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Isolation, characterization and immunolocalization of a globin-like antigen from *Ostertagia ostertagi* adults

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SUMMARY

Western blot analysis using an anti-globin rabbit serum Rb94 revealed a major band of 17 kDa in extracts of *Ostertagia ostertagi* adults and 4th-stage larvae. The adult stage globin-like antigen (OoAdGlb) was purified from total worm extracts by liquid chromatography. The protein has an estimated molecular mass of 36 kDa under non-reducing conditions, suggesting a dimeric structure containing 2 non-covalently linked 17 kDa monomers. Tryptic peptides were sequenced and showed strong similarities with the globins of free-living and parasitic nematodes. Immunolocalization revealed the presence of this globin-like antigen in the body wall musculature and/or the cuticle of *O. ostertagi* adults. An enzyme-linked immunosorbent assay based on the purified OoAdGlb showed no differences in response between calves infected by *O. ostertagi* and/or *Cooperia oncophora* and the negative controls.

Key words: nematode, *Ostertagia ostertagi*, haemoglobin.

INTRODUCTION

Ostertagia ostertagi is a highly pathogenic gastrointestinal nematode of cattle. Outbreaks of clinical ostertagiosis are common in calves under 2 years of age and provoke important production losses. The development of a protective immune response against this abomasum-infecting species occurs rather slowly and leads to non-sterile resistance. The control of gastrointestinal nematodes in livestock animals is achieved mainly by anthelmintic drugs. However, the multiple use of these therapeutics has many drawbacks: the occurrence of residues in meat and milk, the development of resistance and the contamination of the environment. These disadvantages could be overcome by the development of a vaccine.

Attempts to vaccinate calves against *O. ostertagi* using exoantigens, cultured larval stages (Herlich & Douvres, 1979), X-ray (Burger *et al.* 1968) and ultraviolet attenuated larvae (Herlich & Tromba, 1982), infectious larvae administered intravenously (Williams, Roberts & Todd, 1974), and somatic and metabolic antigens of the fourth (L4) larval stage

(Hilderson *et al.* 1995) have so far been without success.

At present, the search for candidate protective antigens against gastrointestinal nematodes is focused on (1) antigens recognized by the host immune system (2) immunomodulatory molecules which assist the parasite to survive in the hostile immunological environment and (3) substances which are not immunogenic during natural infection but nevertheless vital for the parasites' invasion, differentiation, persistence and propagation in the host. Where *O. ostertagi* is concerned, the antigens recognized by antibodies from immune calves have been described (Mansour *et al.* 1990; Canals & Gasbarre, 1990; Hilderson *et al.* 1993). Cross & Klesius (1989) isolated a distinct fraction from *O. ostertagi* L3 extracts which can down-regulate humoral and cellular immune functions in mice. The surface polypeptide profiles of 3rd (L3) and 4th-stage larvae (L4) have been examined (Keith *et al.* 1990). The proteolytic enzymes present in, and secreted by, the different life-cycle stages of *O. ostertagi* were partially characterized (De Cock *et al.* 1993). Putative cysteine protease genes were isolated from an *O. ostertagi* genomic library (Pratt, Boisvenue & Cox, 1992). Recently, a cDNA clone encoding an excretory/secretory (ES) product with multiple tandem repeats was isolated and sequenced (de Graaf *et al.* 1995).

This paper is focused on a 17 kDa immunodominant antigen that drew our attention during

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screening of adult extracts for antigens with diagnostic potential (de Graaf *et al.* 1994). It was recognized by both *O. ostertagi* and *Cooperia oncophora* antiserum. Based on its molecular weight, we suggest that it corresponds to the host-protective globin-like antigen from *Trichostrongylus colubriformis* (Frenkel *et al.* 1992).

MATERIALS AND METHODS

Parasites

Infective larvae (L3) were obtained by culturing the faeces of calves infected with a laboratory strain of *O. ostertagi*. L4 larvae and adults were collected 9 and 21 days respectively after infection of a worm-free calf with 500 000 L3 of *O. ostertagi*. The L4 larvae were harvested by opening the abomasum and placing it on a Baermann apparatus in phosphate-buffered saline (PBS) with penicillin (500 IU/ml) and streptomycin (1 mg/ml) at 37 °C for 5 h. Adults were collected from abomasal washings.

Somatic extracts

After 3 washings in Tris-HCl buffer (0.05 M, pH 8.0) containing 0.2 M NaCl and 1.0% (v/v) Triton X-100, the larval and adult worms were homogenized in a glass-Teflon homogenizer on ice. Insoluble fragments were spun down (25 000 g, 4 °C) and the clear supernatant fractions were filter sterilized (0.2 µm filter; Costar).

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis

SDS-PAGE was performed with crude extracts and purified samples on 12% acrylamide gels (Laemmli, 1970). Proteins were stained using a silver nitrate method (Ansorge, 1985) or transferred to a polyvinylidene difluoride (PVDF) sheet (Immobilon, Millipore) by a semi-dry electrophoretic method (Towbin, Staehelin & Gordon, 1979). The sheets were stained by an indirect immunoperoxidase method using rabbit serum at a dilution of 1:2000 in blot wash buffer (BWB = 0.01 M Tris-HCl buffer, pH 7.4, containing 0.15 M NaCl and 0.05% Tween-20). Anti-rabbit IgG peroxidase (Sigma) (1:17 500 in BWB) was used as a conjugate with diaminobenzidine as substrate.

Sera

Rb94 was an anti-globin rabbit serum developed by immunization with *Nippostrongylus brasiliensis* globin purified by preparative gel electrophoresis (Blaxter, Ingram & Tweedie, 1994a).

Antisera from *O. ostertagi*-, *C. oncophora*- or mixed-(*Ostertagia*+*Cooperia*) infected calves were

collected after an experimental infection period of 17 weeks (20000 L3/week), starting at the age of 7–8 months. Parasite-naive serum was obtained from 8 uninfected animals of the same age.

Globin purification

For the purification of the adult-stage globin, worm extracts were prepared in Tris-HCl buffer (0.05 M, pH 8.5) containing 0.15 M NaCl, 1 mM EDTA and 0.5% (v/v) Triton X-100. Samples of 1 ml were loaded on a Sephacryl S-200 gel filtration (GF) column (800 × 16 mm) and eluted with buffer A (0.05 M ethanolamine-HCl buffer, pH 9.5, containing 1 mM EDTA). Spectrophotometric detection was done at 405 nm and 280 nm. The fractions of interest were concentrated on an Amicon-YM10 ultrafiltration membrane and injected onto a Mono Q HR 5/5 ion-exchange (IE) column (Pharmacia Biotech). The bound fractions were eluted by a step-wise gradient from 5 to 30% buffer B (= buffer A containing 1 M NaCl). Selected fractions were dialysed against 0.1% trifluoroacetic acid in water (buffer C), loaded on a ProRPC HR 5/10 reversed phase (RP) column (Pharmacia Biotech) and eluted with a step gradient of 25 to 45% buffer D (0.1% trifluoroacetic acid in acetonitrile). The collected fractions were lyophilized and evaluated by Western blot analysis using the anti-globin rabbit serum.

Drabkin's reagent (Sigma) was added to some samples to convert the different forms of the haemoglobin to a single cyanoderivative.

Spectrophotometric analysis

Spectrophotometric analysis of the IE-peaks was performed with an Ultrospec II (Pharmacia Biotech) over the range of 300–600 nm. Measurements were corrected for the corresponding buffers.

Amino acid sequencing

The selected fractions were carboxymethylated and digested with trypsin at 30 °C for 16 h in 0.1 M NH₄HCO₃ at an enzyme to substrate ratio of 1:50. Digestion was stopped by acidification and the peptide mixture was separated by reversed phase HPLC using a Vydac C4 column (4.2 × 25 mm) developed in a TFA/CH₃CN system (De Baere *et al.* 1992; Blaxter *et al.* 1994a). Selected peptides were sequenced using an ABI 473 sequencer operated as recommended by the manufacturer.

Database search

Resulting sequence data were aligned with the public databases using the BLAST algorithm (Pearson & Lipman, 1988).

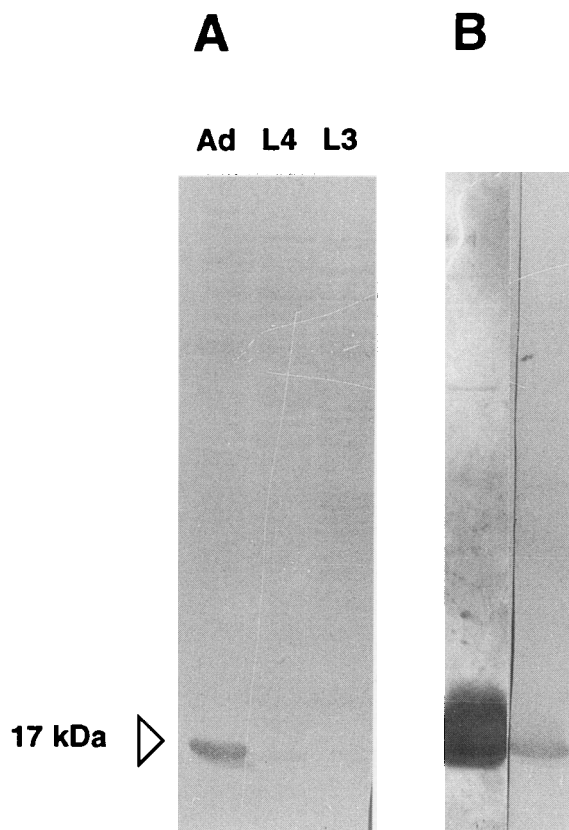


Fig. 1. (A) Western blots of the somatic extracts from *Ostertagia ostertagi* L3 and L4 larvae and adult worms using an anti-globin rabbit serum. A 17 kDa immunoreactive band (arrow) was found in the adult-stage extract. (B) After chromatographic separations the purity of the *O. ostertagi* globin-like antigen OoAdGlb was checked by SDS-PAGE (left) and Western blot using the anti-globin serum (right).

Enzyme-linked immunosorbent assay (ELISA)

The purified fraction was coated overnight (37 °C) at a concentration of 0.5 µg/ml in carbonate buffer (0.025 M, pH 9.6). Diluted bovine sera (1:400) were incubated in phosphate-buffered saline (PBS), pH 7.2, containing 0.5% Tween-20, in duplicate for 1 h. Anti-bovine IgG (whole molecule) peroxidase (Sigma) was added (1:8000). Orthophenylenediamine (0.1%) in citrate buffer (0.038 M, pH 5.0) served as peroxidase substrate. The resulting enzymic reaction was stopped by addition of 2.5 M HCl and absorbance was determined at 492 nm.

Immunolocalization

Sections, 5 µm thick, were made using a cryostat microtome (Microm Heidelberg). They were transferred to coated slides (Nutacon), dried and fixed in acetone (10 min). Sections were stored at -20 °C until further use.

An indirect immunofluorescence assay was performed by incubating the sections in 25 µl anti-

globin rabbit serum diluted (1:100) in PBS (pH 7.2) containing 0.5 M NaCl and 1% bovine serum albumin (PBS/BSA). After incubation in anti-rabbit IgG fluorescein isothiocyanate (Sigma) (1:32 in PBS/BSA) the sections were mounted with buffered glycerin medium and examined. Each incubation lasted 30 min in a humidified atmosphere at 37 °C.

RESULTS

Western blot analysis

Western blot analyses using the anti-globin rabbit serum Rb94 revealed a single major band of 17 kDa in the *O. ostertagi* adult stage (Fig. 1A). At lower serum dilution (1:500) a faint band was observed at the same molecular mass in the 4th-stage larval extract (not shown). No immunoreactive bands were found in the 3rd-stage extract. Since the 17 kDa antigen was predominantly expressed by the adult nematodes, this stage was chosen for further purification of this *O. ostertagi* globin-like protein (OoAdGlb).

Antigen purification

GF-chromatography of total adult extracts revealed 2 absorbance peaks at 405 nm (Fig. 2A). Western blot analysis revealed that the 17 kDa protein was present in both peaks. Based on the elution profile of molecular weight markers, the proteins corresponding to the major (second) peak have an estimated native molecular mass of 36 kDa. The corresponding fractions were retained for further purification on the IE-column. The fractions that passed straight through the IE-column (unbound), just as those that were released at the 5% buffer B-step (bound) (Fig. 2B) showed a 405 nm peak. Both the unbound and bound fractions contained the 17 kDa protein on Western blots and showed almost identical absorbance spectra, with an optimum at 412 nm and 2 minor peaks at 542 and 578 nm (Fig. 3). When Drabkins' reagent was added to the samples before loading on the IE-column, only one 405 nm peak remained (unbound fraction). Pure OoAdGlb was obtained by loading the unbound IE-fractions on a RP-column, and collecting the 45% buffer D-step (Fig. 2C). Purity was checked on silver nitrate stained SDS-PA gels (Fig. 1B).

Antibody response

An ELISA based on the purified OoAdGlb showed no significant differences in antibody responses between the *Ostertagia*-, *Cooperia*- and mixed-infected animals and the negative controls (Fig. 4).

Partial amino acid sequencing

Amino-terminal sequencing of total OoAdGlb released no significant PTH-amino acids. This re-

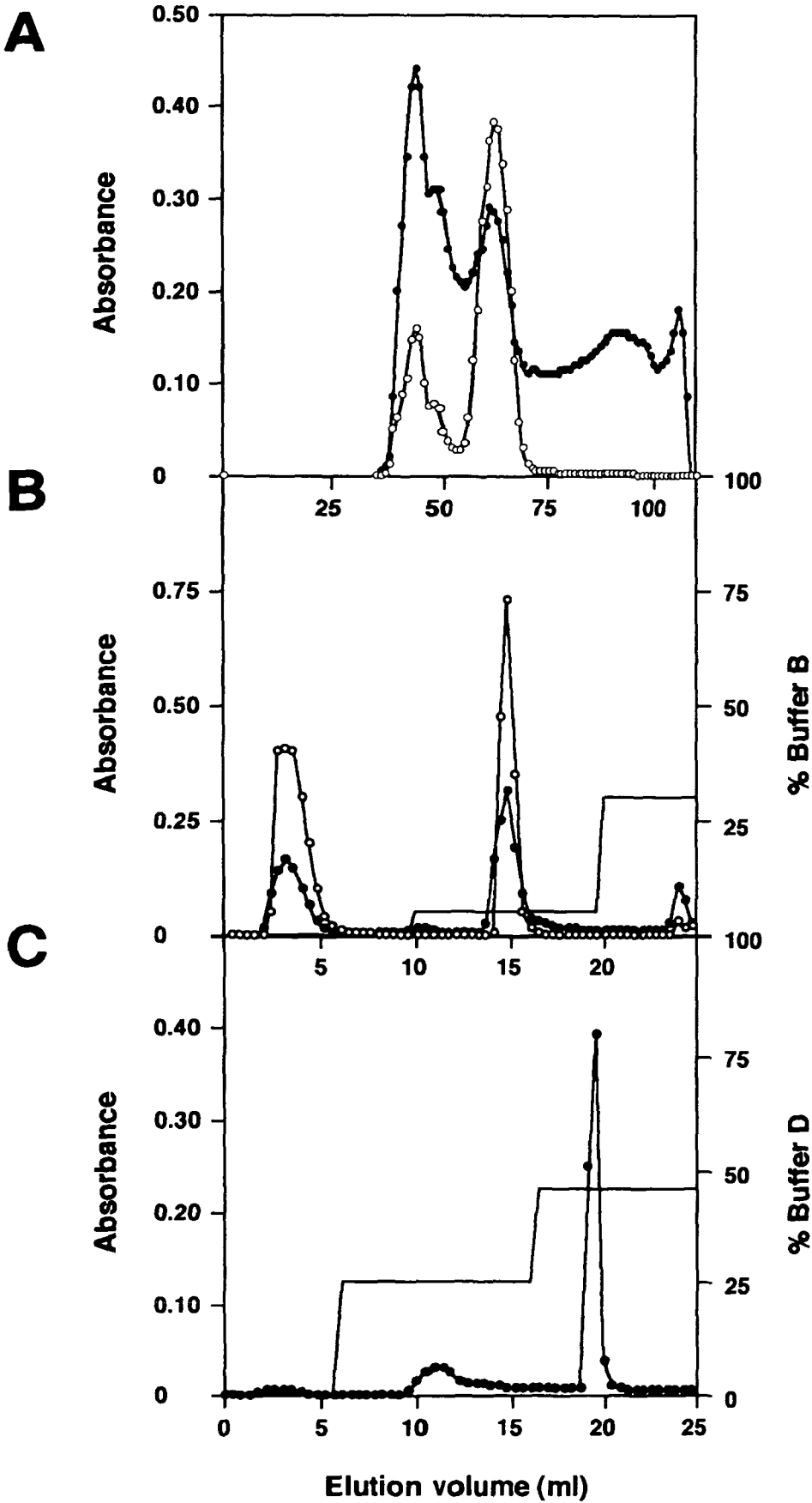


Fig. 2. Elution profile of the FPLC[®] purification of OoAdGlb at 280 (●) and 405 nm (○). (A) Total worm extracts were loaded on a Sephacryl S-200 gel filtration column. (B) Fractions corresponding to the major 405 nm peak were loaded on a Mono Q HR 5/5 ion-exchange column. The unbound fractions (released at 0% buffer B) were chosen for further purification. (C) Finally, OoAdGlb was retained on a ProRPC HR 5/10 column and eluted at 45% buffer D.

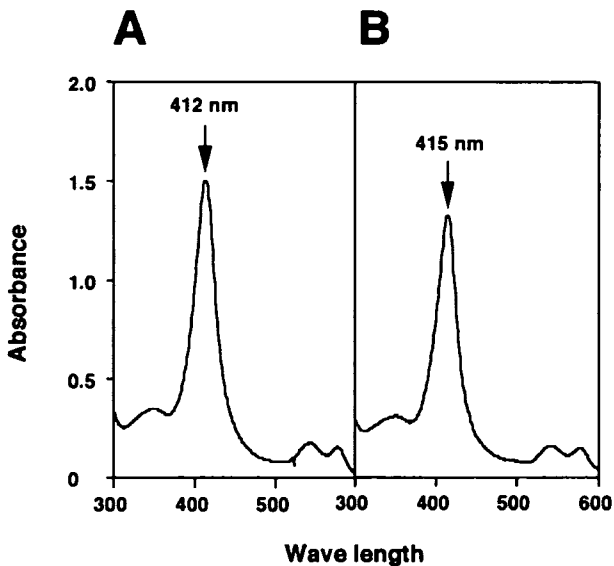


Fig. 3. Both the Mono Q- unbound (A) and the Mono Q-bound fractions (B) show a typical oxy-haemoglobin absorption spectrum, with the Soret (412/415 nm), α (578 nm) and β (542 nm) peaks.

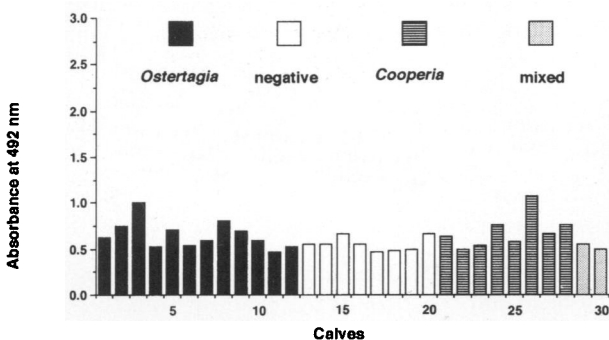


Fig. 4. ELISA based on the purified OoAdGlb using serum from *Ostertagia*- or *Cooperia*-monoinfected calves, mixed-infected calves or parasite-naive animals.

sistance to Edman degradation suggests a modified amino-terminus. In addition, it illustrates that no contaminating proteins with accessible amino-termini are present in our OoAdGlb preparation.

Therefore, tryptic peptides of OoAdGlb were prepared and sequenced. The amino acid sequences of the selected peptides are aligned with globins of *Nippostrongylus* and *Trichostrongylus* (Fig. 5).

Data search

The tryptic peptides of OoAdGlb were used to search a number of databases for homologous proteins. Strong similarities were found with the globins of free-living (*Caenorhabditis elegans*, Sulston *et al.* (1992)) and parasitic nematodes (*T. colubriformis*, Frenkel *et al.* (1992); *N. brasiliensis*, Blaxter *et al.* (1994a); *Ascaris suum*, De Baere *et al.* (1992) and *Pseudoterranova decipiens*, Dixon *et al.* (1991)) (Fig. 5). The most extensive match was found with the cuticular globin of *N. brasiliensis*. The peptide-sequence Trp11 could

	<i>na</i>	<i>helix A</i>	<i>ab</i>	<i>helix B</i>	<i>helix C</i>	
1	MADVKKNCIASLSLAP	I. SKA	QQAQVGKDFYKFFFTN	HPDLRKY	
2	MRFLLLAAFVAYAYAKS.	DEEIRKDALSALDWP	LGSTP	EKLNGREFYKFFFTN	HQDLRKY	
3	MLWFVAVCFATIASVSAMS	PADVKKHTVESMKAVP	VGR..	DKAQNGIDFYKFFFTH	HKDLRKF	
4				YLFQH HPDLR	
					<----->	
					Trp11	
	<i>cd</i>	<i>helix D</i>	<i>helix E</i>	<i>ef</i>	<i>helix F</i>	<i>fg</i>
1	FKGAENF	TADDVQK	SDRFEKLGSGLLLSVHILAH	TFDNEDVFRAF	CRETIORHVG	RGL
2	FKGAETF	TADDIAK	SDRFKLLGNQLLSVHILAAD	TYDNEMIFRAF	VRDTIORHVD	RGL
3	FKGAENF	GADDVQK	SKRFEKQGTALLLAVHVLAN	VYDNQAVFHGF	VRELMNRHEK	RGV
4		
			GAKQGTALVMHVHIFAN	LYDNDMVFR		
			<----->	<----->		
				Trp23/24		
	<i>helix G</i>	<i>gh</i>	<i>helix H</i>		<i>hc</i>	
1	DPALWKAFW.SVWVAFLESK	G.GV	SGDQKAANDKLGTVFNDECQHLAKH		GLPHL	
2	DPKLWKEFW.SIYQKFLLESK	GKTL	SADQKAADFADIGTRFNDEAQQLAHH		GLPHT	
3	DPKLWKIFFDDVWVVPFLESK	GAKL	SGDAKAAMKELNKNFNSEAQHQLEKL		GLPHA	

Fig. 5. Alignment of the sequenced tryptic peptides from OoAdGlb (4) with known nematode globins: (1) *Nippostrongylus brasiliensis* body wall isoform, (2) *Trichostrongylus colubriformis* globin-like antigen and (3) *N. brasiliensis* cuticular isoform. Helical segments are indicated (A-H); amino-, carboxyterminal and interhelical segments are indicated in small. Identity or similarity with *N. brasiliensis* cuticular isoform is indicated as (:) and (.) respectively.

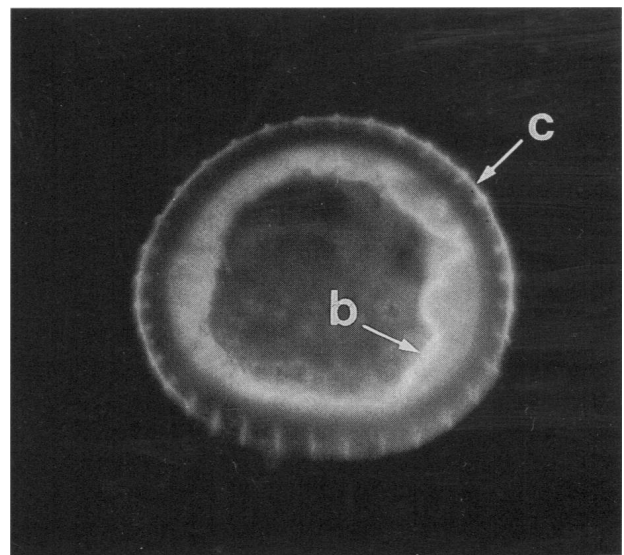


Fig. 6. Immunofluorescence staining of transverse sections of *Ostertagia ostertagi* adults using the anti-globin rabbit serum. Both the body wall musculature (b) and the cuticle (c) were recognized.

be aligned with sequences in the B and C α -helices of the known globins. Trp23/24 matched parts of the E-helix and the ef interhelical region.

Immunolocalization

Immunofluorescence staining of freeze-sections of adult *O. ostertagi* demonstrated that the body wall musculature and the cuticle were recognized by the anti-globin rabbit serum (Fig. 6). When negative control rabbit serum was used, the sections remained unstained (not shown).

DISCUSSION

The purified antigen OoAdGlb is a globin-like protein of adult *O. ostertagi*. This was supported by several features: (1) the recognition by the anti-globin rabbit serum, (2) its apparent molecular mass of 17 kDa under reducing conditions, (3) the similarity of the tryptic peptides with known nematode globins, (4) the absorption spectrum with optimum at 412 nm, and (5) its occurrence in the body wall musculature and/or the cuticle. Two isoforms of globin are known from the strongylids: (1) intracellular myoglobin-like isoforms found in body wall and pharyngeal musculature and (2) extracellular cuticle globins (Blaxter, 1993). The cuticle isoforms are homotetramers (native molecular mass 70 kDa) but the myoglobins have been described as monomers (17 kDa). The purified *O. ostertagi* globin-like protein presented in this paper appears to be a dimer under non-reducing conditions. The *Ascaris* myoglobin is found as a dimer (Blaxter *et al.* 1994b), and further work is required to determine whether the OoAdGlb dimer analysed here is a partially dissociated cuticular tetramer, or the native myoglobin dimer. Cryostat sections immunostained with cross-reactive anti-serum (which identifies both isoforms in *N. brasiliensis*) demonstrated globins in both cuticle and musculature.

IE-chromatography revealed a Mono Q-unbound and a Mono Q-bound 405 nm-peak, both containing the globin-monomer on Western blot. At this moment we do not know why these globin-like antigens show different chromatographic properties. Efforts to resolve this question resulted in conflicting data. The modifications in chromatographic properties caused by the addition of Drabkin's reagent suggested that the two 405 nm peaks at the IE-chromatography represent different haemoglobin derivatives of the same isoform (Drabkin & Austin, 1935). In contrast, both the Mono Q-unbound and the Mono Q-bound fractions have the same typical oxy-haemoglobin absorption spectrum (Di Iorio, 1981).

The OoAdGlb peptides can be aligned unambiguously with the globins of *Nippostrongylus* and *Trichostrongylus*, confirming its globin nature. Highest identity is found with *Nippostrongylus* cuticular isoform. The peptides are part of the structurally important C and E helices, displaying the highly conserved C2 Pro and E7 Gln. Gln in the distal E7 position is typical for nematodes whereas the Leu in *Nippostrongylus* body wall globin and *Trichostrongylus* globin are rather exceptional. The available sequence information allows the synthesis of oligonucleotide probes to isolate and sequence the globin coding cDNA. This work is in progress.

It was interesting to note that OoAdGlb is only expressed in L4 stage and adult *O. ostertagi*. Both

stages live in close contact with the host, respectively the fundus glands of the abomasum and on the surface of the mucosa. In general, the haemoglobins of parasitic nematodes that inhabit the alimentary system have an extraordinarily high oxygen affinity such that they remain oxygenated, even in their near-anoxic environment (Vinogradov, 1985). In contrast to *T. colubriformis* (Frenkel *et al.* 1992) the *O. ostertagi* 3rd larval stage does not contain a globin-like protein. This feature might have been lost during evolution, possibly because the 3rd larval stage lives mainly outside the host in a less oxygen deprived environment.

No significant differences in antibody response against OoAdGlb were found between infected and uninfected calves. Similarly, sheep and guinea-pigs immune to *T. colubriformis* do not contain antibodies against the nematode globin (Frenkel *et al.* 1992). Nevertheless, the *T. colubriformis* globin provided sheep and guinea-pig significant protection against challenge infection. Since the purified OoAdGlb probably represents the *Ostertagia* homologue of this *T. colubriformis* globin-like antigen, it will be interesting to evaluate possible protective capabilities.

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