and histochemistry with the lectins specific for sialic acid also did not show any labeling (Milcheva *et al*., 2009). The absence of particularly α6-silaylation in *T. spiralis* muscle larvae ESP has been reported by Gruden-Movsesijan *et al*. (2002).

We used HPA in the context of its ability to recognize Tn-antigen (Roth, 1984), a feature that resembles the VVL-B_4 affinity (Table 1). Both lectins showed the same pattern of staining on *T. spiralis* muscle larvae tissue sections (Milcheva *et al.*, 2009). The lectin-blots however revealed that HPA and VVL-B_4 labeled very different products and the pattern of labeling was different as well (Fig. 1-a, c). Actually, only the 58 kDa glycoprotein mentioned above interacted with both HPA and VVL-B_4 (Table 2), suggesting that Tn-glycans (GalNAc-αSer/Thr) still are appropriate ligands for HPA. Another intriguing finding was the small number of glycoproteins labeled with HPA and they were common for all the isolates. Thus, the question cropped up to what structure HPA was reactve in *Trichinella*. This lectin is able to bind single α-N-acetylgalactosaminyl residues but it also can interact with α-GalNAc residues located at the nonreducing terminus of O-glycosidically linked oligosaccharides (Roth, 1984). Blood group A antigens (GalNAcα3(α2Fuc)Gal) (Brockhausen, 1999; Varki *et al*., 1999) possess appropriate ligands for HPA but we did not find any common bands labeled with HPA and UEA-I. Theoretically, core 5 (GalNAcα3GalNAc) and core 7 (GalNAcα6GalNAc) structures (Brockhausen, 1999; Varki *et al.*, 1999) also expose suitable ligands for HPA. However, we are currently not able to provide evidence that these oligosaccharides, which are less distributed in mammals, exist in *Trichinella*.

UEA-I was the lectin that interacted with the greatest number of glycoproteins in all five *Trichinella* isolates (Fig. 1-d). We found a lot of specific products but the main set of them seemed to be common for the genus. This lectin is able to bind α2-linked fucose residues (Sugii & Kabat, 1982), which are known to build the ABH-blood group antigens (Watkins, 1966; Varki *et al.*, 1999). The blots showed one common glycoprotein with molecular weight of 111 kDa (Table 2) and several glycoproteins specific for the particular isolates (98, 85, 73, 62 and 52 kDa) (Table 3) that were labeled with both PNA and UEA-I. Herein, another question arose concerning the PNA combining site. This lectin is known to recognize T-antigen (Lotan *et al*., 1975). Terminal α2-fucosylation of T-antigen is a common modification resulting in type 3 H-blood group sequence (Fucα2Galα3GalNAc-Ser/Thr) (Brockhausen, 1999). Once this modification occurs, PNA is not able to interact with the penultimate Gal (Pereira *et al*., 1976). If we accept that PNA has an affinity for T-antigen only, this set of glycoproteins should expose both T-antigens and type 3 H-blood group antigens, suggesting that for some reason not all T-antigens are good substrates for α2-fucosyltransferase in *Trichinella*. On the other hand, Levroney *et al*. (2005) pointed in their report that PNA recognizes the Galβ3GalNAc sequence not only on T-antigen but also on core 2 O-glycans (Galβ3(β6GlcNAc)GalNAc-αSer/Thr). In mammals, α2-fucosyltransferases compete with β3- and β6-N-acetylglucosaminyltransferases and the latter enzyme is responsible for the core 2 biosynthesis (Brockhausen, 1999; Varki *et al.*, 1999). Further, β6-GlcNAc can serve as a substrate for type 1 or 2 chain building and then for type 1 or 2 ABH-blood group antigens respectively. In the light of these considerations it is tempting to assume that the set of glycoproteins listed in Table 3, express type 1 or 2 B-blood group determinants, which display ligands equally attractive to PNA and UEA-I. Based on the results in this study, the synthesis of A-blood group antigen (GalNAcα3(α2Fuc)Gal) is not much likely to occur in *Trichinella* since we did not find any common glycoproteins simultaneously stained with HPA and UEA-I. Concerning the fucosylation in *Trichinella*, both hypotheses proposed here should be further elucidated. Moreover, the fact that all *Trichinella* spp. share a large number of glycoproteins reactive with UEA-I (Table 2) suggests that these products could be implicated in a strategy for successful accommodation and survival in the host that is general for the genus.

Table 3. Molecular masses of glycoproteins in *Trichinella* spp. muscle larvae total extract (kDa) which were labeled with both PNA and UEA-I

98	85	73	62	52
T. pseudospiralis *T. britovi* *T. nelsoni*	*T. spiralis* *T. pseudospiralis*	*T. nelsoni*	*T. spiralis* *T. nelsoni*	*T. spiralis*

The aim of the presented work was to provide initial information about the O-glycosylated proteins in muscle larvae of five *Trichinella* isolates and the experiments were carried out 45 d.p.i., in the condition of asynchronous invasion. Briefly, the results showed that the mucin-type O-glycosylation is widely distributed in *Trichinella* spp. The presence of glycoproteins bearing Tn- and T-antigens and ABH-blood group sequences was demonstrated by their interaction with the lectins VVL-B_4, PNA and UEA-I, however Tn- and T-antigens were not sialylated. The biosynthesis of core 5 and core 7 structures is also expected due to HPA specific pattern of labeling.

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