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Monoclonal Antibodies to the Esophageal Glands and Stylet Secretions of *Heterodera glycines*

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Abstract: Three monoclonal antibodies (MAbs) that bound to secretory granules within the subventral esophageal glands of second-stage juveniles (J2) of the soybean cyst nematode (SCN), *Heterodera glycines,* were developed from intrasplenic immunizations of a mouse with homogenates of SCN J2. Two MAbs to the secretory granules within subventral glands and one MAb to granules within the dorsal esophageal gland of SCN J2 were developed by intrasplenic immunizations with J2 stylet secretions. Stylet secretions, produced in vitro by incubating SCN J2 in 5-methoxy DMT oxalate, were solubilized with a high pH buffer and concentrated for use as antigen. Three of the five MAbs specific to the subventral esophageal glands bound to stylet secretions from SCN J2 in immunofluorescence and ELISA assays. Two of these three MAbs also bound to secretory granules within both the dorsal and subventral esophageal glands of young SCN females. All five of the subventral gland MAbs bound to the subventral glands of *Heterodera schachtii* and one bound to the subventral glands of *Globodera tabacum,* but none bound to any structures in *Meloidogyne incognita* or *Caenorhabditis elegans.*

Key words: Caenorhabditis elegans, esophageal gland, *Heterodera glycines, Heterodera schachtii,* immunoassay, *Meloidogyne incognita,* monoclonal antibody, nematode, plant parasite, soybean, stylet secretions.

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, modifies selected plant root cells into a syncytium that provides nourishment essential for its growth and reproduction. Second-stage juveniles (J2) of SCN penetrate soybean, *Glycine max* (L.) Merr., roots, migrate intracellularly, and establish an initial syncytial cell in the pericycle opposite protophloem or near protoxylem poles (9). A multinucleate syncytium is formed from wall dissolution between cells in contact with the initial syncytial cell (9,16). Syncytia become hypertrophied and contain enlarged nuclei, dense cytoplasm, increased reticulum-like material, vacuoles reduced in size, and a thickened peripheral wall with numerous ingrowths surrounded by the plasma membrane.

The biochemical events involved in syncytium formation are not well understood. Stylet secretions released from secretory granules formed within the esophageal glands of plant-parasitic nematodes control the induction and maintenance of feeding sites by these nematodes (12). Secretory granules formed within the dorsal esophageal gland cell of preparasitic J2 of SCN are small and electron dense, but the granules enlarge and become less electron-dense following the onset of parasitism (8,10). On the other hand, secretory granules within the two subventral esophageal gland cells of preparasitic J2 are large with irregular central cores, but they become smaller and more electron dense during the initial stages of parasitism. Secretions from granules within the dorsal

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gland ampulla of *H. schachtii* are released during syncytium and feeding tube formation (23,24).

Monoclonal antibodies (MAbs) that bind specifically to esophageal gland antigens have been developed for the isolation and characterization of nematode secretions. Atkinson et al. (1) used homogenates of different life stages of SCN to develop MAbs that bind to esophageal gland secretory granules. These MAbs were used to monitor the expression of esophageal gland antigens during SCN parasitism of soybean (2). Monoclonal antibodies that bind to esophageal gland granules and stylet secretions of *Meloidogyne incognita* (Kofoid & White) Chitwood have been developed using intraperitoneal and intrasplenic immunizations of Balb/c mice (6,13). The MAbs to *M. incognita* have been used to isolate one secretory antigen and one gene encoding a secretory antigen, to monitor the developmental expression of secretory antigens, and to demonstrate that dorsal and subventral esophageal gland antigens can be secreted through the stylet (7,15,20). Techniques to induce in vitro production of stylet secretions from nematodes will facilitate the isolation of secretory molecules (3,18). In this study, J2 homogenates and stylet secretions produced in vitro by SCN J2 were used in intrasplenic immunizations (19) to generate MAbs that bound to antigens in J2 stylet secretions.

Materials and Methods

Nematodes: Heterodera glycines (OP50) was supplied by C. H. Opperman, North Carolina State University, and cultured on roots of greenhouse-grown soybean (*G. max* 'Pickett'). Eggs were collected weekly by gently crushing cysts in a ground-glass homogenizer and hatched on Baermann pans. Daily cohorts of hatched J2 were collected and utilized in assays described below. Young, white females of SCN were hand dissected from soybean roots 15 days after inoculation. *Heterodera schachtii* Schmidt was supplied by E. P. Caswell-Chen, University of California at Davis, and cultured on greenhouse-grown sugarbeet (*Beta vulgaris* 'Detroit Red'), and *Globodera tabacum* (Lownsbery & Lownsbery) Behrens was supplied by K. R. Barker, North Carolina State University, and cultured on greenhouse-grown tobacco (*Nicotiana tabacum* L. 'NC 95'). Second-stage juveniles of *H. schachtii* and *G. tabacum* were obtained as described for SCN. Eggs of *M. incognita* were collected from roots of greenhouse-grown tomato (*Lycopersicon esculentum* Mill. 'Rutgers') using 0.5% NaOCl (14) and J2 were hatched on Baermann pans. Mixed life stages of *Caenorhabditis elegans* were collected from agar petri dish cultures (5).

Immunizations: Two Balb/c mice received separate intrasplenic immunizations using immunogen from SCN J2 to generate MAbs as described by Davis et al. (6). Immunogen for the first mouse (M-1) consisted of homogenates of SCN J2 that were stored in 10 mM Tris-NaOH, pH 11, at −80 C. Approximately 1.5 ml of packed J2 were ground in liquid nitrogen to a fine powder with a mortar and pestle. The fine powder was transferred to a ground-glass homogenizer and ground on ice in Tris-NaOH, pH 11, in a final volume of 5 ml. The J2 homogenate was centrifuged for 4 minutes at 1,000*g* to remove nematode cuticles, and the supernatant was stored at −80 C in 100-µl aliquots for subsequent use as immunogen. Two intrasplenic injections of SCN J2 homogenate (100 µl per injection) were administered 11 days apart in M-1, and the spleen was removed for cell fusion 3 days after the second injection. The fusion of spleen lymphocytes and myeloma cells (18), and the culture of resulting hybridomas (11), were conducted by the University of Georgia Monoclonal Antibody Facility. All hybridomas producing desirable antibodies were cloned by limiting dilution for the production of monoclonal antibodies.

Concentrated, solubilized stylet secretions produced in vitro by SCN J2 were utilized as immunogen for the immunization of a second mouse (M-2). Stylet secretions were induced by placing a 24-hour cohort of thousands of SCN J2 in 3 ml of a

solution of 0.2 mg/ml 5-methoxy DMT oxalate (Research Biochemicals Inc., Natick, MA) in distilled water in a polystyrene dish (32 mm × 75 mm) in a moist chamber for 4 hours at room temperature. Accumulation of proteinaceous stylet secretions was monitored by placing an aliquot of J2 in DMT solution containing 0.005% Coomassie brilliant blue G in cavity slides. The viscous stylet secretions were solubilized by adding an equal volume of 20 mM Tris-NaOH, pH 11, to the dish; after 2 minutes, the entire suspension was centrifuged at 10,000*g* at 4 C to pellet the J2. The supernatant fluid containing the solubilized stylet secretions was collected and concentrated to approximately 200 µl using 10,000 MW cutoff Centricons (Amicon, Beverly, MA) at 4 C. A protease inhibitor cocktail (10 µl/ml secretion) consisting of 0.5 mg/ml leupeptin, 1 mM EDTA, 0.7 mg/ml pepstatin A, 0.2 mM phenylmethyl-sulfonyl fluoride (PMSF), and 0.2 mM iodoacetamide, plus 0.02% sodium azide, was added to concentrated stylet secretions for daily storage at 4 C in Centricons. Stylet secretions from SCN J2 were produced daily as described above and concentrated into the same sample for 15 consecutive days. Protease inhibitors, sodium azide, and DMT were removed from the final "15-day" sample of concentrated stylet secretions using 10,000 MW cutoff Ultrafree- MC microcentrifuge filter units (Millipore, Bedford, MA). The concentrated stylet secretions were brought to a final volume of 300 µl with 10 mM Tris-NaOH, pH 11, and stored in 100 µl aliquots at −80 C. Immunization consisted of two intrasplenic injections of 100 µl concentrated stylet secretions administered 11 days apart, and hybridoma production was conducted as described for M-1.

Immunofluorescence: Hybridomas were screened and immunolocalization of antibody-binding sites was confirmed by indirect immunofluorescence microscopy of nematode sections as described previously (1,13). Hatched SCN J2 were fixed overnight at 4 C in 2% paraformaldehyde in phosphate-buffered saline (PBS; 137 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 8.1 mM Na_2HPO_4, pH 7.4), rinsed, and stored at −20 C in M9 buffer (22 mM KH_2PO_4, 42.3 mM Na_2HPO_4, pH 7.0, 85.6 mM NaCl, 1 mM $MgSO_4$). Thawed, fixed J2 were cut into sections under a dissecting microscope, agitated in 2 mg/ml Proteinase K (Sigma, St. Louis, MO) in M9 buffer for 20 minutes at 37 C, the enzyme was removed following centrifugation, and the sections were frozen on dry ice to render them permeable. Permeable J2 sections were treated with methanol for 30 seconds, acetone for 1.5 minutes, and resuspended in 10% goat serum (Sigma) in PBS that contained 0.02% sodium azide and 1 mM PMSF. Nematode sections were stored in 10% goat serum at 4 C for at least 3 days to block nonspecific antibody-binding sites. Mixed life stages of *C. elegans* and J2 of *H. schachtii, G. tabacum,* and *M. incognita* were prepared similarly for immunofluorescence microscopy, except that the Proteinase K treatment of *H. schachtii* was conducted at room temperature. Young females of SCN were stored at 4 C in fixative for 5 days, rinsed in M9, and the anterior ends of females were excised and collected under a dissecting microscope. Fixed SCN female anteriors were made permeable and blocked as described above, substituting a different Proteinase K buffer (100 mM Tris-HCl, pH 7.4, 1 mM calcium chloride, and 0.1% Triton X-100).

Immunofluorescence screening of hybridomas was conducted using blocked, permeable SCN J2 sections and the Multiscreen Assay System (Millipore). Sections of SCN J2 were incubated overnight at room temperature in individual hybridoma supernatants in separate wells of 96-well microfiltration plates. Sections were rinsed three times with PBS and incubated in the dark for 3 hours at room temperature in fluorescein isothiocyanate (FITC)-conjugated second antibody. Second antibody was a mixture of FITC goat anti-mouse IgG and FITC goat anti-mouse IgM (Sigma), each diluted 1:500 in FITC diluent (10 mM Tris-HCl, pH 7.2, 149 mM NaCl, and 0.02% sodium azide). Sections were rinsed three times in PBS and received a final rinse in distilled water.

Treated sections of SCN J2 were transferred in a 10-µl drop of water to individual wells on Multitest slides (ICN-Flow, Horsham, PA) that were previously coated with 2 µl of poly-L-lysine at 1 mg/ml (Sigma). Sections were air-dried, covered with a 2.5 µl drop of antiquenching agent (0.02 mg/ml phenylenediamine in 500 mM carbonate buffer, pH 8.6, mixed 1:1 with nonfluorescent glycerol), and a coverslip was applied. Sections of J2 of *H. schachtii, G. tabacum,* and *M. incognita,* mixed life stages of *C. elegans,* and anteriors of young SCN females were prepared similarly for immunofluorescence microscopy, except that all incubations and rinses were performed in 0.5-ml microcentrifuge tubes. Anteriors of SCN females were transferred directly to drops of antiquenching agent on Multitest slides and a coverslip applied. All specimens were viewed with the 40× oil immersion objective of an Olympus fluorescence microscope.

Preparation of stylet secretions from SCN J2 for immunofluorescence microscopy was conducted as previously described for *M. incognita* (7). Viscous stylet secretions from SCN J2 that had been incubated in DMT and Coomassie brilliant blue were collected under a dissecting microscope with a fine glass needle held by a micromanipulator. Stylet secretions were deposited in a designated area on the surface of a 12,000–14,000 MW cutoff dialysis membrane (Spectrum, Los Angeles, CA) and allowed to adhere to the membrane. All incubations and rinses of stylet secretions were performed by transferring the membrane-carrying stylet secretions to 1.5-ml microcentrifuge tubes that contained appropriate solutions. Secretions were rinsed in PBS for 1 hour and incubated overnight at room temperature in hybridoma supernatant that was diluted 1:10 in PBS. Secretions were rinsed three times in PBS and incubated in the dark for 3 hours in FITC-conjugated second antibody as described above. Secretions then were rinsed two times in PBS and finally in distilled water. The membrane carrying secretions was placed on a microscope slide, covered with a coverslip, and observed by fluorescence microscopy as described above.

Enzyme-linked immunosorbent assay (ELISA): Monoclonal antibodies that bound to the esophageal glands of SCN J2 were tested by ELISA for their ability to bind to solubilized stylet secretions of J2. Concentrated, solubilized stylet secretions from SCN J2 were produced and collected as described above. Wells of Immulon 2 microtiter plates (Dynatech Laboratories) were coated overnight at room temperature with 20 µl of stylet secretions diluted to 100 µl with borate buffer saline (100 mM boric acid, 24.9 mM $Na_2B_4O_7 \cdot 10H_2O$, 75.7 mM NaCl, pH 8.5). Wells were rinsed three times with PBS and a MAb diluted 1:10 with ELISA diluent (PBS plus 1.0% bovine serum albumin, 0.05% Tween 20, and 0.02% sodium azide) was added to each well. After 1 hour at 37 C MAbs were removed, wells were rinsed three times with PBS, and 100 µl of alkaline phosphatase-conjugated second antibody (a mixture of goat anti-mouse IgG and goat anti-mouse IgM, each diluted 1:1000 with ELISA diluent) was added to each well. Second antibody was removed after 1 hour incubation at 37 C, wells were rinsed twice with PBS, and a final rinse with alkaline phosphatase substrate buffer (0.2 mM $MgCl_2 \cdot 6H_2O$, 0.9 M diethanolamine, pH 9.8). One hundred microliters of p-nitrophenyl phosphate substrate (1 mg/ml in substrate buffer) was added to each well, and the plate was incubated for 30 minutes in the dark. Absorbance was measured at 405 nm on an automated microplate reader (Titertek). Negative controls consisted of nonimmune mouse serum and second antibody only. Polyclonal serum derived from the blood of the M-2 mouse served as the positive ELISA control. Selected MAbs that bound to SCN J2 were tested similarly by ELISA using solubilized stylet secretions from resorcinol-treated J2 of *M. incognita* as antigen (7).

Results

Three MAbs, $9C_2$, $6A_3$, and $1D_9$ (Table 1), with desired specificity were selected from 746 hybridomas produced from the

TABLE 1. Binding specificity of monoclonal antibodies to the esophageal glands[a] of second-stage juveniles (J2) and young females of *Heterodera glycines*, J2 of *Heterodera schachtii*, *Globodera tabacum*, and *Meloidogyne incognita*, mixed life stages of *Caenorhabditis elegans*, and to stylet secretions of J2 of *H. glycines*.

Monoclonal antibody	Antibody class	*H. glycines* J2	*H. glycines* female	Secretions[b] (FITC)	Secretions[b] (ELISA)	*H. schachtii*	*G. tabacum*	*M. incognita*	*C. elegans*
$9C_2$	IgM	SvG	Svg, DG	+ + +	+ + +	SvG	ND	ND	ND
$6A_3$	IgM	SvG	SvG, DG	+ + +	+ + +	SvG	ND	ND	ND
$1D_9$	$IgG2_b$	SvG	ND	I	ND	SvG	ND	ND	ND
$3H_5$	$IgG2_a$	SvG	ND	+	+ + +	SvG	ND	ND	ND
$9H_{12}$	IgM	SvG	ND	I	ND	SvG	SvG	ND	ND
$5B_9$	IgG_3	DG	ND	I	ND	ND	ND	ND	ND

[a] Antibody binding was specific to secretory granules within the dorsal (DG) or subventral esophageal glands (SvG), or was not detected (ND) by indirect immunofluorescence microscopy using fluorescein isothiocyanate (FITC)-conjugated anti-mouse second antibody.

[b] Strong (+ + +), weak (+), inconsistent (I), or no detection (ND) of antibody binding to stylet secretions collected, in vitro, from J2 of SCN that were incubated in 5-methoxy DMT oxalate. Antibody binding was confirmed by indirect immunofluorescence (FITC) microscopy of intact J2 secretions and enzyme-linked immunosorbent assay (ELISA) utilizing solubilized stylet secretions as antigen.

mouse (M-1) immunized with homogenates of SCN J2. Three MAbs, $3H_5$, $9H_{12}$, and $5B_9$, were selected from 873 hybridomas produced from the mouse (M-2) immunized with solubilized stylet secretions from SCN J2. Five of the six MAbs bound specifically to secretory granules within the subventral esophageal glands of SCN J2. Binding of $9C_2$ and $6A_3$ to the subventral glands was strong, and often antibody binding to the subventral gland valves was observed. Binding of $3H_5$ (Fig. 1A) and $9H_{12}$ to subventral gland secretory granules was moderately strong, with the binding of $3H_5$ revealing coarser granules than $9H_{12}$. The intensity of binding of $1D_9$ to the subventral glands (Fig. 1B) was less and appeared more granular than the other MAbs. The MAbs that bound to subventral glands of SCN J2 also bound to the subventral glands of J2 of *H. schachtii,* but only one, $9H_{12}$, bound to subventral glands of *G. tabacum* (Table 1). None of the subventral gland MAbs bound to any structure in J2 of *M. incognita* or mixed-life stages of *C. elegans.* The $5B_9$ MAb bound specifically to secretory granules in the dorsal esophageal gland of SCN J2 (Fig. 1C) but did not bind to young adult SCN females or the other nematode species. In young adult SCN females, MAbs $9C_2$ (Fig. 1D) and $6A_3$ bound to secretory granules within both the dorsal and subventral glands.

Relatively large amounts of stylet secretions were produced by SCN J2 incubated in DMT (Fig. 2A). In immunofluorescence assays, $6A_3$ (Fig. 2B) and $9C_2$ bound strongly and $3H_5$ bound relatively weakly to viscous stylet secretions of SCN J2 that were placed on dialysis membranes. Binding of $1D_9$, $9H_{12}$, and $5B_9$ to J2 stylet secretions was observed by fluorescence microscopy, but binding was inconsistent among several assays. Stylet secretions from SCN J2 dissolved readily in pH 11 buffer and were detected by several of the MAbs in ELISA assays. The MAbs $9C_2$, $6A_3$, and $3H_5$ were strongly positive ($A_{405} > 1.500$) in the ELISA assay, whereas the other three MAbs did not bind ($A_{405} < 0.100$) to solubilized stylet secretions. The MAbs, $9C_2$, $6A_3$, and $1D_9$, did not bind to solubilized stylet secretions from J2 of *M. incognita* in ELISA assays.

Discussion

MAbs that bound to secretory granules in the esophageal glands also bound to antigens in stylet secretions of J2 of *H. glycines.* The binding of MAbs to stylet secretions from J2 indicated that antigens synthesized in the subventral glands can be secreted through the stylet. Binding of MAbs to stylet secretions from subventral glands has been recently reported for J2 of *M. incognita* (7). Although MAbs to antigens in esophageal glands of *H. glycines* have been raised previously (1), it was not established whether those antigens were present in stylet secretions. The MAb binding pattern in the esophageal glands may not be indicative of whether the antigen will be secreted through the nematode's stylet, as illustrated by the MAbs in Figure 1A and B. Some secretory granule antigens may only have a role in the secretory process; thus, they may not be discharged from the esophageal gland (12). Presumably, only esophageal gland compounds that are secreted through the stylet can be directly involved in plant–nematode interactions. The six MAbs reported here that are specific to the esophageal glands of SCN J2 bound to stylet secretions in immunofluorescence assays, but three of the MAbs did not bind consistently enough to confirm them as positive. In addition, these three MAbs did not bind to solubilized stylet secretions in ELISA assays, while the other three MAbs were strongly positive in this assay. The ELISA assay was a useful confirmation of antibody-binding to stylet secretions because strong positive reactions only occurred with specific MAbs and no nonspecific binding occurred with *M. incognita* secretions or negative controls. It must be emphasized, however, that the stylet secretion assays do not compensate for changes in epitope conformation or loss of appropriate secretory antigens.

Intrasplenic immunizations of mice with

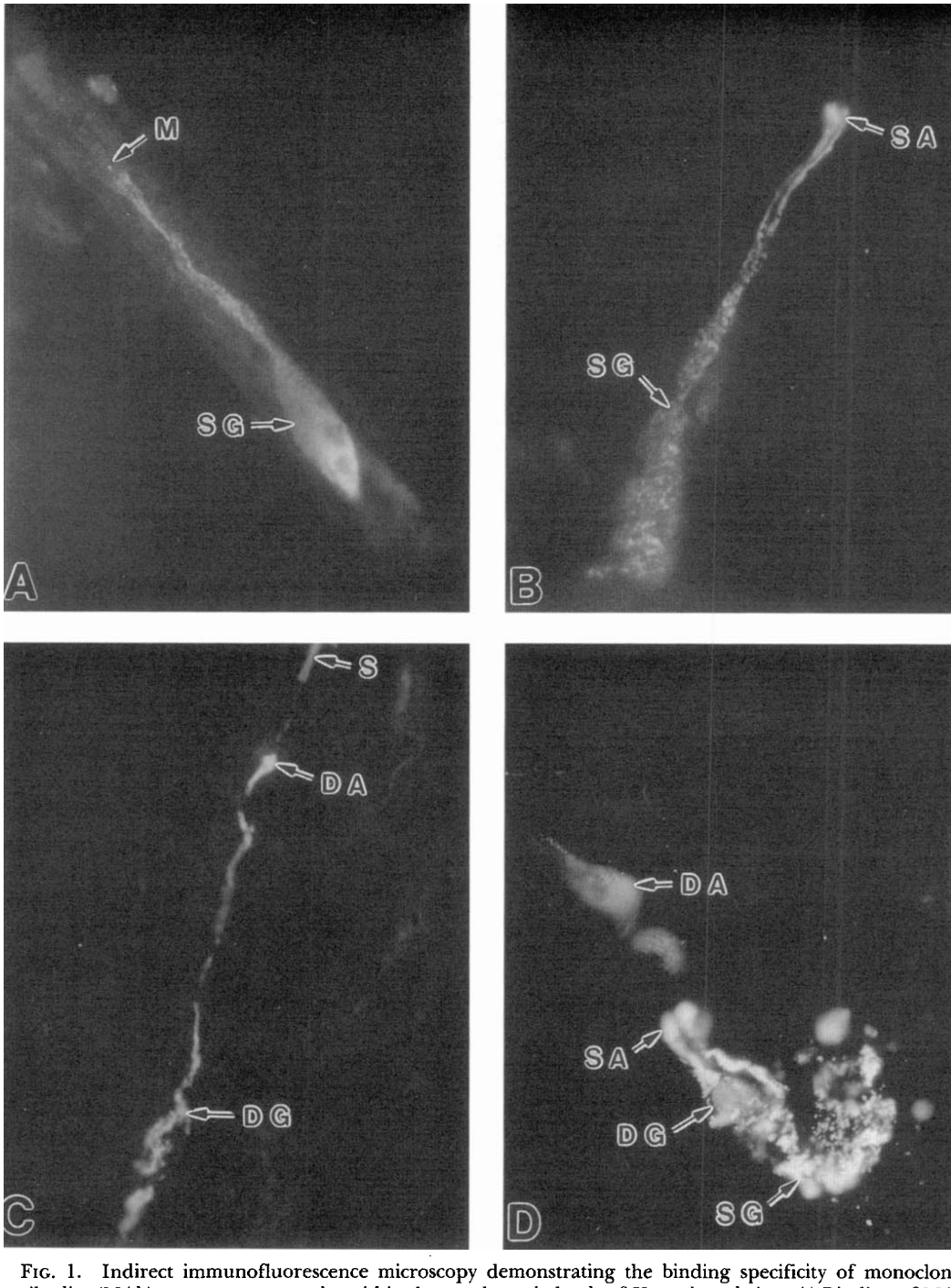

FIG. 1. Indirect immunofluorescence microscopy demonstrating the binding specificity of monoclonal antibodies (MAb) to secretory granules within the esophageal glands of *Heterodera glycines*. A) Binding of MAb $3H_5$ to the two subventral glands (SG) of a second-stage juvenile. M = metacorpus (543×). B) Binding of MAb $1D_9$ to the subventral glands (SG) and subventral gland ampullae (SA) of a second-stage juvenile (644×). C) Binding of MAb $5B_9$ to the dorsal gland (DG) and dorsal gland ampulla (DA) of a second-stage juvenile. S = stylet (731×). D) Binding of MAb $9C_2$ to the dorsal (DG) and subventral glands (SG) of a young female. DA = dorsal gland ampulla, SA = subventral gland ampulla (538×).

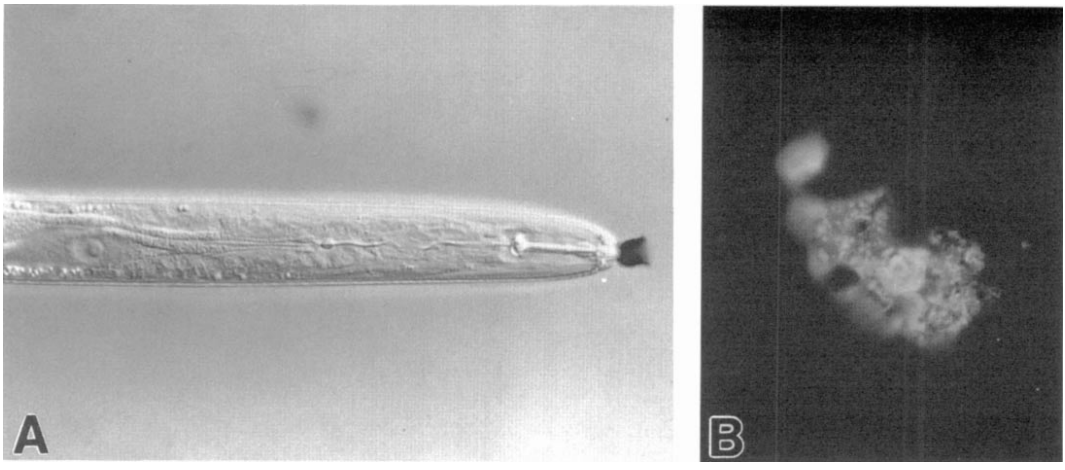

FIG. 2. Stylet secretions produced by second-stage juveniles of *Heterodera glycines* incubated in 5-methoxy DMT oxalate. A) Stylet secretions stained with Coomassie brilliant blue G (318×). B) Indirect immunofluorescence microscopy demonstrating binding of monoclonal antibody $6A_3$ to stylet secretions immobilized on a dialysis membrane (637×).

homogenates of SCN J2 or solubilized stylet secretions were both successful in stimulating the production of antibodies to esophageal gland antigens. The in vitro stimulation of stylet secretions from *H. schachtii* J2 has been reported (18), but the serotonin agonist, 5-methoxy DMT oxalate, was superior to resorcinol for stimulation of stylet secretions from SCN J2. Second-stage juveniles of SCN incubated in DMT produced relatively large quantities of stylet secretions, remained active, and were capable of infecting soybean in greenhouse tests (data not reported). An alkaline buffer was used for solubilization of SCN J2 stylet secretions. In preliminary studies with stylet secretions from J2 and females of *M. incognita,* several biological detergents, chaotropic agents, and solutions of different ionic strengths were unable to solubilize viscous stylet secretions produced in vitro (Davis and Hussey, unpubl.). Results of these studies (not reported) also indicate that other proteins, perhaps from the *M. incognita* J2 cuticle surface or excretory system, can be solubilized from intact J2 in high pH buffer. Nevertheless, the positive ELISA assays and the stimulation of antibodies to SCN esophageal gland antigens confirm the presence of stylet secretions in the solubilization buffer. The potential loss of soluble secretory antigens below 10,000 daltons during concentration of the stylet secretions was not investigated and should be addressed in future experiments.

Binding of esophageal gland-specific MAbs to young females of SCN was limited. Only two MAbs bound to secretory granules within the esophageal glands of SCN females and both MAbs bound to the dorsal and subventral glands. These results are consistent with developmental expression of esophageal gland antigens and changes in esophageal gland form and function reported elsewhere (2,4,7,8).

Several distinguishing biological and morphological differences separate *Heterodera* and *Globodera* (21), and in our study, only one SCN subventral gland specific MAb, $9H_{12}$, which did not bind to stylet secretions, bound to the subventral glands in *G. tabacum.* All of the antigens recognized by the esophageal-gland-specific MAbs appear to be specific to cyst nematodes, since no binding occurred with *M. incognita* or *C. elegans.* Binding of MAb $5B_9$ to secretory granules in the dorsal gland was relatively weak overall and specific to SCN J2.

Techniques developed to produce, solubilize, and concentrate relatively large quantities of stylet secretions from J2 of plant-parasitic nematodes, in vitro, will fa-

cilitate the analysis of secretory components. Direct analysis of stylet secretions has only been reported once (22). It is acknowledged that secretions produced in vitro may not truly reflect secretions produced during infection of plants by nematodes, but this remains to be confirmed. The MAbs to SCN developed here, and those produced previously (1), and the detection of some of their antigens in stylet secretions of J2, will enable the isolation of biologically significant secretions produced by SCN. The identification of nematode secretions essential for plant parasitism will facilitate the development of novel strategies to interfere with nematode infection of plants and reduce nematode-related crop damage.

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