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Monoclonal Antibodies to Secretory Granules in Esophageal Glands of *Meloidogyne* Species[1]

R. S. Hussey[2]

Abstract: Monoclonal antibodies to secretory granules in the dorsal or subventral esophageal glands were generated by injecting BALB/c mice with immunogens from preparasitic second-stage juveniles (J2) of *Meloidogyne incognita.* Antibodies specific for secretory granules in the J2 subventral esophageal glands or the dorsal gland were identified by indirect immunofluorescence microscopy. Only antibodies that reacted with granules in the J2 dorsal gland reacted with the esophageal gland lobe of *M. incognita* adult females. The antibodies also reacted with secretory granules in both types of esophageal glands in *M. javanica* and *M. arenaria* J2 but not with granules in esophageal glands of *Heterodera glycines* J2.

Key words: esophageal gland, immunocytochemistry, immunofluorescence, *Meloidogyne incognita,* monoclonal antibody, root-knot nematode, secretion, secretory granule.

Infective second-stage juveniles (J2) of *Meloidogyne incognita* have three large and complex esophageal glands, one dorsal and two subventral, where membrane-bound secretory granules (dense-core vesicles) are formed. Materials sequestered in the secretory granules in juveniles presumably transform recipient root cells of susceptible plants into elaborate permanent feeding sites called giant cells (11). The nematode secretions that induce and maintain this unique host response have not been purified; consequently, their biologically active molecules have not been identified, nor is the role of the secretions in this specialized host–parasite relationship understood. Likewise, the origin of secretions involved in the host–parasite interaction, whether from the dorsal or the subventral esophageal glands or both, has not been resolved (11).

Although the morphology of the esophageal glands in J2 and adult females of *Meloidogyne* species has been studied (2–4,9,17), little data are available regarding the composition of the matrix of secretory granules. Recent ultrastructural cytochemical analyses for enzyme activity and nucleic acids performed on such granules from *M. incognita* showed positive activity for acid phosphatase in granules in the subventral glands of J2, whereas granules in the dorsal gland of adult females were positive for peroxidase and nucleic acid (16).

The most promising means of identifying and characterizing nematode secretions and their involvement in pathogenesis may come from monoclonal antibody research on materials sequestered in secretory granules. Epitope specificity of monoclonal antibodies provides a means of identifying and purifying secretions. Antibodies specific for secretory granules could be used as immunocytochemical probes to identify gland products secreted through the nematode stylet and potentially important in pathogenesis. Antibodies specific for stylet secretions of *Meloidogyne* species could then be used as probes to identify the subcellular site(s) in giant cells where secretions accumulate, thereby providing some insight into the nature of the molecular and cellular mechanisms by which secretions affect cell differentiation and growth. Immunoaffinity chromatography employing specific antibodies could aid in purification of secretions for further biochemical characterization and determination of biological activity. Monoclonal

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[2] Professor, Department of Plant Pathology, University of Georgia, Athens, GA 30602.

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antibodies have been raised to various tissues of *Heterodera glycines,* including the esophageal glands (1).

Subcellular granules similar in size and appearance to esophageal gland secretory granules have been isolated from homogenates of preparasitic J2 of *M. incognita* (14). The objective of this study was to generate monoclonal antibodies against secretory granules in the dorsal and subventral esophageal glands of preparasitic *M. incognita* J2. A preliminary report has been published (10).

Materials and Methods

Meloidogyne incognita (Kofoid & White) Chitwood, *M. arenaria* (Neal) Chitwood, and *M. javanica* (Treub) Chitwood were propagated on greenhouse-grown tomato, *Lycoperiscon esculentum* Mill. cv. Rutgers. Eggs were collected and hatched as described previously (14). Eggs of *H. glycines* Ichinohe, propagated on soybean, *Glycine max* (L.) Merr. cv. Wright, were collected as described previously (5) and hatched on a 25-μm-pore sieve in the same manner as *Meloidogyne* eggs but in 10 mM $ZnSO_4$. Second-stage juveniles were collected daily and stored in water at 15 C for up to 7 days. To obtain adult females of known age, 3-week-old tomato plants growing in 400-ml styrofoam cups were inoculated with 10,000 freshly hatched J2 of *M. incognita.* After 48 hours the roots were washed free of soil and the plants were transplanted into styrofoam cups containing sterilized soil. Adult females were collected at specific time intervals by washing the infected roots free of soil and carefully dissecting females from galls.

Immunogen preparation: Two different immunogen preparations from *M. incognita* were used for monoclonal antibody production. Subcellular granules isolated from J2 served as one type of immunogen (14). A second immunogen was prepared by suspending J2 (1:1 v/v) in 0.1% Triton X-100 in phosphate-buffered, pH 7.4, saline (PBS) (137 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8.1 mM Na_2HPO_4) (1). Nematodes were homogenized in a motorized glass homogenizer on ice until 90% of the nematodes were broken. The homogenate was centrifuged at 4,400 *g* for 10 minutes at 4 C and the supernatant fluid served as the immunogen. Protein concentration in extracts was determined by the method of Bradford (6).

Monoclonal antibody production: Monoclonal antibodies were produced at the University of Georgia hybridoma facility. BALB/c mice were injected intraperitoneally with granule protein (150 μg protein in 200 μl/injection) emulsified with an equal volume of Freund's complete adjuvant. Three subsequent injections with immunogen mixed with Freund's incomplete adjuvant were given at intervals of 90, 107, and 40 days. A final injection without adjuvant was given 20 days later. Mice immunized with the second immunogen were injected intraperitoneally with protein (60 μg protein in 200 μl/injection) emulsified with an equal volume of Freund's complete adjuvant. A second injection with Freund's incomplete adjuvant was made 14 days later. A third injection of the immunogen without adjuvant was given 37 days later both in the tail vein and intraperitoneally. Mice were killed 3 days after receiving the last injection and the spleens were removed aseptically for fusion.

Other procedures for monoclonal antibody production were according to the methods of Cordonnier et al. (8).

Screening: Hybridomas were screened by indirect immunofluorescence staining according to Atkinson et al. (1) and Okamoto and Thomson (12) with minor modifications. The J2 were fixed overnight at 4 C in 2% paraformaldehyde in PBS. Nematodes were washed once in M9 buffer (7) and three times in deionized water and transferred to aluminum foil dishes for drying in a thin layer. Dried nematodes were cut into sections (1), flooded with M9 buffer, and transferred to a 1.5-ml centrifuge tube. The buffer was removed after centrifugation and nematode sections were resuspended and incubated for 20 minutes in 1 ml proteinase K (2 mg/ml, Type XI, P-0390, Sigma Chemical Co., St. Louis,

MO). Following centrifugation, proteinase K was removed and nematodes were frozen for 20 minutes on dry ice. Nematode sections were thawed and treated for 1 minute with −20 C methanol followed by a 2-minute treatment with −20 C acetone. Nonspecific binding sites were blocked by gently agitating the sections for 1 hour on an orbital mixer in PBS with 10% goat serum (S-2007, Sigma Chemical Co.) and 1 mM phenylmethylsulfonyl fluoride.

Hybridoma medium was screened in Millititer 96-well filtration plates (Millipore Corp., Bedford, MA) (1). Each well contained 80–100 µl hybridoma medium and 10 µl nematode suspension adjusted to contain approximately 200 sections. After incubation overnight at room temperature in a humid chamber, sections were washed three times for 15 minutes each in PBS. Forty microliters fluorescein isothiocyanate (FITC)-IgG goat anti-mouse conjugate (F-9006, Sigma Chemical Co.) diluted 1:500 with Tris-saline containing 0.2% Triton X-100 and 3% bovine serum albumin (BSA) were added per well, and the plates were incubated for 2 hours in the dark at room temperature. Nematodes were washed three times in PBS followed by a final distilled water wash. Nematode sections in each well were transferred in 20 µl distilled water to wells on Multitest slides (Flow Laboratories, McLean, VA) coated with 1 µl of 1% polylysine per well. After the nematodes air dried, 1.8 µl of phenylenediamine (2 mg/100 ml buffer) in carbonate-glycerin buffer was added per well. A coverslip was placed on the slide and sections were viewed with the 40× oil immersion objective of an Olympus fluorescence microscope. Controls included either replacing hybridoma medium with 10 µl nonimmune mouse serum and 80 µl PBS with 1% BSA, 2% Triton X-100, and 0.1% sodium azide or only the PBS solution. Nonimmune mouse serum was obtained from BALB/c, C57, and C3H mouse strains, a pet store mouse, and commercial mouse serum (I-5381, Sigma Chemical Co.).

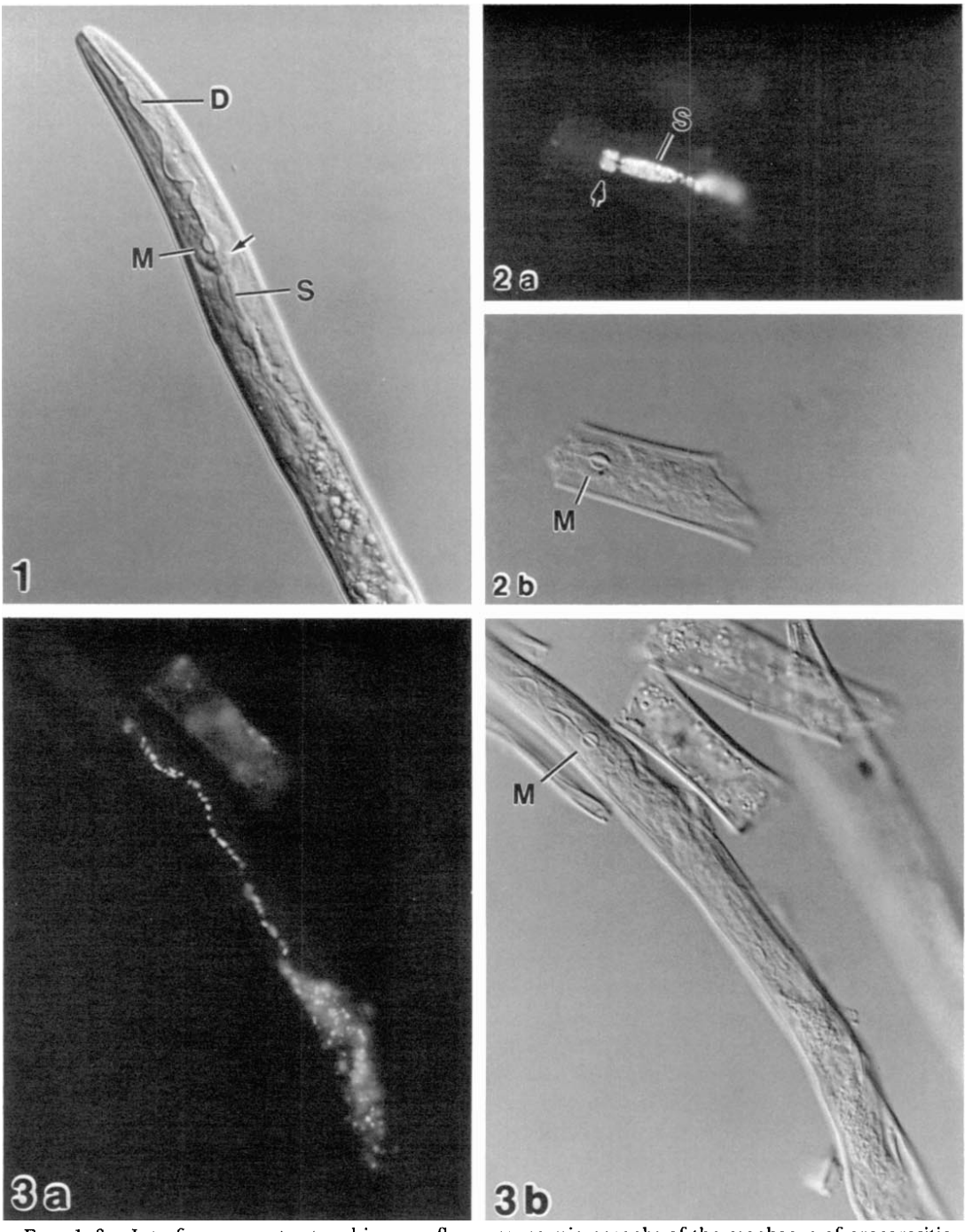
Positive cell lines were grown in 24-well plates, and the hybridoma medium was rescreened using the immunofluorescence procedure already described. Cell lines producing antibodies to secretory granules in the dorsal or subventral glands were cloned by limiting-dilution, preserved in liquid nitrogen, and grown to produce 1 liter of spent hybridoma medium. Antibodies from 1 liter of medium were concentrated 20-fold by precipitation for 15 minutes with 50% saturated ammonium sulfate and centrifugation at 15,300 *g*. The protein pellets were dissolved in buffer (1 mM sodium phosphate, 1 mM EDTA, pH 7.8) and stored at −20 C in 0.5 ml aliquants (13).

Adult females of *M. incognita* (25–33 days old) were fixed overnight at 4 C in 2% paraformaldehyde in PBS. The body of each female was cut into two pieces, and the section with the esophagus was washed in M9 buffer in 1.5-ml centrifuge tubes and centrifuged at 3,250 *g*. Sections were processed in centrifuge tubes for immunofluorescence staining with selected monoclonal antibodies following the procedure used for the juveniles.

Results

The cytoplasmic extension of the dorsal esophageal gland of preparasitic J2 terminates near the base of the stylet, whereas the extensions of the two subventral glands terminate immediately behind the pump chamber in the metacorpus (Fig. 1). The different locations of the ducts of the two types of glands permitted antibodies specific for granules in each gland type to be easily identified by immunofluorescence staining. In preparasitic juveniles, the subventral gland extensions usually contained many secretory granules, whereas fewer granules were present in the dorsal gland extension.

Two hybridoma clones secreting antibodies specific for secretory granules in the subventral esophageal glands of *M. incognita* J2 were identified from products of two separate fusions. In sections where the subventral gland extensions and the ampullae were distended with secretory granules, the immunofluorescence staining was



FIGS. 1–3. Interference contrast and immunofluorescence micrographs of the esophagus of preparasitic second-stage juveniles of *Meloidogyne incognita* (×270). 1) Interference contrast micrograph showing the dorsal gland duct (D), metacorpus (M), and subventral gland extensions (S) distended with secretory granules and ampullae of the subventral glands (arrow). 2, 3) Paired fluorescence (a) and interference contrast (b) micrographs of esophageal regions of nematode sections with indirect staining with FITC of a monoclonal antibody. 2) Fluorescent staining of secretory granules in the ampullae (arrow) within the metacorpus (M) and the two subventral gland extensions (S). 3) Fluorescent staining of individual secretory granules in a subventral gland and its cytoplasmic extension which terminates in the metacorpus (M).

intense (Fig. 2). In sections containing subventral glands with fewer secretory granules, individual granules were stained and easily observed (Fig. 3).

Antibodies reacting with secretory granules in the dorsal esophageal gland also were generated. These were identified by their binding with granules in the cytoplasmic extension of the dorsal gland anterior to the metacorpus in adult females and J2 (Figs. 4, 5). Secretory granules throughout the esophageal gland lobe in the adult female reacted with these antibodies (Fig. 4). In some preparations, the antibody that reacted with the dorsal gland granules appeared to bind weakly to granules in the subventral glands.

Nonimmune mouse sera from various sources gave different immunofluorescence staining patterns. While nonimmune sera obtained commercially or from a pet store mouse did not produce any specific staining, varied results were produced with nonimmune sera from inbred mice strains. Nonimmune sera from BALB/c mice reacted strongly with secretory granules in the subventral esophageal glands, whereas sera from the C57 and C3H strains did not. Similar results were obtained with nonimmune sera from three different sources of BALB/c mice. The fluorescence staining pattern from the BALB/c sera differed from that obtained with monoclonal antibodies hybridomas. Immunofluorescence staining of individual granules with the nonimmune sera gave a ring pattern, whereas the pattern from the antibodies was a solid sphere. Nonimmune BALB/c mouse sera produced similar staining patterns in J2 of the three *Meloidogyne* species but failed to react with *H. glycines.*

Three monoclonal antibodies, two specific for subventral gland granules and one for dorsal granules, were tested against J2 of *M. javanica, M. arenaria,* and *H. glycines,* and adult females of *M. incognita.* None of the antibodies reacted with secretory granules in the esophageal glands of J2 of *H. glycines* (Table 1). All of the antibodies reacted with granules in the glands of J2 of *M. javanica* and *M. arenaria*; however, only the antibody that reacted with secretory granules in the dorsal gland of J2 reacted with the esophageal gland lobe in adult females of *M. incognita.*

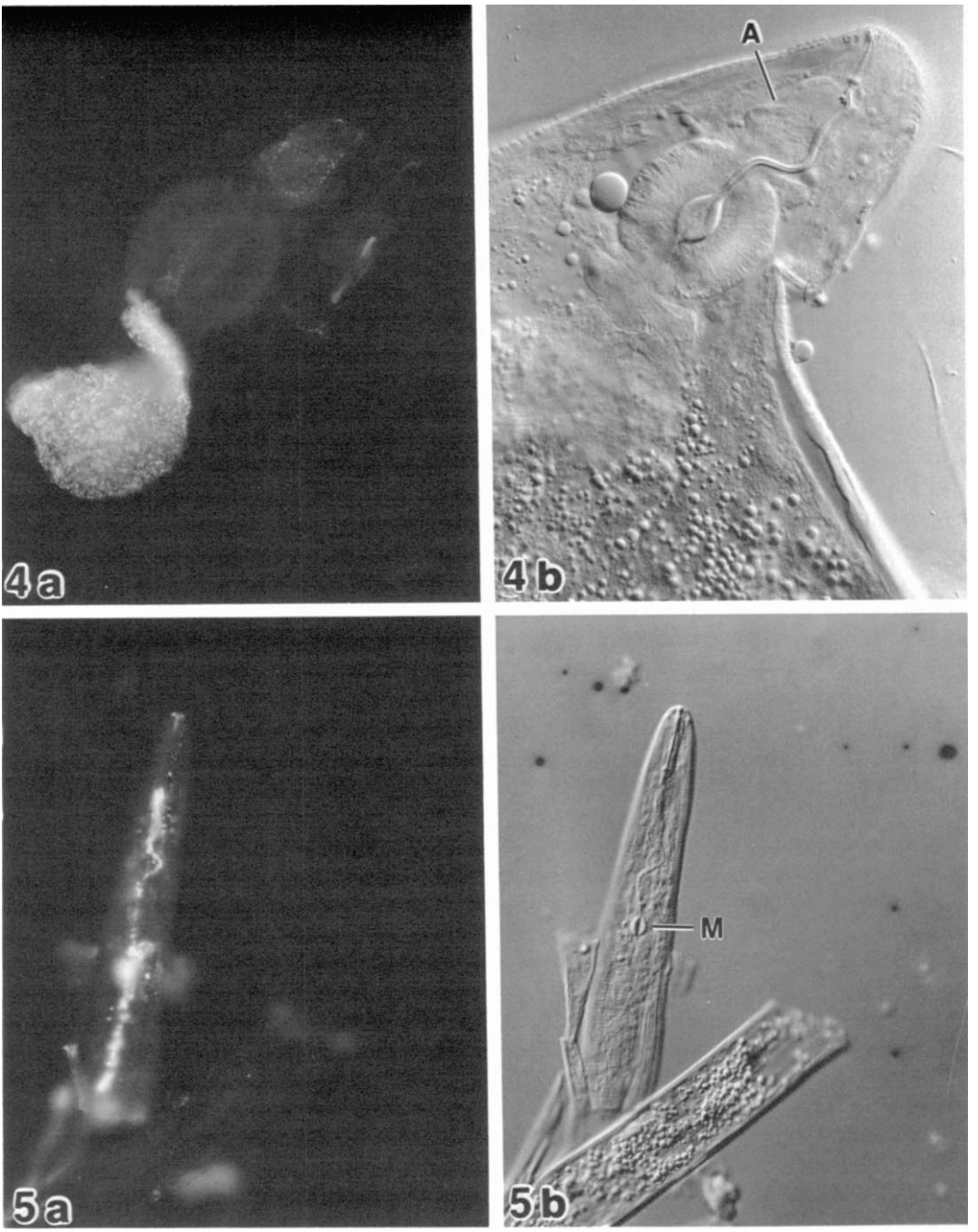
Discussion

Production of monoclonal antibodies to secretory granules formed in the esophageal glands will enable secretions of plant parasitic nematodes involved in pathogenesis to be identified and characterized. Antibodies specific for granules formed in the esophageal glands of *Meloidogyne* species were identified using an indirect immunofluorescence staining procedure developed by Atkinson et al. (1). The fluorescence staining pattern indicated the antibodies were specific for the matrix of the granules and not their limiting membranes. Distribution of the antigens within granules will need to be determined by electron microscopic immunocytochemistry using immunogold labelled antibodies (15).

Both preparations of immunogens were successful in generating antibodies to secretory granules. Nevertheless, preparation of total homogenates was more practical and less laborious than granule preparations and therefore would be the preferred manner for immunogen preparation. In this study, however, considerably fewer antibodies specific for the esophageal glands in *M. incognita* were produced than in a study with *H. glycines* where total homogenates were used as immunogens (1).

Reaction of nonimmune serum from BALB/c mice with secretory granules in the subventral glands of *M. incognita* was unexpected. Nonetheless, the apparent specificity of nonimmune sera for the limiting membrane of the granules allows these antibodies to be distinguished from those specific for the granule matrix during the screening procedure.

Results reported herein indicate that antigens synthesized in the dorsal esophageal gland of J2 are also produced later in the life cycle in adult females. Although the antibody that reacted with granules in the dorsal gland appeared to have some activity against granules in the subventral



FIGS. 4, 5. Paired fluorescence (a) and interference contrast (b) micrographs of esophageal regions of an adult female and a preparasitic second-stage juvenile of *Meloidogyne incognita* with indirect staining with FITC of a monoclonal antibody (×270). 4) Immunofluorescent staining of secretory granules in the dorsal gland lobe and ampulla (A) of an adult female nematode. 5) Immunofluorescent staining of granules in the dorsal gland extension anterior to the metacorpus (M) in a second-stage juvenile.

glands, the fluorescence staining was more intense in the dorsal gland. Immunolocalization of this antigen by electron microscopy will be necessary to firmly establish the distribution of the antigen in the esophageal glands. Failure of the antibodies specific for antigens in the J2 subventral glands to react with antigens in the esoph-

TABLE 1. Specificity of three monoclonal antibodies raised against secretory granules in the esophageal glands† of second-stage juveniles (J2) of *Meloidogyne incognita*.

Nematode and stage	Monoclonal antibody		
	$6D_4$/ DG	$3H_{11}$/ SvG	$5G_{11}$/ SvG
M. incognita J2	+	+	+
M. incognita adult	+	−	−
M. javanica J2	+	+	+
M. arenaria J2	+	+	+
Heterodera glycines J2	−	−	−

+ = immunostaining; − = no staining.
† DG = dorsal gland; SvG = subventral glands.

ageal gland lobe of adult females indicates that these glands were inactive in females or that the antigen was no longer being synthesized in this stage. Similar results were obtained with J2 and adult females of *H. glycines* (1). Reaction of the antibodies raised with *M. incognita* immunogens against antigens in *M. javanica* and *M. arenaria*, but not with granules in *H. glycines*, indicates that these antibodies have intergeneric specificity but lack interspecific specificity.

Identifying molecules of nematode origin that are involved in pathogenesis and characterizing their biological activity should provide the knowledge required to make novel advances in managing nematode diseases of plants. Monoclonal antibodies to nematode secretions provide a means to identify secretions involved in pathogenesis. Research is in progress using these antibodies to localize nematode secretions in giant cells to determine their role in the host–parasite interaction.

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