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Origin of a *Meloidogyne incognita* Surface Coat Antigen[1]

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Abstract: The surface coat (SC) of plant nematodes is thought to originate either from the living hypodermis or from secretory glands associated with the excretory system or nervous system. In this study, we investigated the origin of the SC of *Meloidogyne incognita* by immunolocalization with a monoclonal antibody raised against the surface coat of the preparasitic juveniles (J2). Under the electron microscope, strong labeling was found on the cuticular surface and in the rectal dilation of the J2, while labeling was absent in other parts of the nematode, including the hypodermis, excretory system, nervous system, and digestive system. Because the rectal glands are known to be the origin of the gelatinous egg matrix produced by adult females of *Meloidogyne,* we also examined sections of mature females from monoxenic cultures of *Arabidopsis thaliana.* Labeling of the female occurred in the rectal glands and in the gelatinous matrix exuded from the anus. At the ultrastructural level, gold particles were mainly deposited in multivesicular bodies that appeared to be associated with the Golgi bodies of the rectal glands. Our results suggest that at least one component of the J2 SC originates from the rectal gland cells and that the SC of the J2 shares common epitopes with the gelatinous egg matrix of mature females.

Key words: antibody, cuticle, electron microscopy, immunocytochemistry, *Meloidogyne incognita,* nematode, rectal gland, root-knot, surface coat, ultrastructure.

The surface coat (SC) of nematodes is an amorphous, extra-cellular layer overlaying the epicuticle. In animal parasitic nematodes, the SC displays dynamic properties related to evasion of host immune response (Blaxter et al., 1992), and it has been suggested that the properties of the SC in plant-parasitic nematodes enable the nematodes to avoid recognition phenomena that might lead to a resistance response by the host plant (Spiegel and McClure, 1995). The SC of *Meloidogyne incognita* preparasitic juveniles (J2) is about 5–30 nm thick and is characterized by the presence of carbohydrate binding domains (Spiegel et al., 1995) and fucosyl-bearing glycoproteins (Gravato-Nobre et al., 1995; Lin and McClure, 1996). The origin of the SC is obscure. One hypothesis is that the SC of the plant-parasitic nematodes originates from secretions released via the secretory-excretory pore (Bird and Zuckerman, 1989), while others postulate that SC may be partially assembled in the hypodermis and reach the surface of the nematode via a transcuticular route (Preston-Meek and Pritchard, 1991). Transcuticular transport may be facilitated by cuticular pores or channels (Pritchard, 1986), but these structures appear to be lacking in *M. incognita* and other tylenchida. The SC of some nematodes may contain host materials that coat the nematodes as they emerge from the eggs (Spiegel and McClure, 1991).

The objective of this study was to investigate the origin of the *Meloidogyne incognita* SC with a monoclonal antibody (MISC) raised in our laboratory against the surface coat of *M. incognita* J2.

Materials and Methods

Nematodes and plant materials: Preparasitic juveniles of *M. incognita* race 3 were hatched from eggs harvested from eggplant (*Solanum melongena* L. 'Black Beauty'). Hatched J2 for inoculation of *Arabidopsis thaliana* (L.) Heynh. 'Landsberg' were used immediately, whereas those for electron microscopy were stored at 14 °C for 48 hours for SC to accumulate. Seeds of *A. thaliana* were surface-sterilized with undiluted household bleach, followed by two washes of double-distilled water. Sterile seeds were germinated in 9.5-cm by 9.5-cm petri dishes containing 9 ml of Gamborg's B5 basal medium with minimal organics (Sigma, St. Louis, MO), 0.8% agar and 1.5% sucrose. When the radicles were 2–3 mm long, the seedlings were transferred to new petri dishes containing the same me-

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dium. The dishes were sealed with plastic film and kept at 23 °C with a light/dark cycle of 16/8 hours. Two-week-old plants with well-developed root systems were inoculated with axenized J2 (McClure and Viglierchio, 1966). Four weeks after inoculation, the plants were harvested and 2-mm-long root segments, containing mature females with egg masses, were processed for electron microscopy.

Tissue processing for light and electron microscopy: Preparasitic J2 and excised root segments of infected *A. thaliana* were fixed overnight in 3% glutaraldehyde in 0.05 M sodium cacodylate buffer (pH 7.2). Fixed materials were washed twice in cacodylate buffer, dehydrated in ethanol (10% increments), and embedded in either Eponate-Araldite resin or LR White resin (Ted Pella, Redding, CA). Sections of embedded tissues, 1 µm thick, were cut with a glass knife and mounted on glass microscope slides for light microscopy. Sections 70 nm thick were cut with a diamond knife and collected on naked 200-mesh nickel grids for electron microscopy.

Immunogold labeling: Monoclonal antibody (MISC) to the SC of *M. incognita* was used to localize SC epitopes. The development and characteristics of MISC have been described previously (Gravato-Nobre et al., 1999). Ultrathin sections on nickel grids were transferred to one drop of 0.01 M phosphate-buffered saline (PBS, pH 7.2) containing 0.2% (w/v) polyethylene glycol 20,000 (PEG 20,000) for 5 minutes at room temperature. The sections then were incubated in a drop of blocking solution consisting of 0.01 M PBS (pH 7.2), 10% (v/v) fetal bovine serum, 1% (w/v) bovine serum albumin (BSA), 0.05% (v/v) Tween-20, and 0.2% (w/v) sodium azide for 30 minutes, followed by incubation in a drop of primary antibody (MISC) in a moist chamber at 23 °C for 2 hours. Unbound MISC was removed by washing in several drops of buffer (0.01 M PBS, pH 7.2, containing 1% BSA and 0.05% Tween-20), and the sections were transferred to a drop of colloidal gold-conjugated goat antimouse immunoglobulins (GAM-gold antibody) diluted 1:20 in PBS-BSA buffer for 1 hour at room temperature. Gold-labeled sections were washed with 0.01 M PBS (pH 7.2), fixed in 1% glutaraldehyde for 2 minutes, rinsed with double-distilled water, and stained with uranyl acetate and lead citrate. Labeled and stained sections were photographed with a Philips 420 transmission electron microscope at 80kV. Observations were made on sections of 23 individual J2.

Silver-enhanced labeling of thick sections: Sections, 1 µm thick, mounted on glass microscope slides, were incubated with 1 ml of blocking solution for 30 minutes at 23 °C. Blocked sections were incubated in 1 ml of MISC supernatant for 2 hours followed by five washes with PBS, pH 7.2, and incubation for 1 hour in GAM-gold antibody diluted 1:20 in a PBS-BSA buffer. Gold labeling was enhanced by incubating the sections in a silver-containing solution according to the directions of the manufacturer (BBI international, Cardiff, UK). Sections from 11 specimens were examined.

Immunocytochemical controls: Controls for both light and electron microscopy consisted of sections incubated with PBS-BSA-MISC, followed by incubation in the gold-conjugated secondary antibody. For silver enhancement of gold-labeled thick sections, an additional control was incubation of the sections in PBS-BSA lacking the secondary antibody.

Results

Surface-coat epitopes in preparasitic juveniles: The SC of J2 embedded in LR White resin was consistently labeled with MISC-colloidal gold (Fig. 1A). In contrast, the surface coat of J2 embedded in Eponate-Araldite was only sparsely labeled (not shown). Sections through the rectum and anus of J2 revealed intense labeling of material filling the rectal dilation and the rectum proper (Figs. 1B, 2). Tissues surrounding the rectal dilation also were labeled by MISC (Fig. 3). Surface coat epitopes were not detected elsewhere in the body wall in either LR White or Eponate-Araldite resins (Fig. 1A). Nor was labeling observed in the stomodeum, pseudocoelom,

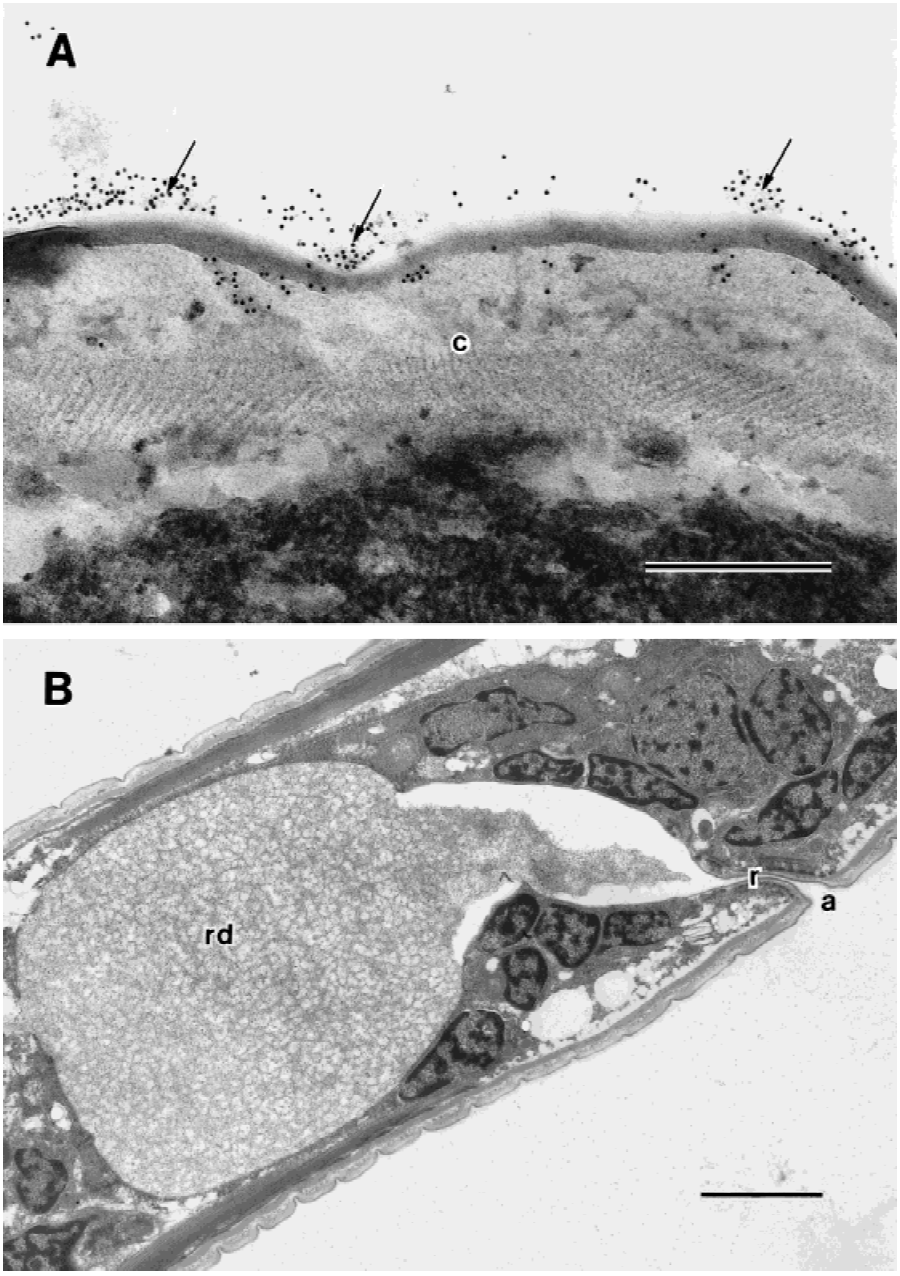

FIG. 1. Longitudinal sections through *Meloidogyne incognita* preparasitic juveniles. A) Specimen embedded in LR White resin and probed with a primary monoclonal antibody (MISC) followed by a secondary antibody conjugated to colloidal gold. Only the surface coat (arrows) is labeled. Labeling was not found in the hypodermis or other layers of the body wall (c, cuticle). Scale = 0.5 µm. B) Specimen embedded in Eponate-Araldite resin and probed with the same antibodies as above. Only the rectal gland cells and the contents of the rectal dilation were labeled (see Figs. 2 and 3 for high-magnification details) (a, anus; r, rectum; rd, rectal dilation). Scale = 5.0 µm.

secretory-excretory system (Fig. 5B), or nervous system of J2.

Surface-coat epitopes in adult females: Under the light microscope, labeling of Eponate-Araldite sections was observed in the rectal glands and egg mass matrix of adult females treated with MISC (Fig. 4A). By electron microscopy, antibody was found bound mainly

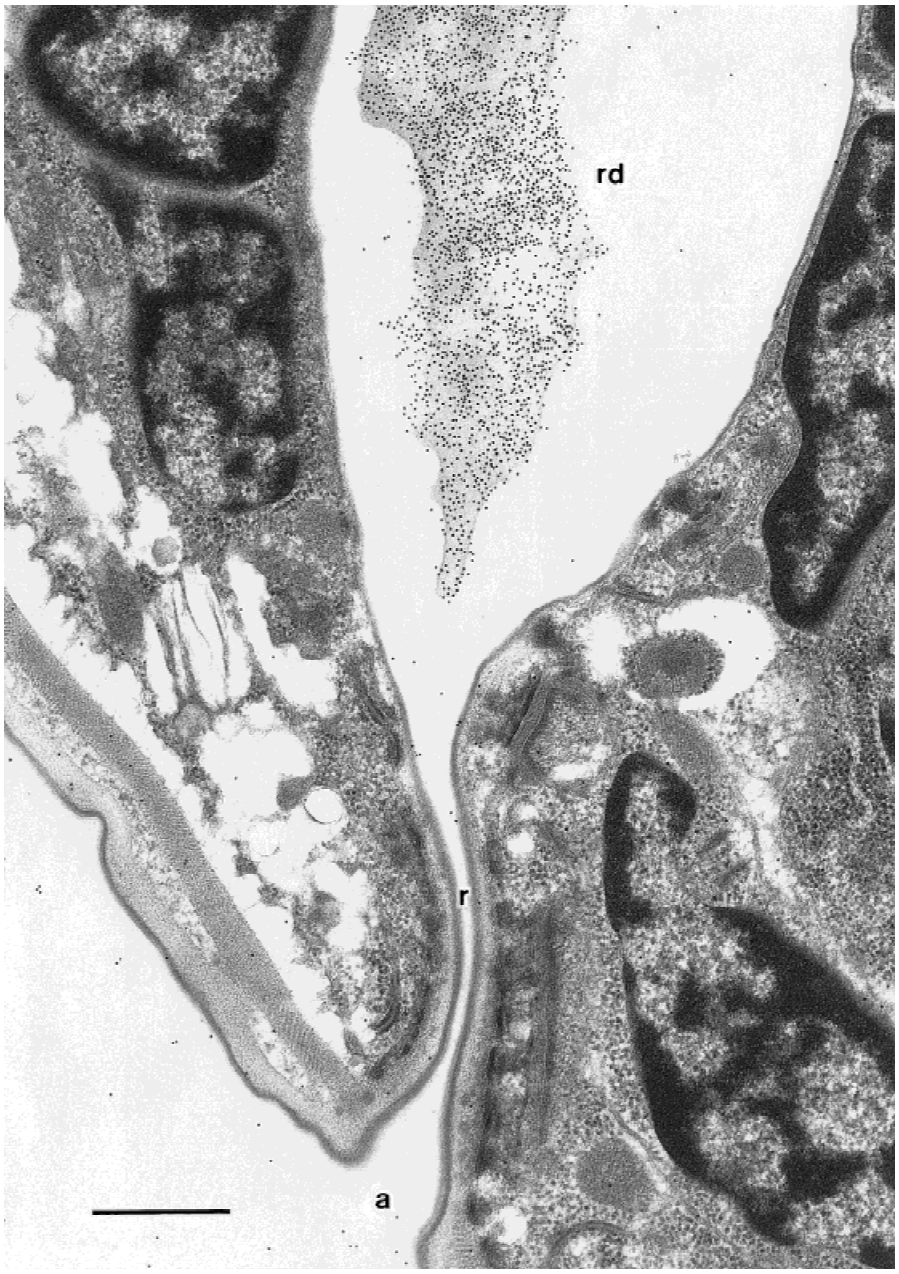

FIG. 2. Longitudinal section through the rectum and anus of *Meloidogyne incognita* preparasitic juvenile. Specimen was embedded in Eponate-Araldite and probed with a primary monoclonal antibody (MISC) followed by a secondary antibody conjugated to colloidal gold. Only the contents of the rectal dilation are labeled (a, anus; r, rectum; rd, rectal dilation). Scale = 0.5 μm.

to Golgi-associated multivesicular structures (Fig. 4B) and to the egg mass matrix (Fig. 5A).

Immunocytochemical controls: Controls incubated in PBS-BSA, either in the absence of the primary antibody (MISC) or in the absence of the secondary antibody (GAM-gold antibody), were completely free of labeling.

DISCUSSION

The SC appears to be a common feature of nematodes (Spiegel and McClure, 1995), yet its origin in many species is a matter of speculation. Although the evidence is mostly circumstantial, investigations thus far seem

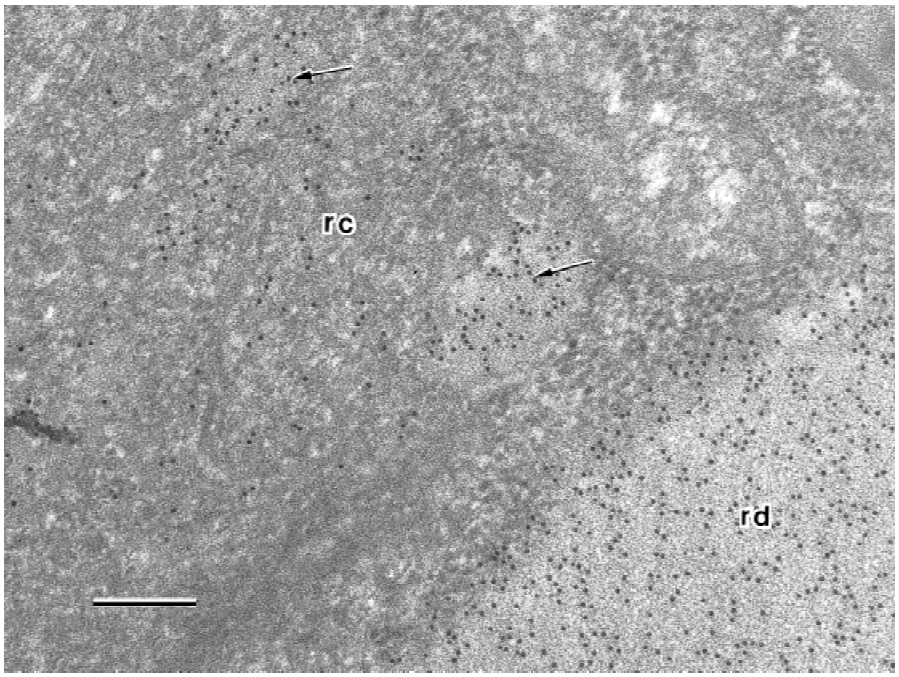

FIG. 3. Longitudinal section through the rectal dilation of *Meloidogyne incognita* preparasitic juvenile. Specimen was embedded in Eponate-Araldite, and the section was probed with a primary monoclonal antibody (MISC) followed by a secondary antibody conjugated to colloidal gold. Contents of the rectal dilation are heavily labeled. Label also was detected in the cells surrounding the rectal dilation (arrows) (rd, rectal dilation; rc, rectal gland cell). Scale = 0.2 μm.

to support the hypothesis that SC originates from the secretory-excretory system (Blaxter et al., 1992; Duncan et al., 1997; Lopez De Mendoza et al., 1999; Spiegel and McClure, 1995; Spiegel and Robertson, 1988). A protein-specific dye, Brilliant Blue-G, stained secretions from the excretory pore, amphids, and phasmids of *M. incognita,* but staining of the surface surrounding these openings was limited (Premachandran et al., 1988). Duncan et al. (1997) prepared a polyclonal antibody to the secretions from *Globodera pallida* and observed that some of the secretory material was surface-associated, but the origin of the secretions was not determined. Lin and McClure (1996) prepared a polyclonal antibody to SC of *M. incognita* that bound to the body contents of ruptured nematodes, but they were unable to localize the site of SC synthesis. Our observations do not support the proposal that the SC is a product of the secretory-excretory system, released through the excretory pore; neither do they suggest a hypodermal origin (Preston-Meek and Pritchard, 1991) since SC epitope was not detected in the hypodermis or inner layers of the cuticle. In our investigation, MISC bound specifically to the SC and the contents of the rectal dilation of *M. incognita* J2. Because no other part of the J2 was labeled, we propose that the MISC-specific component of the *M. incognita* SC originates from the rectal dilation and its associated gland cells. That other components may have a different origin was not explored. Rectal glands appear to be lacking in other Tylenchida, including *Heterodera, Meloidodera,* and *Globodera.* In these nematodes, an alternate source for the SC must be postulated.

Intensity of SC labeling was dependent upon the medium used to embed the nematodes for electron microscopy. Labeling of J2 SC was more intense in LR White medium than in Eponate-Araldite. Page et al. (1992) were able to extract surface components from *Toxocara canis* with 35–100% ethanol. Because we used ethanolic dehydration for

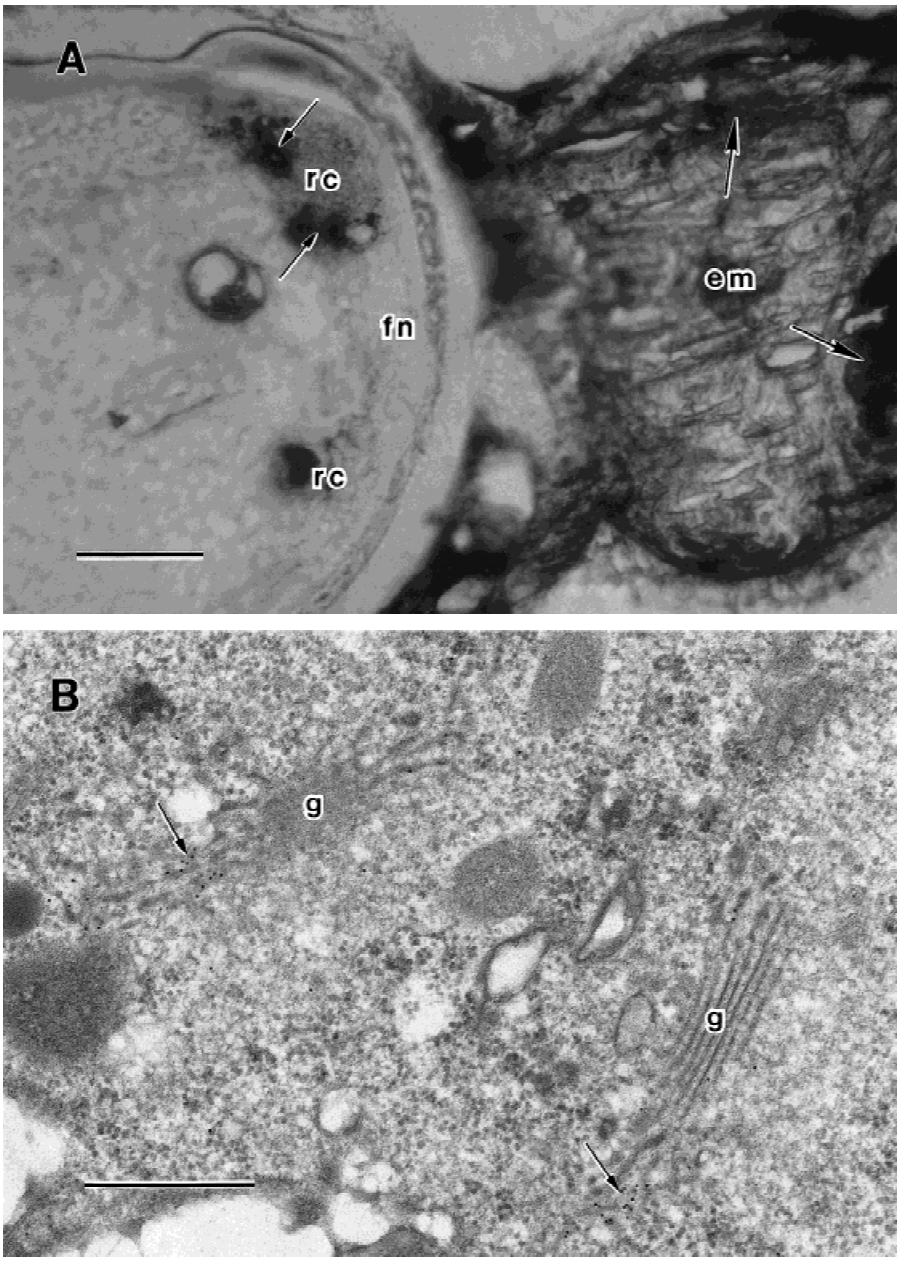

FIG. 4. Longitudinal Eponate-Araldite sections through an adult female and gelatinous egg mass of *Meloidogyne incognita* in an *Arabidopsis thaliana* root. A) Light micrograph of a 1-µ-thick section through the female. Section was probed with a primary monoclonal antibody (MISC) followed by a secondary antibody conjugated to colloidal gold. Gold labeling was intensified with silver (dark deposits, indicated by arrows). Two rectal glands visible in this section and the gelatinous matrix exuded from the anus are heavily labeled. Except for weak labeling on the cuticle surface, labeling was not detected in any other parts of the female (fn, posterior of female nematode; rc, rectal gland cell; em, gelatinous egg mass). Scale = 100 µm. B) Electron micrograph of an ultrathin section through a rectal gland cell in an adult female. Gold labeling (arrows) was associated with vesicles of the Golgi bodies (g, Golgi body). Scale = 0.5 µm.

J2 embedded in both media, ethanolic extraction seems an unlikely reason for the reduction in colloidal gold label on J2 embedded in Eponate-Araldite. An alternative explanation is that the SC is removed by Eponate-Araldite or that Eponate-Araldite

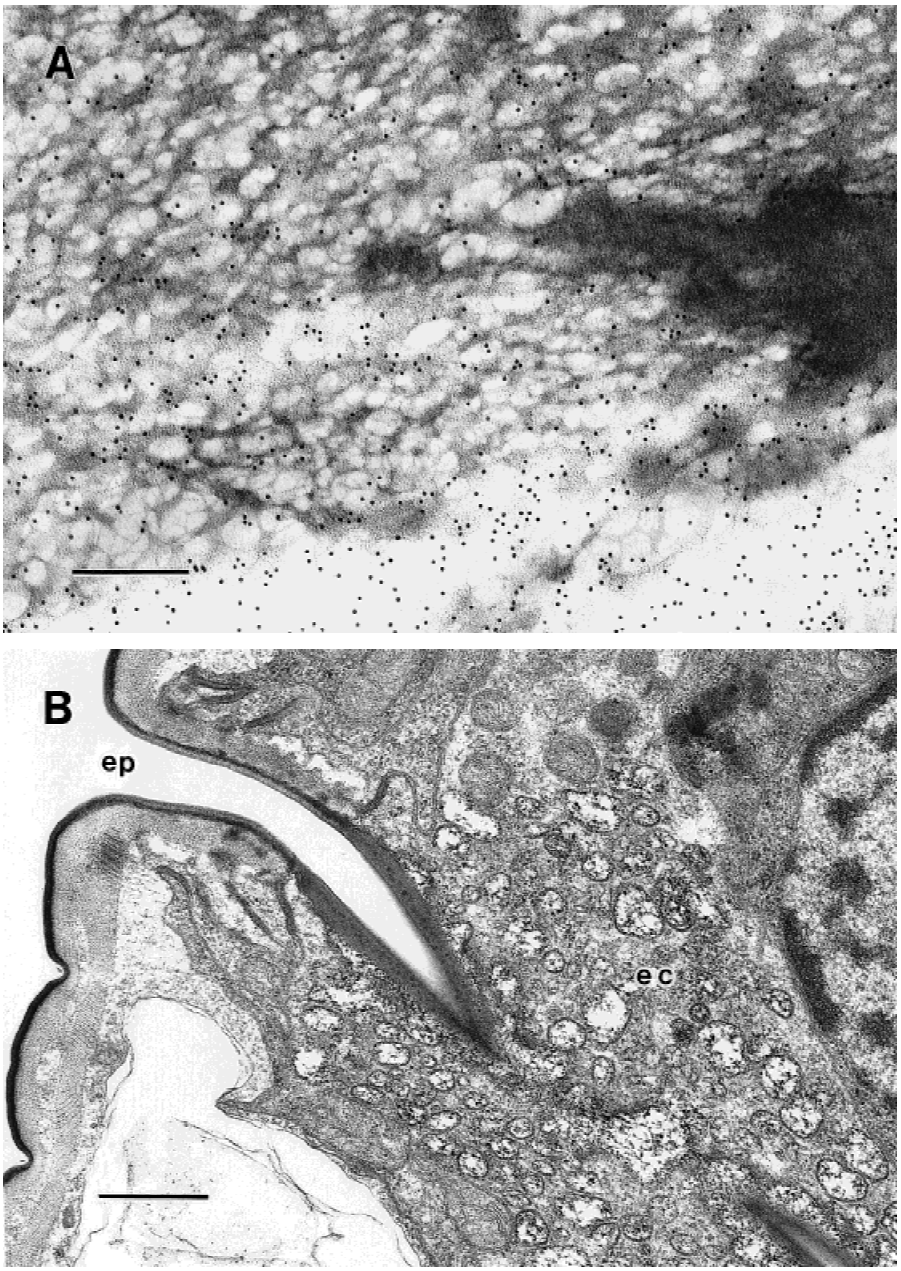

FIG. 5. A) Electron micrograph of an Eponate-Araldite section through a portion of the gelatinous matrix exuded from an adult female of *Meloidogyne incognita* in an *Arabidopsis thaliana* root. The section was probed with a primary monoclonal antibody (MISC) followed by a secondary antibody conjugated to colloidal gold. Gold particles are uniformly distributed over the gelatinous matrix. Scale = 0.25 µm. B) Longitudinal section through the excretory pore of a preparasitic juvenile of *Meloidogyne incognita* embedded in Eponate-Araldite. The section was probed with a primary monoclonal antibody (MISC) followed by a secondary antibody conjugated to colloidal gold. Neither the excretory gland cell nor the contents of the excretory duct were labeled by the antibody (ep, excretory pore; ec, excretory gland cell). Scale = 0.5 µm.

masks or destroys the antigenicity of the SC.

The rectal dilation and its associated gland cells have been described by Bird (1979), who noted structural similarities between its contents and that of the gelatinous matrix produced by the rectal glands of the

adult female. We found that, in addition to the SC and the rectal gland of the J2, MISC also bound to the rectal glands of adult females and to the gelatinous egg mass they produce—a result we interpret as the occurrence of common epitopes. The significance of this commonality has yet to be determined.

Few studies have dealt with the structure, composition, and functions of the gelatinous matrix (Orion, 1995; Orion and Franck, 1990; Sharon and Spiegel, 1993). Maggenti and Allen (1960) first noted that the matrix produced by the six large rectal glands is secreted through the anus, via the rectum, to the exterior of the female where it forms a mass surrounding the vulva. The rectal gland cells contain a large number of Golgi and multivesicular bodies (Bird and Rogers, 1965). In our study, the Golgi and multivesicular bodies were labeled by MISC, evidence that these structures are the site of SC epitope synthesis. The means by which the synthesized materials are mobilized and transported to the rectal dilation are not known. Bird (1979) observed desmosomes in the rectal gland of the J2 and hypothesized that they may be involved in pinocytotic transport of rectal gland glycoproteins. We also observed desmosomes but, because none were labeled with MISC, we conclude that the desmosomes must serve other functions.

Recently, Gravato-Nobre et al. (1999) showed that the SC of *M. incognita* is shed as the J2 penetrates and migrates through host tissue, leaving behind a trail of SC material that binds to cell walls of the host, and that may be transported in the phloem sap. Based on these findings, they propose a functional role for the SC in the host-parasite relationship. An understanding of the origin of the SC should help to guide future studies of this kind.

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