Characterization of Sialyl and Galactosyl Residues on the Body Wall of Different Plant Parasitic Nematodes[1]

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Abstract: The plant parasitic nematodes *Helicotylenchus multicinctus, Meloidogyne javanica, Tylenchulus semipenetrans,* and *Xiphinema index,* differing in their host specificity and parasitic habits, were analyzed as to their cuticle surface sialyl, galactosyl, and/or N-acetylgalactosaminyl residues. The procedure involved the selective oxidation of sialic acid and galactose/N-acetylgalactosamine residues using periodate and galactose oxidase, respectively, to form reactive aldehyde groups. These functional groups were coupled directly with a new hydrazide-containing compound, the fluorescent reagent lissamine rhodamine-β-alanine hydrazide, or they were utilized to introduce DPN-groups to the nematode cuticle. The distribution of the DNP-tagged glycoconjugates was visualized by treating the nematodes with rabbit anti-DNP antibody and staining with fluorescein isothiocyanate (FITC)-labeled goat antirabbit IgG. Sialo residues were observed along the entire outer body wall of the first three aforementioned nematodes, but there were some differences in reaction among the various life stages within the species. In *X. index,* sialo residues were sited in the tail and head areas, mainly on the lips, oral opening, amphid apertures and stylet. Galactose oxidase treatments revealed galactose on N-acytylgalactosamine residues on *T. semipenetrans* and *X. index,* but there were no indications that their presence was dependent on the developmental stage. Trypsin, pronase, and neuraminidase pretreatment completely abolished the fluorescence in *T. semipenetrans* but did not alter the sialo residue binding reaction in *H. multicinctus* or *M. javanica,* indicating possible differences in the outer body wall saccharide structure and composition between these nematodes. The existence and nature of sugar residues on the cuticle surface of nematodes could contribute to an understanding of the specific recognition by phytophagous nematodes of their host, and perhaps also of the virus transmission mechanism in those nematodes which serve as vectors. *Key words: Helicotylenchus multicinctus, Meloidogyne javanica, Tylenchulus semipenetrans, Xiphinema index,* fluorescent reagent, lissamine rhodamine-β-alanine hydrazide, double antibody technique, glactose oxidase, enzymes, neuraminidase, chymotrypsin, trypsin, host-parasite recognition.

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Phytopathologists have recently become interested in surface associated glycoconjugates because of the unique functions attributed to them (4). Surface sugars have been implicated in recognition phenomena, such as intercellular communication and host-parasite relationships (7). However, there is little information concerning the nature of carbohydrates of the outer body wall of nematodes, and especially of the plant parasitic forms.

Recently, it was shown by Himmelhoch *et al.* (6) that ruthenium red staining of the cuticle surface of *Caenorhabditis briggsae* suggested the presence of acid mucopolysaccharides but not of neuraminic, hyaluronic, or glycoronic acids. Histochemical techniques were used by Sood and Kalra (13) to compare outer body wall of a ruminant nematode *Haemonchus contortus* with that of the plant-parasitic nematode, *Xiphinema insigne.* In *H. contortus* they found indications of the presence of proteins associated with acid mucopolysaccharides and in *X. insigne* the cuticle consisted of weakly acidic mucopolysaccharides. In addition, the presence of galactose, glucose, mannose, and N-acetylglucosamine was indicated on *C. briggsae* and *C. elegans* by using three iodinated plant lectins (20).

The existence and nature of sugar residues on the cuticle surface of nematodes could contribute to an understanding of the specific recognition by phytophagous nema-

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todes of their host, and perhaps also of the virus transmission mechanism in those nematodes which serve as vectors.

The object of the current study was to characterize and map the cuticle surface glycoconjugates of three tylenchid plant parasitic nematodes *Helicotylenchus multicinctus,* a migratory endoparasitic pathogen of bananas in Israel; *Meloidogyne javanica,* a sedentary polyphagous root gall-forming nematode; and *Tylenchulus semipenetrans,* a sedentary semiendoparasitic pathogen of citrus (9), and of the dorylaimid plant-parasitic nematode, *Xiphinema index,* which is relatively specific in its host range among ecto-parasitic plant forms and also acts as vector of the fanleaf virus disease in grapevine (3).

MATERIALS AND METHODS

Abbreviations: DNP, 2,4, Dinitrophenyl; DNP-DOBH ⁴N Dinitrophenyl-L-2,4 diaminobutyric acid hydrazide; FITC, Fluorescein isothiocyanate; PBS, Phosphate-buffer saline (pH-7.4); SBA, Soybean agglutinin.

Nematodes: *Helicotylenchus multicinctus*-infected banana roots, galled tomato roots infected with *M. javanica,* and citrus roots with heavy *T. semipenetrans* infection symptoms were incubated for 24 h at room temperature. Larvae, males and females of *H. multicinctus* and larvae and males of *M. javanica* and *T. semipenetrans* were collected by the Baermann funnel technique. Larvae, males and females of *X. index* were extracted from soil around fig trees and washed three times with PBS. The nematodes were used immediately or kept in water at 4–5 C, but no more than 48 h.

Chemicals: DNP-DABH and purified rabbit anti-DNP were provided by M. Wilchek, Department of Biophysics, Weizmann Institute of Science, Rehovot, Israel. Fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG was purchased from Miles Laboratories, Rehovot, Israel. Galactose oxidase (150 units/mg) and pronase from *Streptomyces griseus* (0.7-1.0 units/ml) were purchased from Sigma Chemical Co., St. Louis, Mo., U.S.A. Lissamine rhodamine-β-alanine hydrazide was provided by M. Wilchek. Neuraminidase (500 units/ml) was purchased from Behringwerke, Marburg, West Germany. Potassium borohydride and sodium periodate were obtained from BDH Chemicals Ltd., Pool, England. Trypsin and chymotrypsin from bovine pancrease were obtained from Worthington Biochemicals, Corp., Freehold, N.J., U.S.A.

Sialic acid labelings: 1. PBS-washed nematodes were treated with sodium periodate (1 m*M*) for 30 min in ice-water bath (0 C), centrifuged, washed, and resuspended in PBS containing lissamine rhodamine-β-alanine hydrazide (0.5 mg/ml) for 30 min at room temperature. The nematodes were then thoroughly washed with PBS, mounted on slides, examined by epifluorescence microscopy, and photographed. To verify the specificity of the observed binding, parallel control experiments were conducted in which periodate oxidation was excluded.

2. Suspended nematodes in PBS were treated with sodium periodate (1 m*M*) for 30 min in ice-water bath (0 C), washed with PBS, and resuspended in PBS containing DNP-DABH (0.5 mM). After incubation for 30 min at room temperature, the nematodes were washed again with PBS and treated with potassium borohydride (1 m*M*) for 10 min at room temperature; washed nematodes were then incubated with rabbit anti-DNP IgG (400 μg/ml) in 0.2 ml PBS for 30 min at 0 C (in ice-water bath). A twice-washed nematode suspension was then incubated with fluorescein-labeled 1 mg/ml goat anti-rabbit IgG at 0 C for 30 min, washed again with PBS, and examined under a Zeiss fluorescence microscope, as described above.

D-galactose and N-acetylgalactosamine labelings: PBS (free of Ca^{++} and Mg^{++})-suspended nematodes were treated with galactose oxidase (5 untis/ml) for 30 min at 37 C. After incubation, the nematodes were washed once with 0.2 M D-galactose and three times with PBC, and then treated with lissamine rhodamine-β-alanine hydrazide, or treated by the double antibody technique and examined under fluorescence microscope as described above for sialic acid labeling. Control experiments in which the galactose oxidase treatment was excluded were run in parallel to prove the binding specificity.

Enzyme pretreatments: Trypsinization, chymotrypsinization, and pronase treatment of PBS-washed nematodes were accomplished in a concentration of 1 mg of crystaline trypsin, chymotrypsin, or pronase per ml of PBS at 37 C for 20 min.

Fifty units/ml of neuraminidase in a medium containing 0.145 M NaCl, 0.003 M $CaCl_2$, and 0.004 M $NaHCO_3$, pH 6.5 (12) were applied to washed nematodes for 60 min at 37 C before sialic acid and D-galactose labelings.

RESULTS AND DISCUSSION

Development of techniques for assaying nematodes: While information concerning the sugar character in the outer cuticle of nematodes is sorely lacking and techniques for studying this field are poorly developed, novel methods recently have been introduced for the localization of membrane-bound sialic acid sites on blood cells (5). These methods are based on periodate selective oxidation of sialic acid, resulting in formation of the C-7 aldehyde derivatives (17). Further reaction of these aldehydes with reagents containing hydrazide groups can be utilized to attach the appropriate chemical group to the cell (15).

A new fluorescent reagent, lissamine rhodamine-β-alanine hydrazide, was developed and synthesized by us in cooperation with other workers (18) for the labeling of cell surface glycoconjugates. This reagent contains hydrazide which couples directly with the aldehyde formed after oxidation and the complex is immediately ready for the fluorescence microscopy observation. We could not use this reagent for labeling glycoconjugates on the outer cuticle of *X. index* because this nematode exhibited a strong autofluorescence on the same wavelength as the fluorescence emission of rhodamine. It was also not possible to apply on *X. index* the parallel fluorescein-β-alanine hydrazide reagent because it was found to penetrate slightly into the nematode, thus giving a reaction also with non-oxidized nematodes. Thus, in *X. index*, DNP groups were attached to the nematode body wall sialo-carbohydrates by reacting DNP-DABH with aldehyde formed upon mild periodate oxidation of the nematode (Fig. 1). In order to study the distribution of the DNP-modified sialic acid residues, we employed the double antibody technique using IgG to DNP, followed by fluorescein-labeled goat anti-rabbit IgG (15).

Nematode reactions (to treatments): Results of the fluorescent labeling of the three species examined with lissamine rhodamine-β-alanine hydrazide (*H. multicinctus, M. javanica,* and *T. semipenetrans*) revealed differences both between the nematode species as well as between their life stages. Using the sialic acid labeling procedure with *H. multicinctus,* males and females were fluoresceinated all over the body wall,

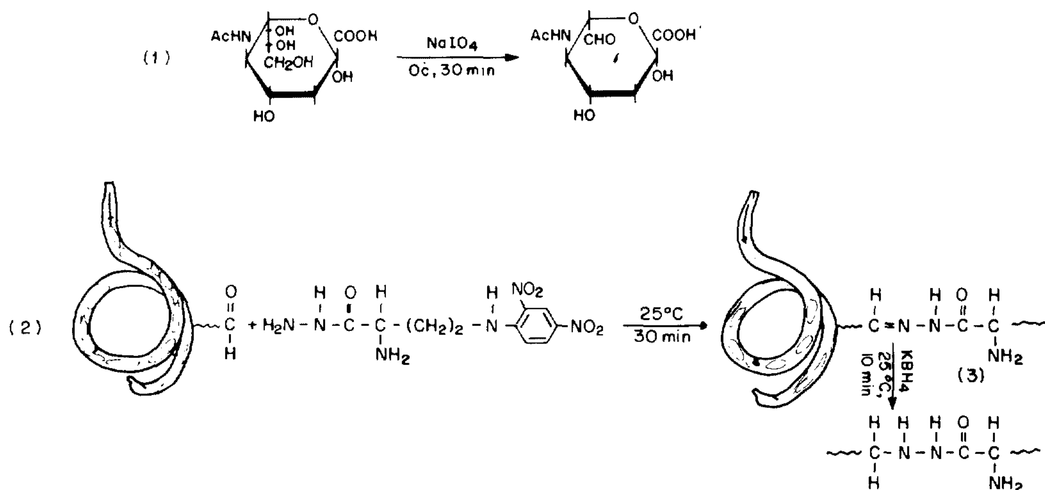

Fig. 1. First steps in nematode sialic acid labeling: (1). Periodate oxidation of sialic acid. (2) DNP-coupling with an aldehyde residue formed in step (1), on the nematode surface. (3) Hydrogenation of the (–C=N–) bond by potassium borohydride.

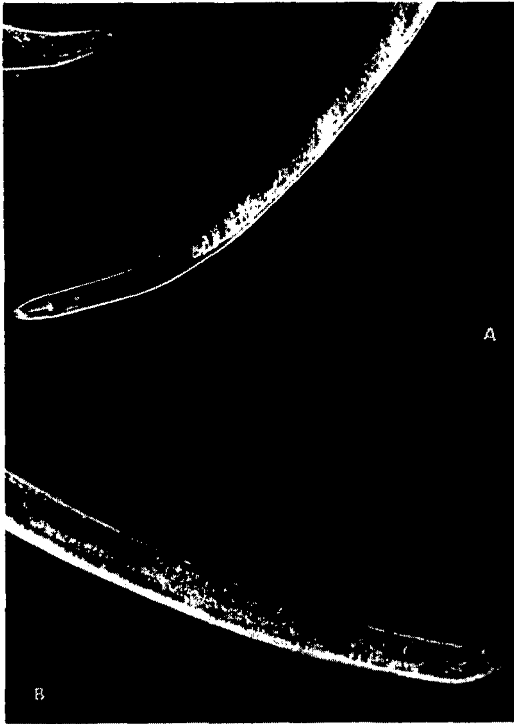

Fig. 2. Surface fluorescence pattern of the *Helicotylenchus multicinctus* body wall after labeling for sialic acid with lissamine rhodamine-β-alanine hydrazide. (A) Fluorescence of the anterior end region. (B) Fluorescence of the posterior region. (× 200).

especially in the region of the spicules and vulva (Fig. 2). Larvae of *H. multicinctus*, however, showed negative results after these treatments.

Both larvae and males of *M. javanica* reacted positively with the fluorescent reagent after periodate treatment and revealed sialic acid residues all over the body wall. The fluorescence of the males was even more intense than in that of the larva stages. When periodate-treated *T. semipenetrans* larvae and males reacted with lissamine rhodamine-β-alanine hydrazide, their surface became highly fluorescent (Fig. 3).

As can be seen in Figure 4, sialic acid-labeling in *X. index* gave a strong tail and head area fluorescence, mainly on the lips, oral opening, amphid apertures, and stylet. The described labeling was a result of a specific interaction between the DNP derivatives and periodate oxidized sialyl residues. This conclusion is based on the following evidence: 1. Only sequential treatments of nematodes with periodate and DNP-DABH gave rise to intense fluorescence. A different reaction combination, which did not result in DNP coupling, failed to induce this fluorescence. 2. No fluorescence was observed in the presence of unrelated normal rabbit IgG instead of rabbit anti-DNP IgG.

Thus, the sugar residue which has hitherto been consistently observed by us in all the phytophagous nematodes studies is sialic acid. Reversal of fluorescence labeling by neuraminidase, trypsin, or pronase pretreatment in *T. semipenetrans*, in contrast

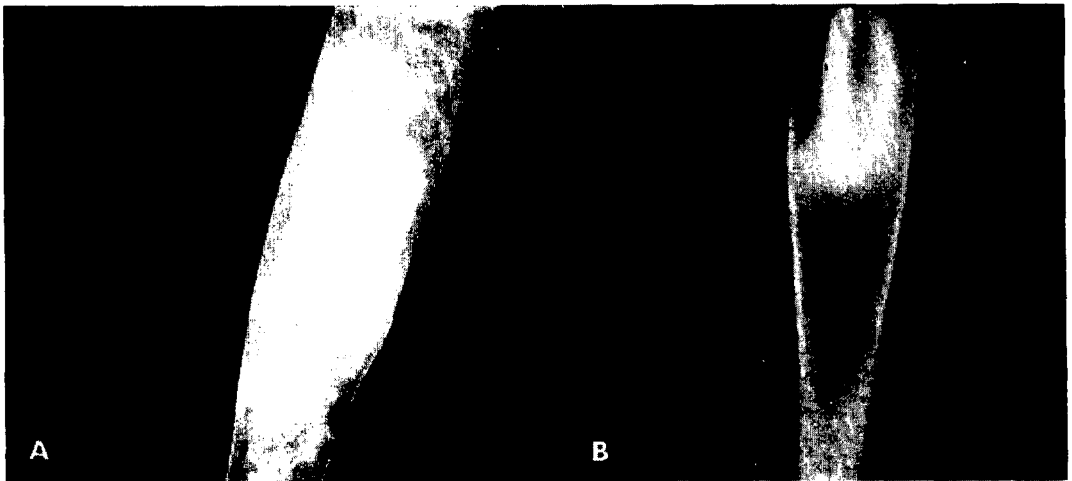

Fig. 3. Surface fluorescence pattern of the *Tylenchulus semipenetrans* body wall after labeling with lissamine rhodamine-β-alanine hydrazide. (A) Reaction for sialic acid; tail area. (B) Reaction for galactose residues; tail area. (× 1250).

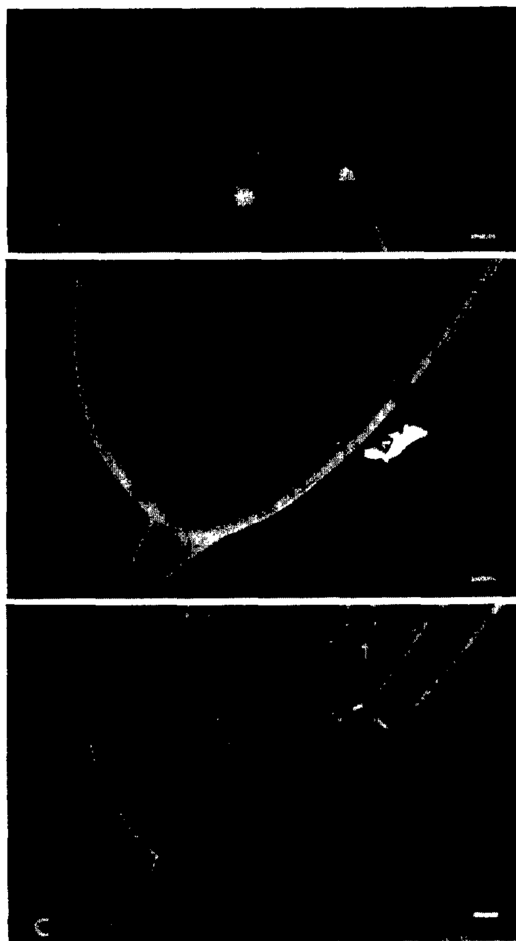

Fig. 4. Surface fluorescence pattern of *Xiphinema index* body wall after sialic acid labeling: (A) Fluorescence of the head region. (B) Fluorescence of the tail region. (C) Same area as (B) under phase optics. (× 1250). Bar = 3.3 μm.

to *H. multicinctus* and *M. javanica,* suggests that in the former nematode the sialo residues are possibly part of sialoglyco-proteins (2,19) or that in the two latter nematodes the peptides of the glycoproteins are inaccesible to the enzymes. It can be speculated that this finding may be related to the differences in the host preference of these nematodes: while *H. multicinctus* and *M. javanica* are polyphagous on a wide range of plant species, *T. semipenetrans* is a specialized parasite limited more or less to citrus species. The nature of the sialoglycoconjugates on the surface of *H. multicinctus* and *M. javanica* nematodes may be regarded either as a part of sialoglycolipid or as a part of glycoprotein-peptide.

The dorylaimid nematode *X. index* exhibited sialo residues conspicuously confined to specific body areas, in contrast to the tylenchid nematodes where the sialo residues appeared all over the body wall. These differences cannot be attributed to differences in labeling techniques, because *M. javanica* larvae and males, for instance, treated with periodate followed by coupling to a DNP-ligand stained with rabbit anti-DNP-antibody and rhodamine labeled goat anti-rabbit IgG, showed the same results as were obtained with lissamine rhodamine-β-alanine hydrazide. Sialic acid fluorescence binding studies indicated also clear differences between some of the nematode life stages within species, as to the structure and organization of the body wall glycoproteins and/or glycolipids. These findings support the possibility that development of larvae into adults may be accompanied by changes in cell surface saccharides (19).

The presence of sialic acid on the head area of *X. index,* associated closely with nematode sense and host-finding organs, may have a special significance regarding virus transmission by plant parasite nematodes, because sialic acid is considered to be one of the main components of the contributors to virus-receptor constitution (8). Furthermore, the retention sites of virus particles in all known nematode vectors of plant viruses occur along the cuticular surface of the stylet and esophagus within the nematode (16).

Galactose oxidase was used as a tool to identify galactose/N-acetylgalactosamine residues on the nematode body wall. Galactose/N-acetylgalactosamine moieties exposed on the nematode surface could be oxidized at the C-6 position by galactose oxidase to yield the 6-aldehydo-analogue (1) (Fig. 5).

The aldehyde thus formed could be

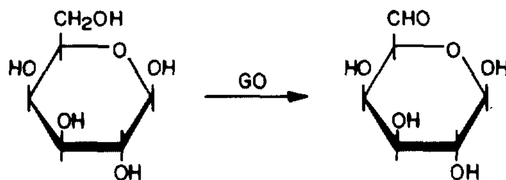

Fig. 5. Conversion of D-galactose to D-galacto-hexodialdose by galactose oxidase. GO = Galactose oxidase.

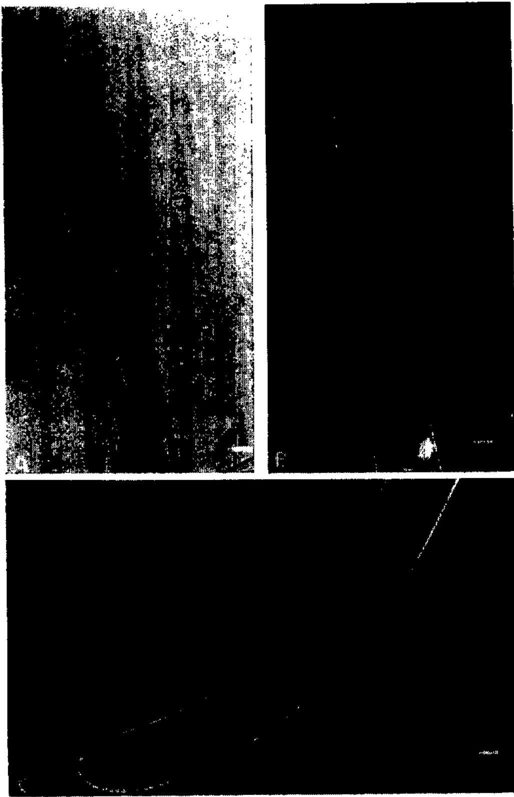

Fig. 6. Surface fluorescence pattern of *Xiphinema index* body wall after galactose residues labeling by galactose oxidase pretreatment. (A) The anterior end region under phase optics. (B) Same area as (A) under fluorescent optics. (C) Fluorescence of the posterior end region. (× 200). Bar = 20 μm.

labeled as previously described in the sialic acid labeling procedure. *Helicotylenchus multicinctus* larvae, males, and females and *M. javanica* larvae and males gave no fluorescence after galactose oxidase treatment. However, larvae and males of *T. semipenetrans* (Fig. 3b) and larvae, males, and females of *X. index* (Fig. 6), revealed a strong fluorescence all over the nematode body wall after galactose oxidase treatment. Trypsinization and chymotrypsinization did not alter the fluorescence created by the galactose/N-acetylgalactosamine labeling, suggesting at least two possibilities for the nature of these glycoconjugates: 1. The sugar residues are part of the polysaccharide or glycolipid rather than glycoprotein. 2. the peptides of the glycoproteins are inaccessible to the enzymes (absence of digestible glycoprotein).

As we noted for sialic acid residue findings, it is possible that the differences found among the four genera in galactose/N-acetylgalactosamine experiments may be related to the differences in host-preference of these nematodes.

Using SBA lectin (14) further confirmed the presence of galactose and/or N-acetyl galactosamine, revealed by the galactose oxidase method. The finding that these sugars were bound by a lectin which is widely distributed in plants suggests that the specific recognition of the host by the nematode parasite could be accomplished by interaction between the parasite sugar residue and the host lectin.

The last possibility gains support from some positive results with *Rhizobium* bacteria and other pathogenic bacteria and fungi as well (11), and lately from Nordbring-Hertz and Mattiason (10) who presented evidence as to the presence of a lectin on the traps of a nematode-trapping fungus which binds to a carbohydrate on the nematode surface.

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