Novel Immunization Procedures Used to Develop Monoclonal Antibodies that Bind to Specific Structures in *Meloidogyne* spp.

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ABSTRACT

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Homogenates of the anterior regions of *Meloidogyne incognita* adult females injected into the spleen of Balb/c mice stimulated the production of nine monoclonal antibodies (MAbs) that bound to secretory granules formed in the nematode dorsal esophageal gland. Five of these nine MAbs were female-specific and bound to secretory granules in *M. incognita, M. javanica,* and *M. arenaria,* but not to those in *M. hapla* or *Heterodera glycines.* None of these nine MAbs recognized antigens in the dorsal gland of second-stage juveniles of the *Meloidogyne* spp. Two MAbs that bound to secretory granules within the subventral glands of females of *M. incognita, M. javanica,* and *M. arenaria,* but not to those in *M. hapla* or *H. glycines,* were obtained from an intrasplenic nitrocellulose implant carrying stylet secretions from *M. incognita* females. No MAbs to nematode esophageal glands were produced by a subcutaneous implant of nematode stylet secretions on nitrocellulose or through intraperitoneal injection using cyclophosphamide to favor the immune response to homogenates of the anterior region of *M. incognita* females. Antibodies to nematode nuclei, amphids, esophago-intestinal cells, muscle, and other structures also were produced. All antibody binding was confirmed by indirect immunofluorescence microscopy of nematode specimens. Intrasplenic immunizations required the least time, used small quantities of immunogen, and produced the most desirable MAbs.

Additional keywords: Caenorhabditis elegans, immunology, root-knot nematode, soybean cyst nematode.

Members of the genus *Meloidogyne* (Chitwood), the root-knot nematodes, are an agronomically important group of pathogens that establish a complex parasitic relationship with a wide range of host plants (24). Substances produced in the esophageal glands are secreted through the stylets of feeding root-knot nematodes, modifying several root protophloem cells into elaborate feeding sites called giant-cells (13,19). Nutrients from the giant-cells are required for the growth and pronounced morphological changes that occur during nematode development from a motile, vermiform juvenile to an enlarged, rounded, sedentary adult female. These changes include a reduction in size of the two subventral esophageal gland cells and an increase in size of the single dorsal esophageal gland cell as the nematode develops to an adult female (3). Secretory granules formed in the dorsal and subventral glands differ in size and apparent composition, and the number of granules increases dramatically in the dorsal gland and decreases in the subventral glands after giant-cell initiation and nematode development (16).

The composition and, hence, the specific physiological roles of *Meloidogyne* esophageal gland secretions are unknown. Numerous potential functions of these secretions (13), in particular the initiation and regulation of giant-cells and the formation of elaborate feeding tubes within the giant-cells (17), suggest that they contain several different components. Analysis of stylet exudate of *M. incognita* (Kofoid and White) Chitwood females by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis revealed nine major protein bands, three of which were glycoproteins (25). Development of monoclonal antibodies (MAbs) to secretory granules formed in the esophageal glands of *M. incognita* and the soybean cyst nematode, *Heterodera glycines* Ichinohe, represents significant progress toward the isolation and characterization of biologically important secretions (1,14). One MAb has been used to isolate a high molecular weight secretory glycoprotein present in the esophageal glands of *M. incognita* (18). These advances developed slowly, because the minute amounts of secretory material recovered from root-knot nematodes make direct analysis or development of specific MAbs an

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extremely difficult task.

Several novel immunization strategies have been developed to increase the production of desired antibodies. The use of the immunosuppressant drug, cyclophosphamide, has been used to favor antibody production to the esophageal region of *H. glycines* (1). Intrasplenic and subcutaneous immunizations allow the use of minute quantities of immunogen for development of specific antibodies (6,21). We have used these methods to create a panel of MAbs specific to secretory granules formed in the esophageal glands of *Meloidogyne*. Antibodies specific to other nematode structures also were generated in the process.

MATERIALS AND METHODS

M. incognita, M. javanica (Treub) Chitwood, *M. arenaria* (Neal) Chitwood, and *M. hapla* Chitwood were propagated on greenhouse-grown tomatoes (*Lycopersicon esculentum* Mill. 'Rutgers'). All adult *Meloidogyne* females used in this study were dissected from host roots 30–35 days after inoculation with eggs, except when large numbers of females were freeze-dried. The method described to recover large numbers of *Meloidogyne* females from host roots (11) was modified because Pectinol 59L (6-5890, Genecor, San Francisco, CA) was no longer available for purchase. Roots of okra (*Abelmoschus esculentus* (L.) Moench) were harvested 35 days after inoculation with *M. incognita* and cut into 1-cm pieces. Roots were incubated on a rotary platform shaker at room temperature in 4% pectinase (31660, Serva, Paramus, NJ) in water for 24 h. Digested roots were deposited on a 0.84-mm-opening sieve placed over a 0.25-mm-opening sieve, and the pectinase solution was recovered. Roots on the top sieve were sprayed with water, and adult female nematodes were retained on the bottom sieve. The remaining intact galls were further digested overnight in the recovered pectinase solution to yield more viable females. The majority of digested root debris was pelleted away from females by layering the slurry over a cushion of $MgSO_4 \cdot 7H_2O$ (250 g/L in water) in 250-ml glass centrifuge bottles and centrifuging at 1,000 *g*. Females were rinsed with water and placed in 0.9% NaCl. Viability was confirmed by observing nematode metacorpal activity with an Olympus inverted microscope (Olympus, New Hyde Park, NY). The remaining root debris was removed with forceps under a dissecting microscope, and females were transferred in distilled water to a 1.5-ml microcentrifuge tube for freeze-drying. Freeze-dried females were stored at −80 C.

Second-stage juveniles (J2) of *Meloidogyne* were hatched on sieves from eggs collected from host roots using 0.5% NaOCl (15), and J2 were collected every 24 h. Eggs of *H. glycines* were recovered from disrupted cysts, and J2 were hatched and collected similarly to the process for *Meloidogyne* J2. Young females of *H. glycines* were dissected from roots of greenhouse-grown soybean (*Glycine max* (L.) Merr. 'Lee') 15 days after inoculation with eggs. The cuticle of older cyst nematode females hindered fluorescence microscope observation of internal structures using the protocols described below. Mixed life stages of *Caenorhabditis elegans* were collected on Baermann funnels from cultures grown on agar plates at room temperature (5).

Immunizations. Immunogen originating from adult females of *M. incognita* was used in all immunization protocols described below. Freund's adjuvant mixed 1:1 with immunogen was used only for intraperitoneal (IP) immunizations. Complete Freund's adjuvant was used for primary immunization and incomplete Freund's adjuvant (F-5881 and F-5506, respectively, Sigma, St. Louis, MO) was used for subsequent IP injections. The inhalant general anesthetic isoflurane (NDC-10019-773-40, Anaquest, Madison, WI) was used for surgical intrasplenic and subcutaneous immunizations.

The anterior portions excised from *M. incognita* females that were fixed in 2% paraformaldehyde in phosphate-buffered saline (PBS) at 4 C for 7 days, were used in the first immunization protocol (IS2). Hundreds of nematode anteriors were homogenized in a grinding slide (22) in 10-μl portions (150 μl total) of PBS (137 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, and 8.1 mM Na_2HPO_4), pH 7.4, containing 0.1% Triton X-100. The homogenate was microcentrifuged at low speed (100 *g*) to remove nematode cuticles and coarse particulates. Using sterile technique, a small incision was made in the skin and peritoneum of an anesthetized mouse, and approximately 100 μl of nematode anterior homogenate was injected directly into the exposed mouse spleen. The peritoneum and skin of the mouse were sutured with 4.0 Chromic Gut on a tapered SH-1 needle (G-181H, Ethicon, Summerville, NJ). The mouse was allowed to recover from anesthesia and was active within several minutes. A second, similar intrasplenic immunization was made 11 days later. Three days after the second immunization, the mouse was euthanized and the spleen was removed to produce hybridomas by cell fusion as previously described (7). This same procedure was used for another immunization protocol (FDIS1) that used the homogenate of several hundred freeze-dried *M. incognita* female anteriors instead of fixed female anteriors.

Secretions obtained from the stylets of adult *M. incognita* females as previously reported (25) were used as immunogen in several immunization protocols. Viable females were incubated for 3 days at room temperature in cavity slides containing an antibiotic-saline solution composed of 0.9% NaCl, 1.5 mg/ml gentamycin sulfate (16051, U.S. Biochemicals, Cleveland, OH), 0.05 mg/ml nystatin (19770, U.S. Biochemicals), and 0.01% Triton X-100. Viscous stylet secretions formed on the lip region of the nematodes and were collected with a siliconized, fine glass needle held by a micromanipulator. Collected stylet secretions in antibiotic-saline were deposited on a 4-mm^2 piece of nitrocellulose. Nitrocellulose carrying stylet secretions from at least 300 *M. incognita* females was implanted (IM3) in a small incision made in the spleen of a mouse opened surgically as described above. A second intrasplenic implant of stylet secretions was made 11 days later, and the fusion was performed 3 days after the second implant.

Solubilized nematode stylet secretions were used in the next immunization protocol (ISDS1). Nitrocellulose carrying at least 300 stylet secretions was boiled for 5 min in 10 μl of PBS that contained 2% SDS. The nitrocellulose was then removed, and the solution of solubilized stylet secretions was brought to 150 μl with PBS for injection into a mouse spleen as described above. A second similar intrasplenic injection of solubilized stylet secretions was administered 11 days later, and the fusion was performed 3 days after the second immunization.

The stylet secretions remaining on SDS-treated nitrocellulose were used to initiate subcutaneous immunization of mice (SUB1). The side of the nitrocellulose that carried the stylet secretions was placed toward the body cavity under the skin of an anesthetized mouse. Nitrocellulose pieces carrying SDS-treated stylet secretions were implanted subcutaneously two times at 4-wk intervals and followed at 4-wk intervals by two more implants of stylet secretions on nitrocellulose that was not treated with SDS. Eleven days after the fourth subcutaneous implant, 50 μl of SDS-solubilized stylet secretions in PBS, prepared as described above, was injected into the tail vein of the mouse. The fusion was performed 3 days after this intravenous boost.

In the last immunization protocol (CYCLO1), hundreds of freeze-dried *M. incognita* females were dissected in half under a dissecting microscope. The posterior and anterior regions of the nematodes were homogenized in a grinding slide as described above as separate groups of material in 100 μl (total) of PBS-Triton X-100. Crude female posterior homogenate was administered to Balb/c mice by IP injection at 4-wk intervals followed by IP injection of cyclophosphamide (C-0768, Sigma; 50 μg/g of mouse) 3 days after each immunization (1). Mouse serum antibody titers were reduced 100× after three series of posterior homogenate/cyclophosphamide injections according to enzyme-linked immunosorbent assay (ELISA) using female posterior homogenate as plate-coating antigen. The *M. incognita* female anterior homogenate was microcentrifuged at low speed as described above and used for IP immunization of mice without drug treatment at 4 wk after the third posterior/drug injection. A second immunization with anterior homogenate minus cyclo-

phosphamide 11 days later significantly raised serum antibody titer tested against anterior homogenate. Mice were euthanized 3 days after the second immunization with anterior homogenate, and the fusion was performed.

Immunofluorescence. Screening of all hybridomas and selection of clones were performed by indirect immunofluorescence microscopy using *M. incognita* female anterior sections prepared similarly to methods described previously (1,14). Fresh females of *M. incognita* were fixed in 2% paraformaldehyde in PBS at 4 C for 7 days, rinsed three times in M9 buffer (22 mM KH_2PO_4, 42.3 mM Na_2HPO_4, 85.6 mM NaCl, and 1 mM $MgSO_4$) (5), and stored in M9 at −20 C. Anterior portions were dissected from hundreds of thawed, fixed *M. incognita* females, collected, and agitated for 1 h at 37 C in proteinase K (P-0390, Sigma) at 2 mg/ml of enzyme buffer (100 mM Tris, pH 7.4, 1 mM $CaCl_2$, and 0.1% Triton X-100). Permeabilized female anteriors were microcentrifuged at 3,000 *g*, the enzyme was removed, and the pellet was frozen on dry ice for 20 min. Thawed material was suspended in methanol on dry ice for 30 s, pelleted, suspended for 1.5 min in acetone on dry ice, and pelleted. The acetone was removed, the pellet was brought to near dryness, and the treated female anteriors were suspended in PBS containing 10% goat serum (S-2007, Sigma) to block nonspecific antibody-binding sites, 1 mM phenylmethylsulfonyl fluoride, and 0.02% NaN_3. Permeabilized *M. incognita* female anteriors in goat serum were stored at 4 C for at least 3 days, but no longer than 1 mo, before use in immunofluorescent assays.

Approximately 10 female anteriors prepared for immunofluorescence were placed in 0.5-ml microcentrifuge tubes for hybridoma screenings and MAb assays. Fifty microliters of individual hybridoma culture fluid was added per tube, and up to six hybridomas were tested per tube. Negative controls included no primary antibody, nonimmune Balb/c mouse serum, or a MAb specific to phytochrome (7). The positive control was $6D_4$, a MAb specific to secretory granules formed in root-knot nematode esophageal glands (14). The final volume in each tube was brought to 350 μl with diluent containing PBS, 1.0% bovine serum albumin (A-9647, Sigma), 0.05% Tween 20, and 0.02% NaN_3, and it was agitated overnight at room temperature. Female anteriors treated with primary antibody were pelleted in a microcentrifuge at 3,000 *g*, rinsed three times in PBST (PBS with 0.5% Triton X-100), and resuspended in fluorescein isothiocyanate (FITC) conjugates of both goat anti-mouse IgG and goat anti-mouse IgM (F-0257 and F-9259, respectively, Sigma), each diluted 1:500 in FITC diluent (10 mM Tris, pH 7.2, 149 mM NaCl, 3.0% bovine serum albumin, 0.2% Triton X-100, and 0.02% NaN_3). Specimens were agitated in FITC second antibody at room temperature in the dark for 3 h, rinsed twice in PBST, and rinsed once in distilled water. A dissecting microscope was used to transfer antibody-treated female anteriors to 2.5-μl drops of antiquenching agent (0.02 mg/ml of phenylenediamine in 0.5 M carbonate buffer, pH 8.6, mixed 1:1 with nonfluorescent glycerol) placed in wells on Multitest slides (60-408-05, ICN-Flow, Horsham, PA). A coverslip was placed on the slide, and specimens were viewed with the 40× oil immersion objective of an Olympus fluorescence microscope.

Positive hybridomas were retested to identify individual cell colonies producing antibodies that bound to specific structures in *M. incognita* females. Individual cell lines were cloned by limiting dilution and were grown in large volume to produce large quantities of desired MAb. The immunoglobulin class of each selected antibody was determined using a mouse MAb isotyping kit (RPN.29, Amersham, Arlington Heights, IL). Selected MAbs were tested by immunofluorescence against female anteriors of *M. javanica, M. arenaria, M. hapla,* and *H. glycines,* which were prepared and assayed as described above. The proteinase K treatment of fixed *H. glycines* female anteriors, however, was reduced to 20 min. Juveniles of all the nematode species mentioned here and mixed life stages of *C. elegans* also were tested against the selected MAbs using the immunofluorescence procedure described previously (1,14). All results presented below were determined from at least two experiments.

RESULTS

Intrasplenic immunization using homogenate of fixed *M. incognita* female anterior regions (IS2) produced 412 hybridomas and the greatest number of MAbs that bound to secretory granules within the esophageal glands of *Meloidogyne* spp. (Table 1). Five MAbs produced bound specifically to secretory granules in the dorsal gland lobe, extension, and ampulla of females of *M. incognita* (Fig. 1A), *M. javanica,* and *M. arenaria,* but they did not bind to secretory granules in *M. hapla, H. glycines, C. elegans,* or J2 of any species. Another MAb bound to granules in the dorsal gland of females of the four *Meloidogyne* spp. tested but not to the dorsal gland of females of *H. glycines* or J2 of any of these species. This same MAb bound to the somatic muscles in J2 (Fig. 1B) of all species and in mixed life stages of *C. elegans.* Two MAbs in a rare immunoglobulin class, IgA, bound to granules in the dorsal gland, the excretory duct, and the cuticle of females of the four *Meloidogyne* spp. These IgAs bound only to the cuticle and lateral hypodermal chords of all vermiform nematodes. A final MAb produced in IS2 bound specifically to the two esophago-intestinal cells of females (Fig. 1C) and J2 (Fig.

TABLE 1. Binding specificity[a] of monoclonal antibodies (MAbs), produced by various immunization protocols, to secretory granules within the esophageal glands of *Meloidogyne* spp. and to other structures in *Meloidogyne* spp., *Heterodera glycines*, and *Caenorhabditis elegans*

Immunization protocol[b]	MAb code	Immunoglobulin class	Monoclonal antibody specificity[c]	
			J2	Female
IS2	$1E_{12}$, $1D_9$, $6F_{11}$	IgG	NB	DG
	$4B_6$, $3H_6$	IgM	NB	DG
	$8D_{12}$	IgM	Muscle (#,●)	DG (#)
	$8B_5$, $12C_{10}$	IgA	Cuticle, hypodermal chords (#,●)	DG, extretory duct (#), cuticle
IM3	$7A_9$	IgM	Muscle (7)	SvG
	$3F_4$	IgM	SvG	SvG, amphids, esophageal lumen
ISDS1	$14C_{11}$	IgM	NB	Dorsal gland ampulla
FDIS1	$12H_7$	IgG	Hypodermal chords (#,●)	DG (#)
Control	$6D_4$	IgM	DG, SvG	DG
	$3H_{11}$	IgG	SvG	NB

[a] Determined by indirect immunofluorescence microscopy of nematode specimens using fluorescein isothiocyanate-labeled second antibody.

[b] IS2 = Intrasplenic injections of homogenate of fixed *M. incognita* female anterior regions. IM3 = Intrasplenic implants of *M. incognita* female stylet secretions carried on nitrocellulose. ISDS1 = Intrasplenic injections of sodium dodecyl sulfate-solubilized *M. incognita* female stylet secretions. FDIS1 = Intrasplenic injection of homogenate of freeze-dried *M. incognita* female anterior regions. Control = Control monoclonal antibodies produced previously (14) by intraperitoneal injection of homogenate of *M. incognita* second-stage juveniles (J2) but not previously tested against females of *M. arenaria, M. javanica,* and *H. glycines* or against any life stage of *M. hapla* or *C. elegans.*

[c] Binding was observed for *M. incognita, M. javanica,* and *M. arenaria.* Similar binding to *M. hapla* and *H. glycines* is indicated by # and ●, respectively; otherwise, no binding was observed for those species. Binding to mixed life stages of *C. elegans* was similar to that for J2 of *H. glycines,* except that no binding of $7A_9$ to *C. elegans* was observed and $14C_{11}$ bound to somatic muscles of *C. elegans.* DG = Binding to dorsal gland lobe, extension, and ampulla. SvG = Binding to subventral gland lobes, extensions, and ampullae. NB = No binding observed.

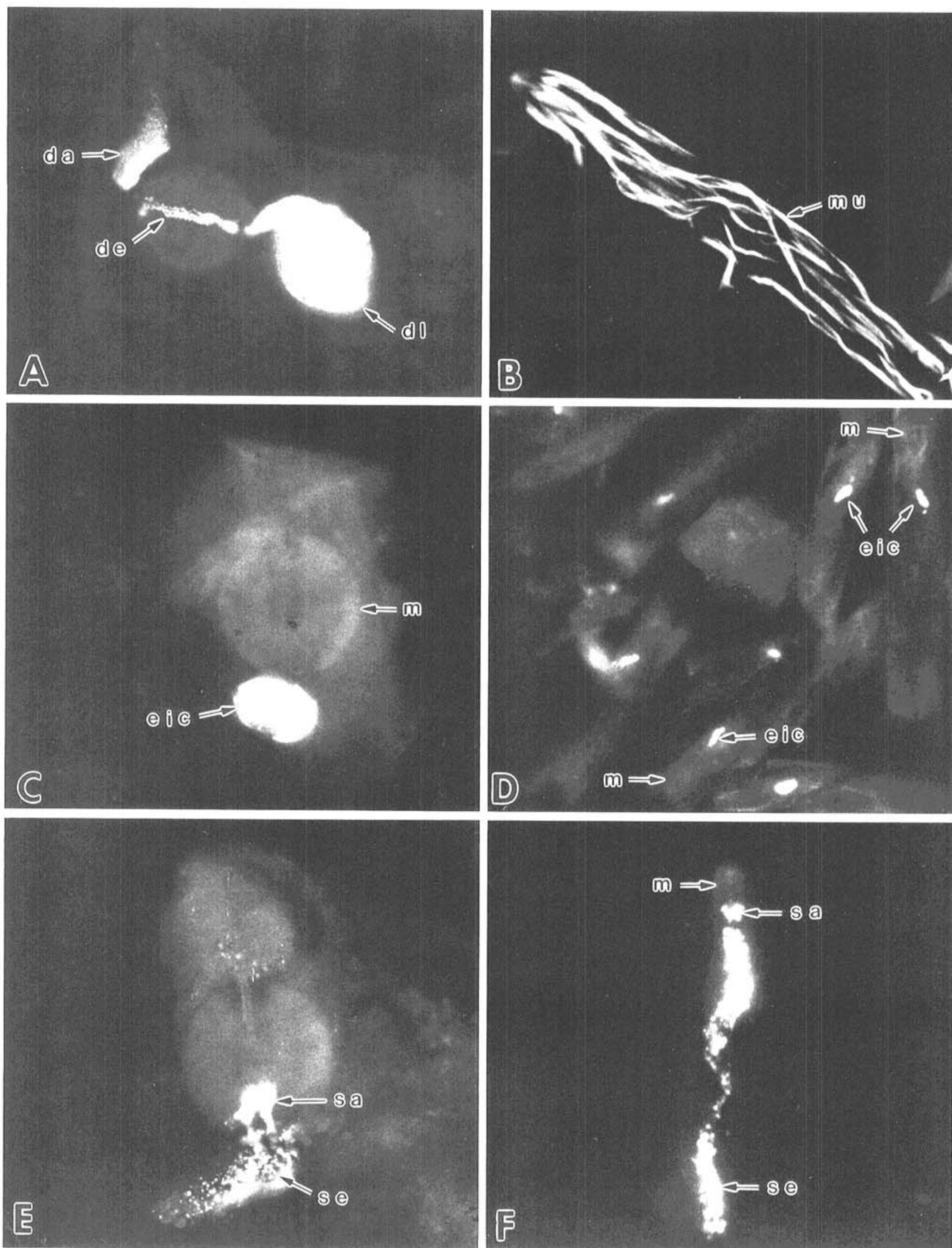

Fig. 1. Micrographs of indirect immunofluorescent staining of specific structures in nematode sections labeled with selected monoclonal antibodies (MAbs) and fluorescein isothiocyanate-conjugated second antibody. **A,** Binding of $4B_6$ MAb to secretory granules within the dorsal esophageal gland lobe, extension, and ampulla in a *Meloidogyne incognita* female. **B,** $8D_{12}$ MAb bound to the somatic muscles in a *Heterodera glycines* second-stage juvenile (J2). **C,** Binding of $7E_1$ MAb to the two esophago-intestinal cells located immediately posterior to the metacorpus in a *M. incognita* female. **D,** $7E_1$ MAb bound to the esophago-intestinal cells located posterior to the metacorpus in several *M. incognita* J2. **E,** Binding of $7A_9$ MAb to secretory granules within the subventral gland extensions and ampullae in a *M. incognita* female. **F,** $3F_4$ MAb bound to secretory granules within the subventral gland extensions and the ampullae in the metacorpus of a *M. incognita* J2. da, Dorsal ampulla; de, dorsal extension; dl, dorsal lobe; eic, esophago-intestinal cell; m, metacorpus; mu, somatic muscle; sa, subventral ampulla; se, subventral extension. (A–E, ×570, F, ×950.)

1D) of all *Meloidogyne* spp., but it did not label these cells in any life stage of *H. glycines* or *C. elegans* (Table 2).

Two MAbs that bound to secretory granules within the subventral esophageal glands of *Meloidogyne* spp. were produced by intrasplenic immunization with *M. incognita* female stylet secretions carried on nitrocellulose (IM3). Only 114 hybridomas were produced by this fusion. One of the positive MAbs bound only to the subventral glands of females of *M. incognita* (Fig. 1E), *M. javanica,* and *M. arenaria,* but not to those of *M. hapla* or *H. glycines.* This MAb bound to the somatic muscles of all vermiform nematodes except *C. elegans* and J2 of *M. hapla.* The second MAb produced bound to the subventral glands, esophageal lumen, and amphids of females of all *Meloidogyne* spp., except *M. hapla,* and to only the subventral glands of J2 (Fig. 1F) of the same *Meloidogyne* spp.

Stylet secretions from *M. incognita* solubilized with SDS and used as immunogen (ISDS1) produced 485 hybridomas and one MAb that bound weakly to secretory granules within the dorsal gland ampulla of females of *M. incognita, M. javanica,* and *M. arenaria* and to the somatic muscles of *C. elegans* mixed life stages. A second MAb bound to muscles within nematodes of all species and life stages tested here.

Homogenate of freeze-dried *M. incognita* female anterior regions used for intrasplenic injection (FDIS1) produced the greatest number (998) of hybridomas in one fusion and yielded two desirable MAbs. One of the MAbs bound to the amphids of females (Fig. 2A) of only the four *Meloidogyne* spp. and to only the lateral hypodermal chords (Fig. 2B) of all vermiform nematodes. The other MAb bound to secretory granules within the dorsal gland of females of only the four *Meloidogyne* spp. and to only the lateral hypodermal chords in all vermiform nematodes.

No MAbs to nematode esophageal glands were identified from 392 hybridomas produced through subcutaneous immunization with *M. incognita* female stylet secretions carried on nitrocellulose (SUB1). One MAb was produced that bound to the excretory canal system within females of *Meloidogyne* spp. (Fig. 2C) but labeled no other species or life stage. A second MAb bound to the metacorpal pump chamber, esophageal lumen, and stylet knobs of females of the four *Meloidogyne* spp. and of *H. glycines* and to only the somatic muscles of all vermiform nematodes.

No MAbs to nematode esophageal glands were obtained from immunizations that used cyclophosphamide to suppress antibody production to the posterior region of *M. incognita* females (CYCLO1), although 825 hybridomas were produced by this fusion. One MAb produced bound to the cephalic, metacorpal, and ovarian muscles in females of all plant parasitic nematodes tested, and it bound to somatic muscles in all vermiform nematodes. A second MAb was obtained that bound specifically to the excretory duct of females of all the *Meloidogyne* spp. tested, but it did not label *H. glycines, C. elegans,* or J2 of any *Meloidogyne* spp. Nuclei throughout the body of all nematodes were labeled by a third MAb (Fig. 2D) produced using cyclophosphamide and, in addition, this antibody bound to the somatic muscles of all vermiform nematodes and muscles in *H. glycines* females.

A MAb developed previously (14), $6D_4$, bound to secretory granules within the dorsal gland of females of *M. javanica* and *M. arenaria,* but it did not label females or J2 of *M. hapla,* females of *H. glycines,* or any life stage of *C. elegans.* The MAb $3H_{11}$, also developed previously (14), did not bind to the subventral glands in J2 of *M. hapla,* females of *Meloidogyne* spp. or *H. glycines,* or any life stage of *C. elegans.*

DISCUSSION

Compared with previous efforts (14), the immunization procedures described here increased the frequency of obtaining MAbs specific to secretory granules within the esophageal glands of *Meloidogyne* spp. However, thousands of hybridomas were screened to yield relatively few desirable MAbs. The low yield may be related to immunogen preparation, potentially poor antigenicity of esophageal gland contents, route of immunization, or a combination of these factors. Intrasplenic immunization using homogenate of the anterior regions of *M. incognita* females was the most effective method for production of MAbs specific to esophageal glands of *Meloidogyne* spp. Immunizations and screening centered around adult females of *Meloidogyne* spp. because one primary objective was to develop MAbs to secretory components that form feeding tubes in giant cells (17). Screening against the anterior region of *M. incognita* females using immunofluorescence microscopy was laborious, but it allowed the rapid identification of MAbs to specific nematode structures and life stages. The occurrence of stage-specific antibodies emphasizes the importance of screening against the original source of immunogen. Early attempts at screening by ELISA proved inefficient and inconclusive. Esophageal gland contents were a minute and inconsistent percentage of nematode homogenate used as ELISA plate-coating antigen, and potential positives were difficult to recognize and still required confirmation by immunofluorescence.

ELISA was useful for monitoring reduction in blood serum titer during cyclophosphamide suppression of mouse immune response to homogenate of *M. incognita* female posterior regions. Suppression of the immune response to the posterior region appeared to last only as long as cyclophosphamide was administered. Most resultant antibodies bound to structures present in both the *M. incognita* female posterior and anterior immunogens, especially to cuticle and muscle. The MAb that labeled nuclei throughout the nematode body and also muscle within vermiform nematodes, $1H_5$, may bind to a cytoskeletal or struc-

TABLE 2. Binding specificity[a] of monoclonal antibodies (MAbs), produced by various immunization protocols, to different structures within second-stage juveniles (J2) and adult females of *Meloidogyne* spp. and *Heterodera glycines*, and within mixed life stages of *Caenorhabditis elegans*

Immunization protocol[b]	MAb code	Immunoglobulin class	Monoclonal antibody specificity[c]	
			J2	Female
IS2	$7E_1$	IgM	Esophago-intestinal cells	Esophago-intestinal cells
ISDS1	$2G_9$	IgM	Muscle (#)	Muscle (#)
FDIS1	$11D_{11}$	IgG	Hypodermal chords (#)	Amphids
SUB1	$11D_4$	IgM	NB	Excretory canals
	$11F_3$	IgM	Muscle (#)	Metacorpal pump chamber (#), esophagal lumen, stylet knobs
CYCLO1	$11H_1$	IgM	Muscle (#)	Muscle (#)
	$3B_3$	IgM	NB	Excretory duct
	$1H_5$	IgM	Nuclei, muscle (#)	Nuclei (#)

[a] Determined by indirect immunofluorescence microscopy of nematode specimens using fluorescein isothiocyanate-labeled second antibody.
[b] IS2 = Intrasplenic injections of homogenate of fixed *M. incognita* female anterior regions. ISDS1 = Intrasplenic injections of sodium dodecyl sulfate-solubilized *M. incognita* female stylet secretions. FDIS1 = Intrasplenic injection of homogenate of freeze-dried *M. incognita* female anterior regions. SUB1 = Subcutaneous implants of *M. incognita* female stylet secretions carried on nitrocellulose. CYCLO1 = Intraperitoneal injections of homogenate of *M. incognita* female anterior regions after immunosuppression to *M. incognita* posterior immunogen with cyclophosphamide.
[c] Binding was observed for *M. incognita, M. javanica, M. arenaria,* and *M. hapla.* Similar binding to *H. glycines* is indicated by (#); otherwise, no binding was observed. Binding to mixed life stages of *C. elegans* was similar to that for J2 of *H. glycines.* NB = No binding observed.

tural antigen such as actin (26). Similar types of antigens may be the targets of two MAbs, $8D_{12}$ and $7A_9$, that bound to muscle in vermiform nematodes and to granules within the dorsal and subventral glands, respectively, in *Meloidogyne* spp. females.

The short immunization period, the minute amount of immunogen required, and the relative efficiency of desired MAb production suggest that intrasplenic immunization is the method of choice for developing antibodies to scarce or weakly immunogenic antigens. This is illustrated by the production of a MAb, $7E_1$, specific to an antigen within the two esophago-intestinal cells. Intrasplenic injection of mixed antigens in homogenate of nematode anteriors stimulated a greater immune response and more MAbs to esophageal glands than did immunization with stylet secretions carried on nitrocellulose or stylet secretions solubilized with SDS. The five MAbs specific to dorsal gland secretory granules within females only of *M. incognita, M. javanica,* and *M. arenaria* may bind to antigens important in the maintenance, but not the initiation, of giant-cells or to feeding tube components. The lack of binding of most MAbs to esophageal glands in *M. hapla* may be further evidence of a more distant phylogenetic and parasitic relationship of *M. hapla* to the three other major *Meloidogyne* spp. (2,12). The two IgAs did bind to the dorsal gland and to other structures of all four *Meloidogyne* spp. and may represent binding to a common epitope such as a carbohydrate moiety.

The poor antigenicity and the relatively few desirable MAbs produced using a more purified preparation, stylet secretions from *M. incognita* females, was disappointing. The stylet secretions carried on nitrocellulose were extremely viscous and insoluble in aqueous solution and were poorly solubilized from the nitrocellulose carrier when boiled in SDS. This may have resulted in poor antigen presentation and, hence, poor antibody response in all immunizations that involved nematode stylet secretions. Solubilization of stylet secretions with other chemical reagents or enzymes should be considered in future studies. The production of MAbs specific to subventral glands of *Meloidogyne* spp. using stylet secretions as an immunogen was unexpected. The valve of the subventral glands is located immediately posterior to the metacorpal pump chamber, which makes it physically improbable that secretions from these glands move anteriorly through the esophagus (13). Also, the reduced size of the subventral glands in adult females of *Meloidogyne* spp. (3) suggests that contents

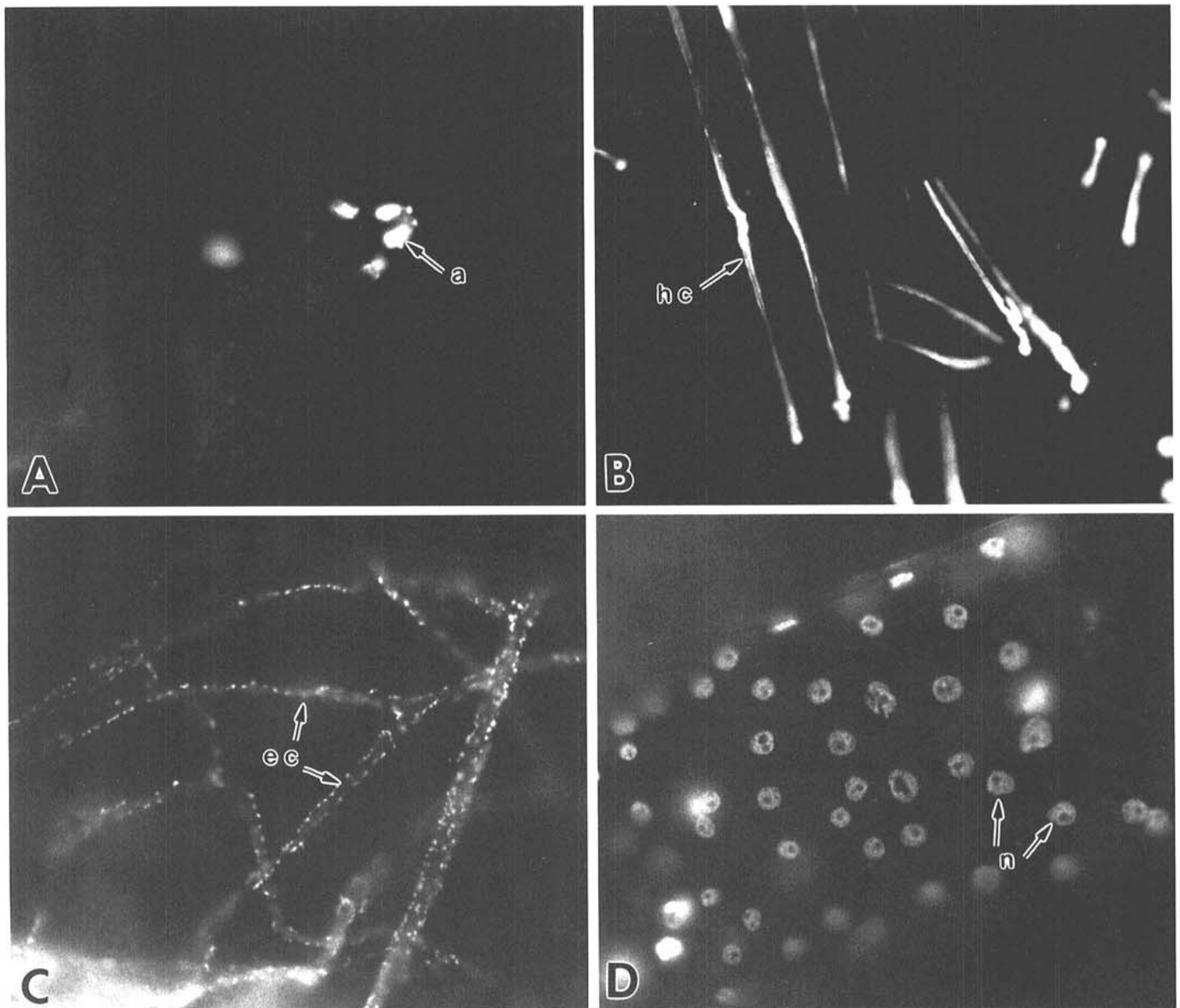

Fig. 2. Micrographs of indirect immunofluorescent staining of specific structures in sections of *Meloidogyne incognita* labeled with selected monoclonal antibodies (MAbs) and fluorescein isothiocyanate-conjugated second antibody (×600). **A,** Binding of $11D_{11}$ MAb to the two amphids in the cephalic region of an adult female. **B,** $11D_{11}$ MAb bound to the lateral hypodermal chords in second-stage juveniles. **C,** Binding of $11D_4$ MAb to an excretory canal system associated with the body wall posterior to the esophagus of an adult female. **D,** $1H_5$ MAb bound to nuclei within the hypodermal portion of the body wall. a, Amphids; hc, hypodermal chords; ec, excretory canals; n, nuclei.

from these glands are not important for giant-cell maintenance. One MAb produced using stylet secretions as immunogen, $3F_4$, bound to the subventral glands of *Meloidogyne* spp. females and J2 and to the amphids of *Meloidogyne* spp. females. It is possible that stylet secretions collected from the oral aperature also contained secretions from the amphids, which have openings in close proximity to the stomatal orifice (25). Secretions from the amphids have been reported to contain protein and carbohydrate (8,20,23).

MAbs to antigens in the amphids, and MAbs specific to other structures within nematodes, will be valuable in studies concerning the localization, development, and function of structures in relation to nematode biology. The $11D_{11}$ MAb recognizes an antigen deep within the amphidial pouches of *Meloidogyne* females that may function in nematode chemoreception. Localization of the esophago-intestinal cells by the $7E_1$ MAb demonstrates precisely the growth and anterior movement of these cells to the base of the metacorpus as *Meloidogyne* spp. develop from J2 to adult female. Previously, the esophago-intestinal cells in an adult female were thought to be related to the esophageal glands (4). Our results indicate that the esophago-intestinal cells in an adult female develop from the esophago-intestinal valve cells in the J2 and probably function during food ingestion similarly to the role of these cells in J2 (9). The extensive excretory canal system that develops in *Meloidogyne* spp. females is difficult to observe with light microscopy, but it has been described previously (10), and a possible connection between this system and the excretory duct has been proposed. The MAbs $11D_4$ and $3B_3$ clearly enhance the localization of the excretory canal system and the excretory duct, respectively, and may help elucidate their function and life stage-specific development.

Investigations are presently being conducted to characterize the esophageal gland antigens that are recognized by the different MAbs presented here. These studies will increase our understanding of the basic biology of root-knot nematodes and their adaptations for plant parasitism and facilitate the development of more target-specific means to reduce nematode-related plant damage.

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