Lectin binding to cuticle exudates of sedentary *Heterodera schachtii* (Nematoda : Heteroderidae) second stage juveniles

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SUMMARY

Over 1 100 sedentary (parasitic) second stage juveniles of *Heterodera schachtii*, reared under monoxenic conditions in agar culture, were dissected from a cruciferous host plant in order to characterize their cuticle exudate by lectin binding. The lectins Con A, WGA, PNA, HPA and UEA-I bound to the exudate of nearly all juveniles examined, but their binding was not carbohydrate-specific. In contrast lectin binding to the amphid exudate was largely inhibited by the specific competitive sugars. LFA did not bind to cuticle or amphid exudates. It is suggested that the lectins bound to fatty acids of the cuticle exudates.

RÉSUMÉ

Liaisons des lectines aux exsudats cuticulaires de juvéniles sédentaires, de deuxième stade, de Heterodera schachtii *(Nematoda : Heteroderidae)*

Plus de 1 100 juvéniles sédentaires de deuxième stade de *Heterodera schachtii* ont été disséqués hors des racines de crucifères hôtes poussant sur agar en condition monoxénique dans le but de caractériser leur exsudat cuticulaire grâce aux liaisons avec diverses lectines. Les lectines Con A, WGA, PNA, HPA et UEA-1 se fixent sur les exsudats de presque tous les juvéniles examinés, mais cette liaison n'est pas spécifique des hydrates de carbone. En revanche, les liaisons des lectines aux exsudats amphidiens sont fortement inhibées par les sucres compétiteurs spécifiques. La lectine LFA ne se lie à aucun exsudat, cuticulaire ou amphidien. Il est suggéré que les lectines sont liées aux acides gras des exsudats cuticulaires.

Sedentary second stage juveniles (J2) of *Heterodera schachtii* produce cuticle exudates soon after they have started feeding from the initial syncytial cell (Zunke, 1985). The exudates form a layer that covers the entire body and were described as " subcrystalline layer " (" subkrystallinische Kruste ") by Schmidt (1871). New exudate layers are formed in all following developmental stages (Zunke, 1985). The cuticle exudates of several *Heterodera* species consist of different fatty acids, e.g. di-, tetra-, and hexacosanoic acid (Brown *et al.*, 1971).

Aumann and Wyss (1988) used lectin binding to examine surface carbohydrates of freshly hatched pre-parasitic *H. schachtii* J2 and observed specific binding of Con A and HPA to the cuticle. We have extended this work by examining possible carbohydrate components of the cuticle exudates of the developing sedentary stages taken from plant roots. The binding of six fluorochrome- and two colloidal gold-conjugated lectins from different specificity groups (Goldstein & Poretz, 1986) to the surface of J2 of *H. schachtii* was examined. The question of cuticle exudate origin and its possible function is discussed in the light of these results.

Materials and methods

MATERIALS

The fluorescein isothiocyanate (FITC) conjugated lectins from *Arachis hypogaea* (PNA) and *Helix pomatia* (HPA) were obtained from Sigma (Deisenhofen, FRG) and LFA-FITC (*Limax flavus* agglutinin) from E. Y. Labs (San Mateo, CA, USA). The tetramethylrhodamine isothiocyanate (TRITC) conjugated lectins from *Canavalia ensiformis* (Con A), from *Triticum vulgare* (WGA), PNA, HPA and from *Ulex europaeus* (UEA-I) were purchased from Sigma. The colloidal gold-conjugated lectins Con A (10 nm gold particles) and HPA (20 nm particles) were also from Sigma. The complementary sugars α-methylmannoside (α-ManMe), N-acetylgalactosamine (GalNAc), and α-L-fucose (α-L-

Fuc) were from Sigma, and D-galactose (D-Gal) and N-acetylneuraminic acid (Neu5Ac) were from Merck (Darmstadt, FRG). Oligomers of N-acetylglucosamine (GlcNAc) were a gift from Dr. J. M. S. Forrest, Scottish Crop Research Institute.

Sedentary second stage juveniles

Cysts of *Heterodera schachtii* from monoxenic cultures were crushed on 3 mmol·l^{-1} $ZnCl_2$ agar plates (Aumann & Wyss, 1988). Freshly hatched J2 from these plates were then transferred to axenic cultures of *Brassica rapa* var. *silvestris* f. *campestris* grown in the dark in a nutrient agar medium (Dropkin & Boone, 1966). After 47 to 53 h sedentary J2 were carefully dissected from the roots with a fine needle. During this process it was unavoidable that some of the cuticle exudate broke off. For LFA binding experiments the dissected juveniles were washed twice with Tris buffered saline (TBS), pH 8.0, with 10 mmol·l^{-1} $CaCl_2$ (for LFA) or phosphate buffered saline (PBS), pH 6.8, with 1 mmol·l^{-1} $CaCl_2$ and $MnCl_2$ (for all other lectins). For binding experiments with gold-conjugated lectins the juveniles were washed twice with PBS plus albumin and Tween (0.15 mol·l^{-1} NaCl, 10 mmol·l^{-1} Na_2HPO_4, 3 mmol·l^{-1} KH_2PO_4, 0.1 mmol·l^{-1} $CaCl_2$ and $MnCl_2$, 0.5 % bovine serum albumin, and 0.05 % Tween-20), pH 6.8.

Lectin binding

Fluorochrome-conjugated lectins (50 and 100 µg·ml^{-1} PBS or 100 µg·ml^{-1} TBS) were applied to the dissected sedentary juveniles for 30 min at room temperature in the dark. Gold-conjugated lectins were diluted 1:20 with PBS plus albumin and Tween (PBSAT) and applied for 2 h in the same way. The nematodes were then washed twice with PBS, TBS or PBSAT. The carbohydrate-specificity controls of fluorescent lectin binding were performed by comparing on the same glass microscope slides the fluorescence intensity between nematodes incubated for 45 min at room temperature in the lectin solutions and in the lectin solutions with the appropriate inhibitory carbohydrates, after both solutions had been incubated for 90 min at 37 °C (Bone & Bottjer, 1985; Aumann & Wyss, 1988). The specificity controls of gold-labelled lectin binding were accomplished under the same conditions, but the nematodes were incubated for 2 h with the lectin-sugar solutions. The inhibitory carbohydrates were used at final concentrations of 200 mmol·l^{-1} (α-ManMe for Con A, D-Gal for PNA, GalNAc for HPA, α-L-Fuc for UEA-I and Neu5Ac for LFA) or 15 mg·ml^{-1} (GlcNAc-oligomers). The pH of the Neu5Ac solution was adjusted to 8.0 with 5 mol·l^{-1} NaOH prior to the incubation with LFA (Davis *et al.*, 1988). Then the nematodes were washed twice with PBS, TBS or PBSAT. The fluorescent lectin binding experiments were repeated two to four times. Specimens labelled with gold-conjugated lectins were processed for electron microscopy as described by Forrest and Robertson (1986).

Microscopy

Fluorescence and differential interference contrast microscopy (Normarski optics) was used as described by Aumann and Wyss (1989). Electron microscopy was according to Forrest and Robertson (1986).

Results

Table 1 shows that the lectins Con A, WGA, PNA, HPA and UEA-I but not LFA bound to the cuticle exudate in most specimens. Typical binding of fluorochrome-conjugated lectins to cuticle exudates is shown in Fig. 1 A-F. However, the lectin binding sites were obviously unspecific for carbohydrates, as the carbohydrate-treated controls also fluoresced.

In addition binding to the amphidial exudate was clearly visible with Con A and WGA, but was less visible with PNA and HPA. UEA-I labelled the amphidial exudate in only a few of the nematodes and LFA did not bind at all. Only a few lectin binding sites were found in the region of the inner labial sensilla (mainly Con A and UEA-I), on the mouth stylet tip (mainly Con A and PNA) and on the excretory pore exudate (Con A and HPA). Most of these binding sites appear to be carbohydrate-specific, because the fluorescence in the controls was lacking. LFA did not bind to the nematode surface.

An examination of ultrathin sections through specimens labelled with either gold-conjugated Con A or HPA confirmed that the lectins labelled the exudate (Fig. 1 G-I) and not the surface of the nematode. However, it was not clear if the labelled exudate attached to the amphidial openings originated from the amphids or through the cuticle or was a mixture of both.

Discussion

According to Brown *et al.* (1971), the cuticle exudates of *Heterodera mani* females consisted of n-tetracosanoic acid and its calcium salt. In *H. avenae*, the exudates also contained hexacosanoic acid, and in *H. trifolii* they were exclusively composed of di-, tetra-, and hexacosanoic acid. We found strong, carbohydrate-unspecific binding of Con A, WGA, PNA, HPA, and UEA-I to the cuticle exudates. Unspecific lectin binding due to hydrophobic interactions with lipids is a common phenomenon (see Ochoa, Sierra & Cordoba, 1981; Roberts & Goldstein, 1983; Grant & Peters, 1984). Con A especially has a well-documented capacity to bind non-carbohydrate

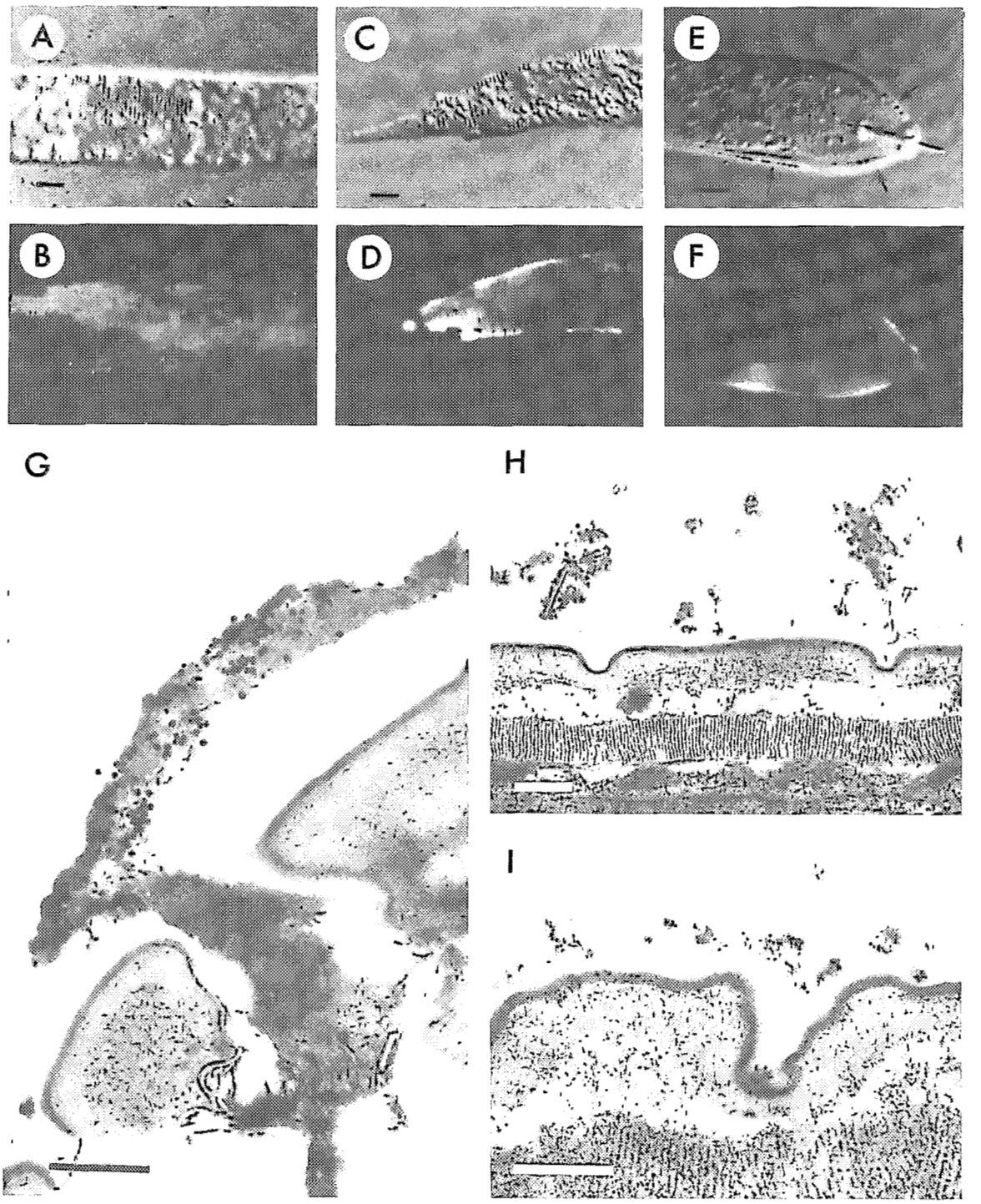

Fig. 1. Binding of fluorochrome- (A-F) and colloidal gold-conjugated (G-I) lectins to the cuticle exudate of *Heterodera schachtii* sedentary J2 A, B : Con A; C, D : WGA; E, F : UEA-I (A, C, E : Differential interference contrast microscopy; B, D, F : Fluorescence microscopy); G : Section through head showing labelling of exudate near amphid with HPA-gold; H : Section showing exudate on side of head labelled with HPA-gold; I : Section showing exudate on side of head labelled with Con A-gold. *(Bar equivalent : A-F = 10 µm; G-I = 250 nm).*

Table 1

Binding of fluochrome-conjugated lectins to the surface of *Heterodera schachtii* sedentary J2 and carbohydrate-specificity controls of lectin binding.

Treatment	*n**	*Lectin binding sites*				
		Cuticle exudate	*Amphid exudate*	*Region of inner labial sensilla*	*Stylet tip*	*Excretory pore exudate*
Con A, 100 $\mu g \cdot ml^{-1}$	81	81 (100 %)	53 (65.4 %)	3	9	22
Con A + α-ManMe	40	36 (90.0 %)	1 (2.5 %)	0	2	0
Con A, 50 $\mu g \cdot ml^{-1}$	58	58 (100 %)	44 (75.9 %)	4	15	5
Con A + α-ManMe	59	53 (89.8 %)	6 (10.2 %)	0	3	0
WGA, 100 $\mu g \cdot ml^{-1}$	67	66 (99.0 %)	46 (68.7 %)	0	1	0
WGA + GlcNAc-olig.	29	29 (100 %)	8 (27.6 %)	0	2	0
WGA, 50 $\mu g \cdot ml^{-1}$	65	65 (100 %)	58 (89.2 %)	1	7	0
WGA + GlcNAc-olig.	64	53 (82.8 %)	16 (25.0 %)	0	6	1
PNA, 100 $\mu g \cdot ml^{-1}$	72	57 (79.2 %)	28 (38.9 %)	0	10	2
PNA + D-Gal	26	9 (34.6 %)	0 (0 %)	0	2	0
PNA, 50 $\mu g \cdot ml^{-1}$	56	50 (89.3 %)	18 (32.1 %)	2	16	0
PNA + D-Gal	58	21 (36.2 %)	3 (5.2 %)	0	0	0
HPA, 100 $\mu g \cdot ml^{-1}$	52	50 (96.2 %)	24 (46.2 %)	0	3	2
HPA + GalNAc	37	31 (83.8 %)	2 (5.4 %)	0	1	0
HPA, 50 $\mu g \cdot ml^{-1}$	51	49 (96.1 %)	29 (56.9 %)	0	3	2
HPA + GalNAc	51	36 (70.6 %)	3 (5.9 %)	0	5	1
UEA-I, 100 $\mu g \cdot ml^{-1}$	86	85 (98.8 %)	5 (5.8 %)	8	4	0
UEA-I + α-L-Fuc	32	30 (93.8 %)	3 (9.4 %)	0	1	0
UEA-I, 50 $\mu g \cdot ml^{-1}$	56	54 (96.4 %)	2 (3.6 %)	9	4	0
UEA-I + α-L-Fuc	53	51 (96.2 %)	1 (1.9 %)	7	4	0
LFA, 100 $\mu g \cdot ml^{-1}$	28	1 (3.6 %)	0 (0 %)	0	0	0
LFA + Neu5Ac	42	0 (0 %)	0 (0 %)	0	0	0

*n = total numbers of J2 examined.

ligands (Davey, Huang & Carter, 1974). Non-carbohydrate specific binding of Con A and WGA has been reported for *Longidorus* spp. (Robertson *et al.*, 1990) and of Con A, WGA and soybean agglutinin on amphidial exudates of J2 of *Meloidogyne* spp. (Davis *et al.*, 1988). Non-carbohydrate binding of PNA and LFA to artificially induced, amphidial exudates of *H. schachtii* males was also recorded by Aumann and Wyss (1989). Barondes (1988) proposed a new definition of lectins; he postulated a second type of binding site which was not carbohydrate dependent. It is most likely that the lectins bound in our experiments to long-chain fatty acids of the *H. schachtii* cuticle exudates. This is supported by preliminary tests in which we showed a strong binding of HPA to the cleavage surface (Fig. 2) of hexacosanoic acid crystals. Shaking the hexacosanoic acid crystals for 24 h in the lectin solution resulted in a nearly complete adsorption of the lectin molecules. Another possible explanation for unspecific lectin binding was given by Wagner (1988). He suggested that at high fluorochrome : lectin ratios the net charge of the molecules would increase resulting in unspecific lectin binding. The cuticle exudates are produced by sedentary J2 after the induction of the initial syncytial cell (Zunke, 1985). Freshly hatched preparasitic juveniles have specific cuticular lectin binding sites for Con A and HPA only (Aumann & Wyss, 1988). Hence, the chemical composition of the surface is altered significantly in feeding *H. schachtii*.

The origin of the cuticle exudates is unknown. A superficial binding of plant products to the nematode surface can be excluded as lectin binding sites are distributed over the entire surface, even in juveniles with only their head inside the root. Bird, Bonig and Bacic (1988) hypothesized that in *Anguina agrostis* the cuticle WGA binding sites are secreted from the excretory system and proposed " a role for the « excretory » system in Secernentea ". However we consider that the

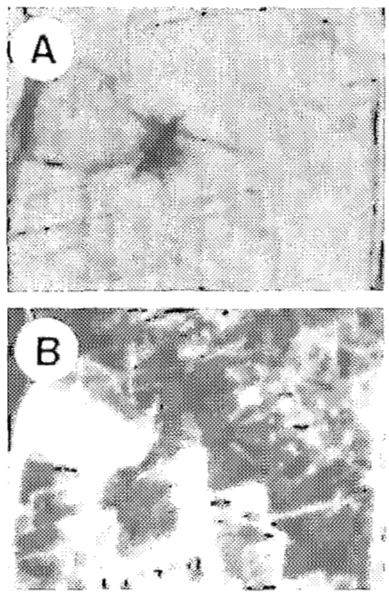

Fig. 2. Binding of TRITC-conjugated HPA to the cleavage surface of a crystal of hexacosanoic acid. A : Differential interference contrast microscopy; B : Fluorescence microscopy.

excretory system is at the most a minor source of the cuticle exudates of sedentary stages of *H. schachtii*, as the excretory pore exudate was labelled in several specimens with Con A and in few cases with HPA, but never with the other lectins that bound to the cuticle exudate. Furthermore it seems unlikely that the excretory pore exudate could be transported in different directions to cover the entire body surface of the immobile sedentary nematodes.

The most probable site of secretion of the cuticle exudates is the cuticle itself. Taylor *et al.* (1970) found cuticular canals in *Longidorus elongatus* and Spiegel *et al.* (1983) stained the cuticular canal system of *Xiphinema index* for carbohydrates. However Martin *et al.* (1987) failed to demonstrate true structural cuticular pores in *Ascaris lumbricoides suum*. Similarly cuticle pores are not discernible in Fig. 1 of Endo's (1987) publication. The electron micrograph shows a longitudinal section through a J2 of *H. glycines* in an early stage of parasitism. The exudates are described by Endo as " uniform layer of fibrillar material oriented perpendicular to the surface ". Forrest, Robertson and Milne (1989*b*) also found fibrillar material surrounding the head of sedentary J2 of *Globodera rostochiensis* and proposed that the fibrils, associated to the annular groves, originated from lacunae in the third layer of the cuticle. In an earlier report Forrest, Robertson and Milne (1989*a*) suggested from specificity tests that this fibrillar material is not identical with the amphid exudate. Bird (1988) visualized cuticle imprints of *Anguina agrostis* second stage dauer larvae on glas slides by labelling with FITC-conjugated WGA. He presumed that these imprints are part of the glycocalyx which is not considered to be part of the cuticle but to be secreted onto it. This secretion may take place continuously and their products may be lost during nematode movement through the soil.

The function of the cuticle exudates is yet a matter of speculation. Kirjanova (1969) suggested that they may protect the cyst nematode females from unfavourable environmental conditions. Shepherd, Clark and Dart (1972) proposed that unwanted components of the " plant cell sap ", like excess sugars, may be excreted through the cuticle. Shepherd and Clark (1978) described " cement " globules encrusting the head end of female cyst nematodes that may seal the gap between the cuticle and the cuticle exudates. Another possible function may be prevention of nematode recognition by the plant. In the trematode *Schistosoma mansoni*, for instance, evidence is accumulating that host antigens mask parasite surface antigens (Harnett, Kusel & Barrowman, 1985). It has been suggested that in the animal parasitic nematode *Dirofilaria immitis* the adsorption of host protein on the cuticle transformed the foreign surface into a host-like one (Kadipasaoglu & Bilge, 1989). However, as sedentary stages of *H. schachtii* produce cuticle exudates in susceptible and resistant host plant cultivars (unpubl.), it appears unlikely that the exudates may mask recognition. As sedentary juveniles of *H. schachtii* defaecate at rather regular intervals at the onset of ingestion periods (Wyss, unpubl.), the exudates are probably not waste products. Their most likely function is thus to provide additional protection to the entire body cuticle.

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