Changing surface antigen and carbohydrate patterns during the development of *Oesophagostomum dentatum*

A. JOACHIM, B. RUTTKOWSKI *and* A. DAUGSCHIES*

Institut für Parasitologie, Tierärztliche Hochschule Hannover, Bünteweg 17, D-30559 Hannover, Germany

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SUMMARY

Living and fixed specimen of *Oesophagostomum dentatum* were labelled *in situ* with serum antibodies or a panel of biotin-labelled lectins. Specific binding of antibodies was observed in all parasitic stages – freshly exsheathed 3rd-stage larvae (L3), 3rd- and 4th-stage (L4) larvae cultured *in vitro* and L3 and L4 and adults isolated from pig intestines. The shedding of the stained layer by motile larvae was inhibited by levamisole-induced paralysis. Larvae cultured *in vitro* exposed serum-derived proteins on their surface which could be labelled with secondary antibody directed against the respective serum donor species. While freshly exsheathed larvae were recognized by *O. dentatum*-positive serum only, older larvae and adults cross-reacted with serum from pigs infected with *O. quadrispinulatum*, a closely related species. Lectin binding varied considerably between stages. While binding was not observed in pre-parasitic stages, Concanavalin A, Soybean Agglutinin, Wheat Germ Agglutinin, *Ricinus communis* Agglutinin and Peanut Agglutinin bound to developing larvae in varying degrees. *Dolichos biflorus* Agglutinin only bound to advanced (luminal) larval stages, while adults generally displayed only weak or partial lectin binding (except with Concanavalin A and Wheat Germ Agglutinin). *Ulex europaeus* Agglutinin only labelled larvae derived from cultures containing 10% pig serum. Cleavage of the carbohydrate residues by sodium periodate treatment resulted in reduction of antibody binding to cultured larvae, but not to freshly exsheathed L3. Concanavalin A, Soybean Agglutinin, and Peanut Agglutinin binding was also reduced by periodate treatment, while binding of Wheat Germ Agglutinin and *Ricinus communis* Agglutinin was inhibited only in early L3, but not in older stages. The different lectin labelling patterns are related to the different stages of the nematode – infective, invasive, histotropic, and luminal – and may serve as a mode of adaptation for the parasite against the host's immune attack by surface glycoprotein variation, together with antigen shedding (as demonstrated by labelling of motile larvae) and a possible acquisition of host molecules at the parasite's surface. Furthermore, a possible role of this developmental variation in surface carbohydrates in parasite–parasite interactions is discussed.

Key words: *Oesophagostomum dentatum*, surface antigens, surface carbohydrates, lectin binding.

INTRODUCTION

The nodular worm *Oesophagostomum dentatum*, a parasite which inhabits the large intestines of pigs, has recently become subject to detailed studies due to its ability to establish and persist in the host in high numbers without causing extensive damage (Pattison, Thomas & Smith, 1980; Hale *et al*. 1981). Several mechanisms of adaptation to the host have been proposed for this species, including population dynamic adaptation of larval development after infections with single high doses (Hass, Brown & Young, 1972; Christensen *et al*. 1995*a*, *b*), immunomodulation by parasite-derived bioactive lipids (Daugschies, 1995, 1996; Daugschies & Ruttkowski, 1998) and immune evasion by stage-specific antigen and glycoprotein expression (Joachim, Ruttkowski & Daugschies, 1998).

Since the parasite's surface is in closest contact with the host it is the natural target for the immune system and was found to be a major source for antigens and glycoproteins in nematodes (Blaxter *et al*. 1992). Developmental variation in the glycoprotein content of surface structures has been demonstrated for other gastrointestinal strongyles such as *Ostertagia* spp. (Keith *et al*. 1990) and *Haemonchus contortus* (Schallig, Baczkowska & Wedrychowicz, 1995; Raleigh, Brandon & Meeusen, 1996). We have demonstrated earlier that stage-specific antigens and glycoproteins are present in culture supernatants and protein homogenates of *O. dentatum* (Joachim *et al*. 1998). In the present paper we investigated the patterns of antibody and lectin binding to the surface of *O. dentatum* specimens of different life-cycle stages.

* Corresponding author. Tel: +49 511 953 8794. Fax: +49 511 953 8870. E-mail: ardaug@parasit.tiho-hannover.de

MATERIALS AND METHODS

Parasite material

The *O. dentatum* strain OD-Hann was used for all studies. Unless stated otherwise worms were fixed in 70% ethanol at 70 °C for 30 min. The following

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stages were investigated: infective (i.e. sheathed) 3rd-stage larvae (L3), freshly exsheathed L3, L3 after 3–24 days of *in vitro* cultivation, 4th-stage larvae (L4c) after 9–24 days of *in vitro* cultivation, L3 derived from pig intestines and mucosal scrapes 2 days after oral infection with 10000 L3, L4 (L4p) from pig intestines 9–11 days after oral infection with 10000 L3, and male and female adults from the intestines of patently infected pigs. Additionally larvae were cultivated for 24 days *in vitro*, then the larger L4 were separated from the smaller L3 by sedimentation (Joachim *et al*. 1998) and rectally transplanted into the colon of a helminth-free pig (Christensen, Grøndahl-Nielsen & Nansen, 1996). After 3 days the pig was killed by captive bolt and exsanguination and the L4 were isolated from the intestines. Worms were recovered from intestinal contents as described by Slotved *et al*. (1996). Exsheathment and *in vitro* cultivation were carried out according to Daugschies & Watzel (1999). To test the influence of culture medium on surface proteins larvae were cultivated for 3, 7, 14 and 21 days in the standard cultivation medium described by Daugschies & Watzel (1999) with varying serum supplements at a concentration of 10%. The first (homologous serum) group was grown in medium containing serum from an *Oesophagostomum*-seropositive pig, in the second group sheep serum from a blood donor sheep, foetal calf serum (FCS; Gibco) or the serum supplement Serum Supreme® (BioWhittaker) were used instead (heterologous serum group), and in the third (no-serum) group the serum supplement was omitted.

Immunostaining

Sera were derived from pigs infected with *O. dentatum* with daily doses of 10000 L3 for 3 consecutive days 8 weeks prior to bleeding or from helminth-naïve pigs which were seronegative in Western blotting as described (Joachim *et al*. 1999). Sheep sera were derived from blood donors kept at our facilities. If not stated otherwise the larvae were fixed and stored at 4 °C. Immediately before labelling they were washed 3 times with TTBS (100 mM Tris, 0·9% sodium chloride, 0·1% Tween 20, pH 7·5). All procedures for labelling and staining were performed at room temperature in 1 ml volumes in 1·5 ml Eppendorf tubes using an ABC kit (Camon, USA) including biotinylated secondary antibodies against pig, sheep or bovine immunoglobulins (IgG) (Camon, USA) and biotinylated horseradish-peroxidase as described previously (Joachim *et al*. 1998). In general, larvae were incubated in TTBS buffer including primary antibody (pAb) for 30 min, washed 3 times with TTBS, and incubated with 7·5 μg/ml secondary antibody (sAb) for 30 min. After 3 washing steps with TTBS the ABC complex was added for 30 min. Then the samples were washed again 3 times and transferred to 24-well macrotitre plates (Renner GmbH), each well containing 1 ml of 3,3′,5,5′-tetramethylbenzidine (TMB) solution (as recommended by the manufacturer) for colour development. As pAb sera from pigs or sheep as above, commercially available serum supplements or medium containing these sera (supplements) were used as described in detail for the respective experiments. TTBS served as buffer control when pAb were omitted from the reaction.

Experiment 1. In the first trial it was investigated whether the type of fixation alters the binding of antibody to the parasite surface, and whether the observed binding of serum antibodies is indeed specific for *O. dentatum*-infected pigs. Three sets each (i.e. unfixed (live), ethanol fixed or fixed in 4% formalin) of freshly exsheathed L3 and L4 cultured *in vitro* (for 14 days in medium with 10% pig serum) were incubated with serum from chronically infected pigs (dilution 1:1000), uninfected pigs (dilution 1:10 or 1:1000) or a buffer control without serum.

Experiment 2. The influence of larval motility on the labelling procedure was tested by paralysing live larvae with levamisole treatment prior to labelling. Levamisole (Sigma, Germany) was added to freshly exsheathed larvae in concentrations of 0·1–10 μg/ml followed by incubation for 1 h. The larval batch incubated in the lowest levamisole concentration was chosen for labelling because most but not all larvae were paralysed, allowing direct comparison of motile and immobilized larvae. An aliquot of the pretreated larvae was washed 3 times and kept in distilled water to test the reversibility of the levamisole effect. The paralysed larvae were labelled and stained in solutions containing 0·1 μg/ml levamisole and compared to untreated live and fixed controls.

Experiment 3. For comparison of the staining intensities and specificities of different stages sheathed L3, freshly exsheathed L3, L4 cultured for 21 days in medium containing 10% pig serum, and L4 and adults recovered from intestinal contents were stained with positive or negative pig serum (dilution 1:1000).

Experiment 4. A possible influence of serum supplements on staining patterns was determined by labelling larvae (3, 7, 14 or 21 days of cultivation) from all medium groups with anti-pig IgG, anti-sheep IgG or anti-bovine IgG without prior incubation in serum (Table 1). Additionally, freshly exsheathed L3 were incubated with sera from chronically infected pigs (dilution 1:1000), from uninfected pigs (dilutions 1:1000 and 1:10) and

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Table 1. Secondary antibody (sAb) binding to larvae of *Oesophagostomum dentatum* cultured *in vitro* for 3–21 days using different serum supplements without incubation with primary antibody (Exp. 4)

(FCS, foetal calf serum; Ig, immunoglobulin.)

		sAb		
Serum supplement	Larval stage	Anti-pig IgG	Anti-sheep IgG	Anti-bovine IgG
None	L3 day 3/7/14*	−	−	−
10% pig serum	L3 day 3/7	+++	++	+++
	L3 day 14/21	+++	+	+++
	L4 day 14/21	+++	−	+
10% sheep serum	L3 day 3/7/14/21	−	+++	++
	L4 day 14/21	−	+++	++
10% FCS	L3 day 3/7/14/21	−	−	−
	L4 day 14/21	−	−	−
Serum Supreme®	L3 day 3/7	−	+++	+++
	L3 day 14/21	−	+	+
	L4 day 14/21	−	++	++

* In cultures without serum supplement no L4 were observed; L3 were only viable until day 14.

Table 2. Binding of serum- and medium-derived primary antibodies (pAb) to freshly exsheathed larvae of *Oesophagostomum dentatum* (Exps 1 and 4)

(FCS, foetal calf serum; Ab, antibody; Ig, immunoglobulin.)

	Secondary Ab		
	Anti-pig IgG	Anti-sheep IgG	Anti-bovine IgG
pAb			
None	−	−	−
Pig serum infected (1:1000)	+++	+++	+++
Pig serum uninfected (1:1000)	−	−	−
Pig serum uninfected (1:10)	+	−	−
Sheep serum (1:1000)	−	−	−
Sheep serum (1:10)	−	+++	+++
FCS (1:10)	−	−	−
Serum Supreme® (1:10)	−	+++	+++
Medium with 10%			
Infected pig serum	+++	++	++
Sheep serum	−	+++	+++
No serum	−	−	−
FCS	−	−	−
Serum Supreme®	−	++	+++

from sheep (dilution 1:10), with FCS (dilution 1:10) and with Serum Supreme® (dilution 1:10) as pAb and the 3 different sAb (Table 2), as well as with fresh media supplemented as described above as pAb and the 3 sAb (Table 2).

Experiment 5. Species specificity of surface antigens was determined for freshly exsheathed L3, larvae from FCS-cultures (24 days old) and adults which were incubated with serum (dilution 1:1000) from a pig patently infected with *O. quadrispinulatum*, a closely related species.

Experiment 6. The role of carbohydrate moieties as epitopes for host antibodies was investigated for freshly exsheathed L3 and for L3 and L4 cultivated in FCS-medium for 24 days by cleavage of sugar residues with sodium periodate in varying concentrations (0, 20, 40, 60, 80 and 100 mM in 100 mM sodium acetate/5 mM EDTA, pH 4·5) for 1 h at room temperature in the dark prior to immunostaining. Controls were incubated in sodium acetate/EDTA buffer without periodate.

Lectin-binding studies

Labelling and staining were carried out as described above, except that after equilibration in TTBS and blocking with a Blocking Kit (Camon, USA) according to the manufacturer's instructions 20 μg/ml of each lectin was added and after incubation for

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Table 3. Lectins used and the respective inhibiting sugars (Exp. 9)

Lectin	Inhibiting sugar
Concanavalin A (Con A)	200 mM methyl-D-mannoside + 200 mM methyl-D-glucoside
Soybean Agglutinin (SBA); *Dolichos biflorus* Agglutinin (DBA)	200 mM *N*-acetyl-D-galactosamine
Wheat Germ Agglutinin (WGA)	500 mM *N*-acetyl-D-glucosamine
Ulex europaeus Agglutinin I (UEA I)	100 mM L-fucose
Ricinus communis Agglutinin I (RCA_{120}); Peanut Agglutinin (PNA)	200 mM D-galactose

Table 4. Surface labelling of different *Oesophagostomum dentatum* stages with various lectins (Exps 7 and 8)

(FCS, foetal calf serum; Con A, Concanavalin A; SBA, Soybean Agglutinin; WGA, Wheat Germ Agglutinin; DBA, *Dolichos biflorus* Agglutinin; UEA I, *Ulex europaeus* Agglutinin I; RCA_{120}, *Ricinus communis* Agglutinin I; PNA, Peanut Agglutinin. −, No staining; +, weak staining; ++, overall dark blue staining; +++, dark blue staining and precipitate formation ('fluffy' surface).)

Stage	Con A	SBA	WGA	DBA	UEA I	RCA_{120}	PNA
L3 sheathed	−	−	−	−	−	−	−
L3 freshly exsheathed	−	−	−	−	−	−	−
Small L3 day 3, pig serum	+++	+++	+++	+++	+++	+++	+++
Big L3 day 21, pig serum	+++	+++	+++	+++	+++	+++	++
Small L4 day 21, pig serum	+++	+++	+++	+++	+++	+++	++
Big L4 day 21, pig serum	+	+++	+++	+	+	+	++
Small L3 day 3, FCS	+++	+++	+++	−	−	+++	−
Small L3 day 7, FCS	+++	++(+)	++(+)	−	−	+++	(+)
Small L3 day 14, FCS	+++	++(+)	++(+)	−	−	+++	++(+)
Big L3 day 14, FCS	+++	+++	+++	−	−	+++	+++
Small L4 day 14, FCS	+++	+++	+++	−	−	+++	+++
Big L4 day 14, FCS	+++	+++	+++	+	−	++(+)	+++
Small L3 day 24, FCS	++(+)	+++	+++	−	−	+++	++
Big L3 day 24, FCS	++(+)	+++	+++	−	−	+++	+++
Small L4 day 24, FCS	++(+)	+++	+++	++	−	+++	++
Big L4 day 24, FCS	++(+)	+++	+++	++	−	+++	+++
L4 rectally transplanted	+++	++(+)	+(+)	−	−	++	++
L3 *ex vivo* (2 days p.i.)	+	++(+)	+(+)	−	−	+(+)	++
L4 *ex vivo* (9–11 days p.i.)	+(+)	++	+(+)/−	+/−	−	++	+/−
Adult females	+(+) ++*	+++*	++	−	++*	+*	++*
Adult males	+(+) ++*	+++*	++	−	++*	+*	++*

* Only the buccal and genital regions.

30 min and 3 washing steps the protocol proceeded with the incubation in ABC complex solution. Seven lectins – Concanavalin A (Con A), Soybean Agglutinin (SBA), Wheat Germ Agglutinin (WGA), *Dolichos biflorus* Agglutinin (DBA), *Ulex europaeus* Agglutinin I (UEA I), *Ricinus communis* Agglutinin 120 (RCA_{120}), and Peanut Agglutinin (PNA) – (Lectin Kit I, Camon, USA) were used (Table 3).

Experiment 7 (*Table 4*). Sheathed and freshly exsheathed L3, larvae cultured for 3 or 21 days in medium with pig serum, larvae cultured for 3–24 days in FCS medium, L3 cultured for 3–7 days in medium with 10% sheep serum, L4 from rectal transplantation and L3, L4 and adults *ex vivo* (all fixed) were incubated with the 7 lectins, as well as living L3 and L4 from cultures containing FCS (after 3 and 14 days of cultivation).

Experiment 8. Since significant differences were found between the binding of DBA, UEA I, and PNA to L3 from cultures with pig serum compared to those from FCS cultures (Exp. 7; Table 4) the influence of serum supplements in the medium on lectin binding was investigated. L3 from FCS, pig serum and sheep serum supplemented cultures and cultures without serum (3 and 7 days) were labelled with DBA, UEA I (the lectins that had previously stained cultured L3 from pig-serum cultures but not

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Table 5. Lectin labelling of *Oesophagostomum dentatum* larvae from different sources pre-incubated with positive pig serum (dilutions 1:10 and 1:1000) or derived from cultures with different serum supplements (Exp. 8)

(FCS, foetal calf serum; Con A, Concanavalin A; SBA, Soybean Agglutinin; WGA, Wheat Germ Agglutinin; DBA, *Dolichos biflorus* Agglutinin; UEA I, *Ulex europaeus* Agglutinin I; RCA_{120}, *Ricinus communis* Agglutinin I; PNA, Peanut Agglutinin. −, No staining; +, weak staining; ++, overall dark blue staining; +++, dark blue staining and precipitate formation ('fluffy' surface).)

	Con A	SBA	WGA	DBA	UEA I	RCA_{120}	PNA
L3 freshly exsheathed; serum pre-incubated	−	−	−	−	−	−	−
L3 day 3, FCS; serum pre-incubated	+++	+++	+++	−	−	+++	(+)
L3 day 7, FCS; serum-pre-incubated	+++	++(+)	++(+)	−	−	+++	++(+)
L3 day 3/7, pig serum	+++	+++	+++	+++	+++	+++	+++
L3 day 3/7, sheep serum	+++	+++	+++	(+)	−	+++	−
L3 day 3/7, FCS	+++	+++	++(+)	−	−	+++	−/(+)
L3 day 3, no serum	−	−	−	−	−	−	−
L3 day 7, no serum	−	−/(+)	−	−	−	−	−

from FCS cultures), ConA, SBA, WGA, RCA_{120} and PNA (Table 5). The influence of serum pre-incubation of fixed worms on subsequent lectin binding was investigated by incubation of freshly exsheathed fixed L3 and fixed L3 from 3- and 7-day FCS cultures with positive pig serum (1:1000 and 1:10) for 30 min followed by labelling with the lectins mentioned above (Table 5).

Experiment 9. To test the specificity of lectin binding lectins were incubated with the respective sugars (Table 3) for 30 min prior to incubation with larvae cultivated in FCS-supplemented medium for 3 and 14 days as described above.

Experiment 10. Binding of all lectins except UEA I to L3 and L4 from FCS cultures (3 and 24 days) after treatment with 50 mM sodium periodate (the lowest effective concentration in immunostaining as described in Exp. 6) prior to blocking with the Blocking Kit (see above) was investigated.

Using a Leica DMLB microscope (Leica, Germany) with 25-, 40- or 100-fold magnification worms were evaluated for motility and surface staining (blue colour) as indicated above. Representative samples were photographed using a TK-1280E colour video camera (JVC; Leica, Germany) attached to the microscope and digitalized using the Quantimet 500+ system (Leica, Germany).

RESULTS

Cultivation of larvae in different media

As observed previously by Daugschies & Watzel (1999) larvae grew best in medium containing pig serum, where L4 were first observed on day 7. Medium with heterologous serum contained the first L4 on day 10. Only slow growth of L3 and no L4 were observed in cultures without serum (data not shown), and the larvae in this group could only be maintained in culture for 14 days. For practical reasons larvae were described as 'small L3' (size of freshly exsheathed larvae or slightly bigger; corresponding to invasive stages), 'big L3' (shortly before ecdysis, size of small L4, corresponding to early histotropic stages), 'small L4' (shortly after ecdysis; corresponding to advanced histotropic stages), or 'big L4' (significantly bigger than early L4; corresponding to the pre-adult luminal stages). In cultures older than 14 days all stages can be found in parallel, with the number of large L4 increasing to 5–10% of the total number of larvae by the end of the third week of cultivation. Pre-cultured L4 recovered from intestinal contents 3 days after rectal transplantation were still at the L4 stage, but bigger. Nodule formation was not detected (data not shown). The larvae recovered from pig intestines 2 days p.i. correspond to 'small L3'; all of them were exsheathed and slightly bigger than freshly exsheathed larvae; those found in mucosal scrapings were occasionally surrounded by lymphoid cells, but nodule formation was not observed (data not shown). Larvae from intestines harvested 9–11 days p.i. had all reached the 'large L4' stage, and were significantly bigger than those recovered from cultures at all cultivation periods (data not shown).

Immunostaining

Staining intensities were generally defined as negative (−), weak or partial (+), strong (++), or 'fluffy' (+++) as shown as examples in Fig. 1A.

Experiment 1. At serum concentrations of 1:1000 freshly exsheathed L3 were stained only with serum

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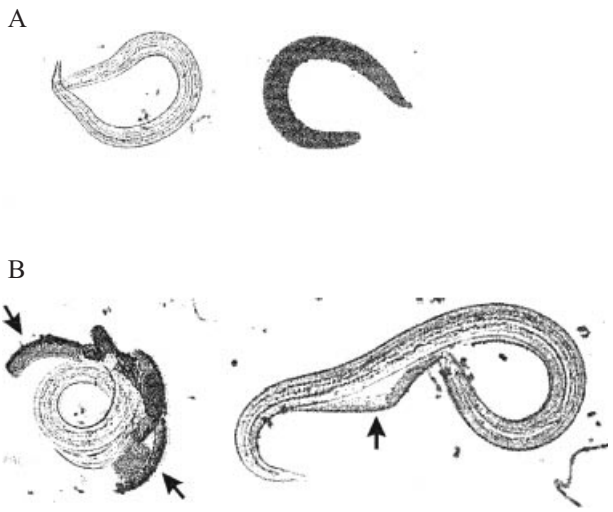


Fig. 1. Examples of 3rd-stage larvae (L3) and 4th-stage larvae (L4) after staining. (A) L3 after 3 days of cultivation. Left, unstained (−); right, strongly stained/'fluffy' (+++). (B) Motile L3 (left) and L4 (right) with partly removed surface layer (arrows).

from infected pigs, whereas with negative serum or buffer control no staining was observed with any of the sAb (Table 2). However, when the serum concentrations were 1:10 staining also occurred with negative and heterologous serum. L4 cultured *in vitro* in medium containing pig serum stained with any pAb and also when the pAb was omitted. While anti-pig IgG did not cross-react with sheep serum or Serum Supreme®, anti-bovine IgG strongly cross-reacted with sheep or positive pig serum, and similarly anti-sheep IgG cross-reacted with positive pig serum and Serum Supreme®. The effect was described by the manufacturer of these 2 biotinylated sAb. Positive larvae were covered with a thick blue precipitate of 'fluffy' appearance, probably due to cross-linking of the biotin–avidin complexes. The type of fixation had no influence on the staining patterns of the larvae; however, in motile larvae it was observed that after staining the outer, blue layer was removed by larval movements and could be found in the vicinity of the larvae as flakes (Fig. 1 B).

Experiment 2. When larvae were immobilized by fixation or levamisole treatment the blue layer stuck to the surface of the larvae. The levamisole-induced paralysis was reversed when larvae were kept in water for 2–3 h. It was noticed that when the fluffy surface was broken up by larval movements, the area underneath the blue layer was completely unstained (Fig. 1 B).

Experiment 3. While binding of serum antibodies was not seen in sheathed L3 the other stages strongly stained at the surface when sera from chronically infected pigs were used. In freshly exsheathed L3, L4 *ex vivo* or adults non-specific binding with negative serum was not observed at that dilution, while larvae grown *in vitro* displayed non-specific binding under the conditions described for Exp. 4.

Experiment 4. After cultivation in medium containing pig or sheep serum or Serum Supreme® (which includes bovine serum) the larvae (L3 and L4) strongly stained when the respective sAb (without incubation in pAb) was used (Table 1). Incubation of freshly exsheathed larvae in pig or sheep serum, Serum Supreme®, or medium containing such sera using the respective sAb indicated that this effect was due to serum antibodies (Table 2). Similar to freshly exsheathed L3 larvae derived from no-serum medium remained negative (Table 1). Larvae derived from medium with FCS or freshly exsheathed larvae incubated with FCS as pAb did not stain when anti-bovine IgG was used as sAb, therefore it was concluded that no antibodies derived from FCS bound to the surface of the larvae, whereas incubation of larvae from FCS-supplemented medium with positive pig serum (1:1000) as pAb resulted in strong staining when anti-pig IgG was used as sAb. Cross-reactions between larvae from the various supplemented media and heterologous sAb were observed as described for Exp. 1 (Table 2).

Experiment 5. While L3 of any cultivation period only stained with serum from the *O. dentatum*-infected pig but not with *O. quadrispinulatum*-positive serum, L4 and adults stained strongly with both sera.

Experiment 6. Antibody binding to freshly exsheathed L3 remained unaffected by sodium periodate treatment while cultured L3 stained only weakly at concentrations of 60 mM sodium periodate or more, and L4 remained unstained at concentrations $\geqslant$40 mM. Antibody binding to controls was unaltered.

Lectin staining (*Tables 4 and 5*)

Experiment 7. None of the 7 lectins tested bound to sheathed or freshly exsheathed L3, whereas strong binding of all lectins was generally observed for the L3 and the small L4 cultured in medium with pig serum for 3–21 days, declining in the pig L4 on day 21 for Con A, DBA, UEA I and RCA_{120}. Since medium-derived molecules (antibodies or non-immunoglobulin glycoproteins) bound to the surface of cultured larvae could falsify the lectin-binding patterns (as observed for the antibody-binding patterns), larvae derived from FCS-supplemented

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Table 6. Surface labelling of different *Oesophagostomum dentatum* stages with various lectins after blocking of lectins with the respective carbohydrates (see Table 3) and after treatment with periodate (Exps 9 and 10)

(FCS, foetal calf serum; Con A, Concanavalin A; SBA, Soybean Agglutinin; WGA, Wheat Germ Agglutinin; DBA, *Dolichos biflorus* Agglutinin; UEA I, *Ulex europaeus* Agglutinin I; RCA_{120}, *Ricinus communis* Agglutinin I; PNA, Peanut Agglutinin. −, No staining; +, weak staining; ++, overall dark blue staining; +++, dark blue staining and precipitate formation ('fluffy' surface).)

Stage	Con A	SBA	WGA	DBA	RCA_{120}	PNA
Sugar-blocked						
L3 day 3/14, FCS	−	−	−	−	−	−
L4 day 14, FCS	(+)	−	(+)	−	(+)	−
Periodate-treated						
L3 day 3 FCS	−	−	(+)	−	−	−
L3 day 24, FCS	+	−	+++	−	++	−
L4 day 24, FCS	+	+	+++	++	++	−
Untreated controls	++(+)	+++	+++	++*/−†	+++	+++†/−‡

* Only L4 day 24.
† All other stages.
‡ L3 day 3.

medium (which were negative for bovine antibodies as described above) were used to screen the lectin patterns of *in vitro* larvae. Strong binding was observed for Con A, SBA, WGA and RCA_{120} throughout the cultivation period, whereas PNA binding was weak at first and increased with the size and stage of the larvae. DBA only bound to big L4 from day 14 and to small and big L4 from day 24. L3 derived from pig intestines 2 days p.i. could be labelled with Con A, SBA, WGA and RCA_{120}, although staining was generally less intense when compared with cultured L3. Additionally, they were also labelled strongly with PNA. L4 *ex vivo* generally stained weaker than cultivated L4, and staining with WGA, DBA and PNA was not observed regularly. The cuticle of the adult stages stained weakly or not at all except with Con A and WGA, whereas staining of varying intensities was observed for all lectins except DBA in the buccal and genital region of both sexes. UEA I only stained larvae from the homologous serum group and the anterior and posterior ends of the adults. It was hypothesized that the binding of UEA I and DBA to L3 derived from medium with pig serum, but not to those from medium with FCS or sheep serum, was due to lectin–serum component interactions (Table 5). There were no differences in staining patterns between fixed and living larvae cultured for 3 or 14 days in FCS (not presented in Table). The shedding of the stained layer (as described in immunostaining) also occurred after lectin binding to motile larvae.

Experiment 8. While larvae derived from cultures containing pig serum strongly stained with all lectins at 3 and 7 days of cultivation larvae from FCS cultures or from cultures with sheep serum only stained with Con A, SBA, WGA and RCA_{120} as described in Tables 4 and 5. Larvae from serum-free cultures displayed no lectin binding except weak staining with SBA only after 7 days of cultivation (Table 5). When freshly exsheathed larvae were pre-incubated with positive pig serum none of the 7 lectins bound, whereas pre-incubated L3 from 3- or 7-day FCS cultures showed staining patterns similar to those without serum pre-incubation, except that binding of PNA to 7-day larvae was more pronounced after pre-incubation (Table 5).

Experiment 9. Lectin-binding to larvae cultured for 3 or 14 days was largely reduced by pre-incubation with the respective sugars (Table 3). Only Con A, WGA and RCA_{120} displayed residual binding to L4 after 14 days of cultivation (Table 6).

Experiment 10. Oxidation of sugar moieties by periodate treatment had no influence on the DBA and WGA binding and little influence on the binding of RCA_{120} to 24-day-old larvae while binding of WGA and RCA_{120} to L3 from 3-day cultures was blocked effectively, as well as the binding of Con A, PNA and SBA to both small L3 and larger L3 and L4 (Table 6). Pre-incubation of larvae in acetate buffer had no effect.

DISCUSSION

Based on previous work on antigen and glycoprotein patterns of *O. dentatum* (Joachim *et al*. 1998) in the present paper we investigated the binding patterns of serum antibodies and of a panel of 7 lectins to different life-cycle stages of the nodular worm. All stages could be labelled with specific serum antibodies from infected, but not from seronegative pigs, and also with sheep serum and commercially avail-

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able bovine serum (Serum Supreme®). It cannot be excluded that these sera contain *O. dentatum*-reactive antibodies, probably due to helminth infections of the donor animals. Cross-reactions between porcine pAb and anti-ruminant IgG and among ruminant pAb and sAb, as we observed, were to be expected since described by the manufacturer, whereas anti-pig IgG did not cross-react with any heterologous serum. It was noticeable that living, motile larvae seemed to be able to shed the stained layer, whereas immobilized larvae (living or fixed) remained stained. This effect of active shedding of surface components *in vitro* has been demonstrated earlier for *Ancylostoma caninum* (Vetter & Klaver-Vesseling, 1978) and *Haemonchus contortus* (Ashman *et al.* 1995) and is very distinct with the staining technique we employed. Since enzyme-labelling cannot be performed at low temperatures to immobilize the parasites, levamisole was used to reversibly paralyse the larvae which resulted in inhibition of this shedding. It is not clear whether this phenomenon is limited to labelling procedures or also occurs *in vivo*. However, there is evidence from other parasites that this may be a mechanism to distract the host's immune defence from the living larva to cellular debris in the parasite's surroundings (as reviewed by Blaxter *et al.* 1992). Incubation of larvae in protein-free medium and subsequent analysis of the excretory–secretory products revealed no antigens or glycoproteins in culture supernatants of L3, while the more advanced stages released a range of proteins into their environment, although only in low amounts compared to other nematodes (Joachim *et al.* 1998). Comparative studies of larvae derived from different cultivation media showed that developing larvae are capable of binding serum-derived antibodies of pigs and other animal species (which do not host *O. dentatum*; Stewart & Gasbarre, 1989) at their surface which seems to influence the binding of antibodies and some lectins (see below). It is possible that this effect plays a role in immune evasion of histotropic stages *in vivo*. However, it is noticeable that mere pre-incubation of fixed larvae with positive serum resulted in antibody, but not in lectin binding; therefore the exposure of serum-derived molecules on the parasite surface (which can be detected by sAb and lectin labelling) is probably not due to antibody–antigen binding but may be based on active association of serum-derived host components with the outer layer of the parasite and subsequent presentation at the surface (McLaren, 1984; Blaxter *et al.* 1992). Host-derived components have also been found on the surface of other helminths (as reviewed by McLaren, 1984, and Philipp & Rumjaneck, 1984) and may serve as molecular mimicry. While on the surface of L3 only *O. dentatum*-specific antigens could be detected, L4 and adults displayed cross-reactivity with *O. quadrispinulatum*-positive serum. In contrast, heterologous sera from other hosts also labelled L3. In *Trichinella spiralis* surface-exposed nematode proteins were found to be highly species specific (Jarvis & Pritchard, 1992), whereas in various other helminths surface antigens bear epitopes cross-reactive between species or even genera (Philipp & Rumjaneck, 1984). Cross-reactivity of host serum between the two species investigated could be meaningful in the parasite's control of heterologous infections. A negative influence of an existing *O. quadrispinulatum* infection on the establishment of *O. dentatum* has been demonstrated *in vivo* (Christensen, Nansen & Barnes, 1997). However, it would be expected that this effect was due to antibodies directed against the invasive rather than the histotropic or luminal stages. The role of carbohydrate moieties in antibody binding was demonstrated by periodate treatment: While binding to the invasive stage remained unaffected, histotropic and post-histotropic stages showed reduced binding of serum antibodies, indicating that, like in *H. contortus* (Schallig & van Leeuwen, 1996), stage-specific antibodies are present in the serum of chronically infected pigs and that the epitopes for those antibodies specific for the advanced parasitic stages contain carbohydrates.

The lectin-labelling studies revealed a developmental variation in the binding of Con A, SBA, WGA, DBA, RCA_{120} and PNA. The common feature of the binding profiles was that, while no binding was observed in the pre-parasitic or early invasive stages, the L3 from 3-day cultures, corresponding to the early histotropic stages (where larvae invade the mucosa but nodule formation has not started) displayed strong binding of Con A, SBA, WGA and RCA_{120}, indicating a highly active expression of the respective sugar-bearing molecules (glycoproteins or glycolipids; Zuckerman, Kahane & Himmelhoch, 1979) which lasts throughout the development of larvae to big L4. Larvae in medium without serum developed poorly, did not moult and bound no lectins at their surface even after 7 days of cultivation. Obviously serum components are essential for the exposition of carbohydrates at the larval surface; however, it could not be determined whether nutritional deficiencies prevent surface maturation or whether the parasites simply incorporate serum components into their surface structure. Since larvae derived from host intestines also displayed lectin binding it can be assumed that, regardless of the underlying mechanism, surface carbohydrate changes also occur *in vivo*. In general, lectin binding to L4 derived from intestines was weaker (similar to the adults) than that to the cultured larvae; however, the parasites derived *ex vivo* are bigger and may be developmentally more advanced. To test this hypothesis we transplanted pre-cultured larvae into the intestines where they did not undergo histotropic development, indicating that these stages had reached the phase of luminal

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dwelling; and their staining had declined for WGA, SBA and RCA_{120}, leaving them in an interim position between *ex vivo* and *in vitro* L4. Similarly, L3 retrieved from intestines after exsheathment but before nodule formation had started the expression of lectin-targets on the surface (or, analogous to the larvae derived from medium containing serum antibodies, the exposure of host-derived proteins at their surface). DBA binding, in contrast, was only found in the advanced L4 corresponding to the late histotropic and the luminal stages. PNA binding was low in the early stages and increased during development until the advanced (luminal) stages where it declined again. Compared to the histotropic stage lectin binding to adults was very restricted; mostly binding was limited to the anterior and posterior ends and may be related to the surfaces of the 'outlets' of the excretory and copulatory organs rather than the worm's cuticular surface. WGA binding, in contrast, is more prominent in early larvae and adults than in pre-adult luminal stages, indicating stage-specific 'no-expression' of certain glycosylation patterns at this stage. Schallig *et al*. (1995) demonstrated that in *H. contortus* the content of glycoproteins was higher in infective larvae than in adults, whereas we could not detect sugar moieties on the surface of infective *O. dentatum*. It is possible that the lectins we employed did not bind to exposed carbohydrates of this stage; this would also indicate a complete change in glycan or glycolipid expression on the larval surface during the development from the infective to the parasitic stage, as it has been described for the surface proteins of *Haemonchus* (Schallig *et al*. 1995; Raleigh *et al*. 1996) and *Ostertagia* (Keith *et al*. 1990), or a masking mechanism for certain carbohydrate binding sites (Preston, Jenkins & McLaren, 1986). In contrast to antibody binding, lectin binding could not completely be prevented by periodate cleavage of the sugar ring in older stages whereas in early parasitic L3 blocking was largely abolished by oxidation, indicating a developmental change of surface carbohydrate patterns despite similar binding of WGA and RCA_{120}. In older larvae DBA, WGA and RCA_{120} binding remained strong although it could be largely blocked by the respective sugars, indicating that these carbohydrate moieties are insensitive to periodate treatment (as described previously by Jarvis & Pritchard, 1992). It can therefore be speculated that the antigens susceptible to periodate contain mainly branched and α-linked D-mannoses, α-linked D-glucose, α-linked *N*-acetyl-D-glucosamine (Con A target glycans), terminal α- or β-linked *N*-acetyl-D-galactosamine (SBA targets), and D-galactose-β-1,3-*N*-acetyl-D-galactosamine (PNA terminal target) as carbohydrate residues, whereas β-linked *N*-acetyl-D-glucosamine (WGA-target) and D-galactose (RCA_{120} target) display differential susceptibility to cleavage.

Developmental variation of surface molecules in nematodes has been described for a variety of nematodes (for references, see McLaren, 1984; Maizels & Selkirk, 1988; Proudfoot *et al*. 1993), and it is now commonly acknowledged that changes in surface molecule expression during parasitic development are part of the parasite's immune evasion strategies. We have demonstrated that antibody binding to the surface of *O. dentatum* is indeed related to stage-specific epitopes, indicating that surface variation can render the immune response ineffective. However, such developmental changes of surface-exposed molecules also mirror the adaptation to habitat changes since they also occur in free-living nematodes such as *Caenorhabditis elegans* (Politz & Philipp, 1992). In parasites they also reflect changes in the environment, e.g. from free-living to invasive, from luminal to histotropic, etc.

Apart from host–parasite interactions stage-specific surface molecules may function as recognition sites in interactions between individual parasites, for example in mating, enabling adults to find a suitable partner. Part of the variation in surface molecules is stage-specific lectin binding as previously observed for various gastrointestinal nematodes (Preston *et al*. 1986; Hill, Fetterer & Urban, 1991; Schallig *et al*. 1995). In mammalian cells carbohydrates play a major role for cell–cell interactions in inflammation, coagulation and other pathophysiological mechanisms that can be evoked in the course of nematode infections (Varki, 1993).

Functional studies on *Schistosoma mansoni* have shown that surface molecules containing carbohydrates attract immune cells and disrupt their function (Caulfield, Korman & Samuelson, 1982). Besides immunomodulation adhesion to host tissue prior to invasion or feeding can be mediated by glycans (Maruyama & Nawa, 1997). However, functional studies concerning stage-specific macromolecules in nematodes are mostly limited to protective antigens.

For the nematode model we employed in this study various techniques (e.g. *in vitro* cultivation of the parasitic stages, rectal transplantation and recovery of parasites from the large intestines of pigs) which have enabled researchers to study in detail the population dynamics and biochemistry (Daugschies, 1995, 1996; Christensen *et al*. 1996; Daugschies & Ruttkowski, 1998). These techniques also helped to produce pre-adult stages without previous contact with the host (by *in vitro* cultivation) and similar stages that dwell in the intestines without the nodular stage (by rectal transplantation of L4). Artificial effects of cultivation techniques, e.g. the binding of serum antibodies from the medium or the weak staining of small L3 in older cultures (which we interpreted as a sign of poor development), are potential pitfalls of such investigations. However, the advantages of these techniques far outweigh the

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disadvantages. Future studies will include the isolation and purification of glycosylated surface proteins by lectin-based affinity chromatography and subsequent electrophoresis in order to reveal details on the biochemistry of these proteins, as well as bioassays which will shed some light on the function of the stage-specific molecules described here.

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