Secretory granule proteins from the subventral oesophageal glands of the potato cyst-nematode identified by monoclonal antibodies to a protein fraction from second-stage juveniles.

Secretory Granule Proteins from the Subventral Esophageal Glands of the Potato Cyst Nematode Identified by Monoclonal Antibodies to a Protein Fraction from Second-Stage Juveniles

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Sodium dodecyl sulfate-extracted proteins from second-stage juveniles (J_2) of the potato cyst nematode *Globodera rostochiensis* were fractionated by preparative continuous flow electrophoresis, and monoclonal antibodies (MAbs) were raised against the 38- to 40.5-kDa protein fraction. Screening of the hybridoma culture fluids by immunofluorescence microscopy of J_2 resulted in the identification of 12 MAbs that bound specifically to the subventral esophageal glands. On Western blots of J_2 these MAbs identified four protein bands with apparent molecular masses of 30, 31, 39, and 49 kDa. Immunoelectron microscopy with one of these MAbs showed an intense labeling of the electron dense core of the secretory granules in the subventral gland cells of J_2. It is concluded that one or more of these proteins are localized within these secretory granules. Immunofluorescence microscopy of J_2 from other plant parasitic nematode species showed that most of these MAbs also bind to the subventral glands of *G. pallida* and *G. tabacum* but not of *Heterodera schachtii, H. glycines, Meloidogyne incognita,* or *M. hapla.*

Additional keywords: host-parasite interaction, virulence factor, host plant transformation, plantibodies.

Potato cyst nematodes are sedentary plant parasites that feed from their host by exploiting a syncytium of metabolically active root cells (Dropkin 1969; Jones and Northcote 1972; Jones 1981; Melillo et al. 1990). Their annual life cycle begins with hatching of second-stage juveniles (J_2) from cysts in the soil. These preparasitic J_2 penetrate the roots behind the growing tips, and following a short migration period within the root, each J_2 selects a cortex cell for feeding site induction. The J_2 inserts its stylet into the cytoplasm of this cortex cell to induce its modification into an initial feeding cell, and, after a short rest period, the juvenile starts feeding (Steinbach

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1972; Steinbach 1973; Wyss and Zunke 1986; Wyss 1992). At this stage the J_2 has already transformed into a sedentary parasite, and the initial feeding cell soon expands to form an elaborate syncytium which transfers nutrients from the vascular tissue to the feeding nematode. After approximately 5 weeks of growth, the adult females have swollen to a globular shape, and they are filled with eggs. When they die, their tanned cuticle forms a protective cyst which harbors the diapausing J_2 of the next generation (Evans and Stone 1977).

Secretory products from the esophageal glands of sedentary plant parasitic nematodes are considered to play a major role in both induction and exploitation of the various types of feeding cells that these parasites establish in the roots of their host (Jones 1981; Hussey 1989a). The production of these salivary secretions takes place in two subventral gland cells and one dorsal gland cell (Endo 1984; Hussey 1989a). The salivary proteins are sequestered in secretory granules, which are transported into the narrow extension of the gland cell. Controlled release of the granule contents in the esophagus occurs at the end of this extension. The subventral gland extensions terminate halfway along the esophagus in the median pump chamber, whereas the dorsal gland extension ends just behind the stylet. It is very likely that some of the secretions from the dorsal gland are responsible for the formation of feeding tubes in the cytoplasm of the feeding cells (Rumpenhorst 1984; Wyss and Zunke 1986; Hussey and Mims 1991; Wyss 1992). The function of the subventral glands, however, is still unclear. It has been suggested that they release cell wall degrading enzymes during root invasion (Wyss et al. 1992) or that they play a role in feeding cell induction (Atkinson et al. 1988). Furthermore, it has been suggested that in parasitic stages subventral gland secretions move posteriorly towards the intestine for the mobilization of lipid reserves (Wyss 1992; Wyss and Grundler 1992), or for internal food digestion (Davis et al. 1994). Immunofluorescence labeling of stylet secretions with monoclonal antibodies has demonstrated that in J_2 and females of root-knot nematodes both gland types are capable of releasing their secre-

tions via the stylet orifice (Hussey et al. 1990; Davis et al. 1994).

Monoclonal antibodies (MAbs) have been used in attempts to identify the esophageal gland secretory proteins of the sedentary plant parasitic nematodes *Heterodera glycines* (Atkinson et al. 1988; Goverse et al. 1994) and *Meloidogyne incognita* (Hussey 1989b; Davis et al. 1992). For both species, screening procedures using immunofluorescence microscopy have identified MAbs that react with secretory granules of the dorsal and subventral gland cells. Various antigens have been used to produce these monoclonals: with *H. glycines* mice were immunized with homogenates from hatched J_2, unhatched J_2, stylet secretions of J_2, and adult females or anterior and posterior parts thereof (Atkinson et al. 1988; Goverse et al. 1994); with *M. incognita* the immunogens were a total protein homogenate of J_2, a subcellular granule fraction from J_2, anterior and posterior parts of females, and stylet secretions of females (Hussey 1989b, Davis et al. 1992). In both species the immunosuppressive drug cyclophosphamide was used to enhance the effect of the immunizations (Atkinson et al. 1988; Davis et al. 1992), and with *M. incognita* intrasplenic immunizations were employed to administer minute quantities of immunogen (Davis et al. 1992). However, the success of these MAbs in identifying esophageal gland secretory proteins, either by immunostaining of gel electrophoresis patterns or by purification with chromatography techniques, has remained limited. Hussey et al. (1990) used a MAb reactive with both the dorsal and subventral glands in *M. incognita* to isolate a secretory component from homogenized J_2. This protein had an apparent molecular mass of more than 212 kDa and was glycosylated. A MAb binding to the subventral esophageal glands of adult females of *M. incognita* was used to isolate a gene from a cDNA expression library (Ray et al. 1994). Because this MAb

also bound to the body-wall muscles in J_2 of *M. incognita* and because the sequence of the isolated gene showed homology with the rod portions of myosin heavy chains, it was suggested that this antigen may be involved in the movement of secretory granules rather than being itself secreted (Ray et al. 1994).

In this study, we report the production of MAbs that bind to secretory granule proteins from the subventral esophageal glands of the potato cyst nematode, *Globodera rostochiensis*. As antigen we used a protein fraction from homogenized J_2, which was collected by preparative continuous flow polyacrylamide gel electrophoresis. Hybridoma cell lines were screened by immunofluorescence microscopy for MAbs reacting with these esophageal glands and these antibodies were subsequently used for identification of the secretory granule proteins on Western blots of J_2. Electron microscopy was used to examine subventral gland morphology and to demonstrate the specificity of binding of one of these MAbs to the secretory granules within these glands. Finally, the MAbs were tested with fluorescence microscopy for cross-reactivity with esophageal glands in other endoparasitic nematode species.

RESULTS

Antibody production.

Homogenized J_2 of *G. rostochiensis* were separated by preparative continuous flow electrophoresis in the presence of sodium dodecyl sulfate (SDS) into 50 protein fractions. The average molecular mass of these proteins fractions ranged from 30 to 52 kDa. Examination of successive fractions by analytical SDS polyacrylamide gel electrophoresis (SDS-PAGE) revealed narrow protein zones, with a very gradual increase in molecular mass (Fig. 1). Therefore the original fractions were pooled to form a representative set of 16 protein samples of increasing molecular mass, which were used for immunizing 16 mice. Antisera collected after the second immunization with these samples were screened with immunofluorescence microscopy for reaction with the esophageal glands of J_2 of *G. rostochiensis*. It was found that the eight mice that were immunized with the successive protein fractions from 36.5 to 52 kDa had produced antisera that showed a strong reaction with the subventral esophageal glands (Fig. 2A). In addition, a very weak reaction with the subventral glands was observed with the antiserum to the 31- to 33-kDa fraction. The mouse that was immunized with the 38- to 40.5-kDa protein sample (Fig. 1) was chosen for monoclonal antibody production.

Immunofluorescence microscopy.

Screening of the hybridoma culture supernatants by immunofluorescence microscopy of nematode sections identified 12 MAbs that bound specifically to the subventral glands of J_2 of *G. rostochiensis* (Fig. 2B—D). The staining of the glands by the MAbs was found to be variable, both between individual J_2 within a single experiment and between repeated experiments with the same antibody. Nevertheless, 10 MAbs (MGR 46, 47, 48, 49, 50, 53, 54, 55, 56, 59) were able to stain the entire contents of subventral gland cells, including the gland extensions (Fig. 2D). Two MAbs (MGR 57 and 60) persistently failed to penetrate the gland cells sufficiently to obtain an even staining reaction.
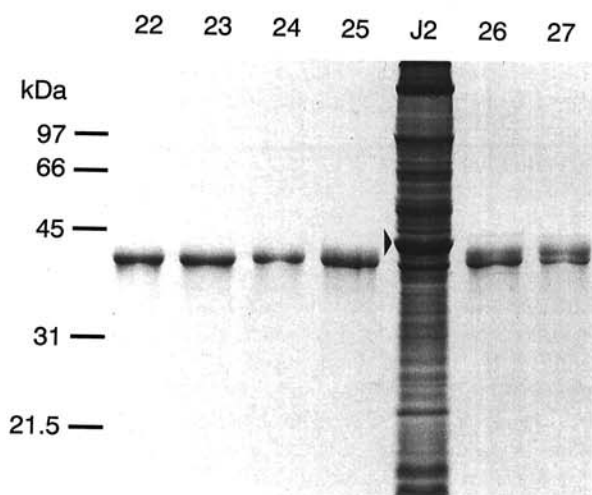
Fig. 1. SDS-polyacrylamide gel electrophoresis pattern of selected protein fractions purified from homogenized second stage juveniles (J_2) of *Globodera rostochiensis* by preparative continuous flow electrophoresis. The lanes numbered 22 to 27 show successive fractions of increasing molecular weight. The mouse that was immunized with the pooled fractions 22 to 25 (molecular mass range 38 to 40.5 kDa) produced antiserum to the subventral esophageal glands, and was used for monoclonal antibody production. The lane labeled J_2 shows a total protein homogenate of J_2. The arrow indicates a characteristic major protein band (presumably actin) which can be used to compare Fig. 1 with Fig. 3.
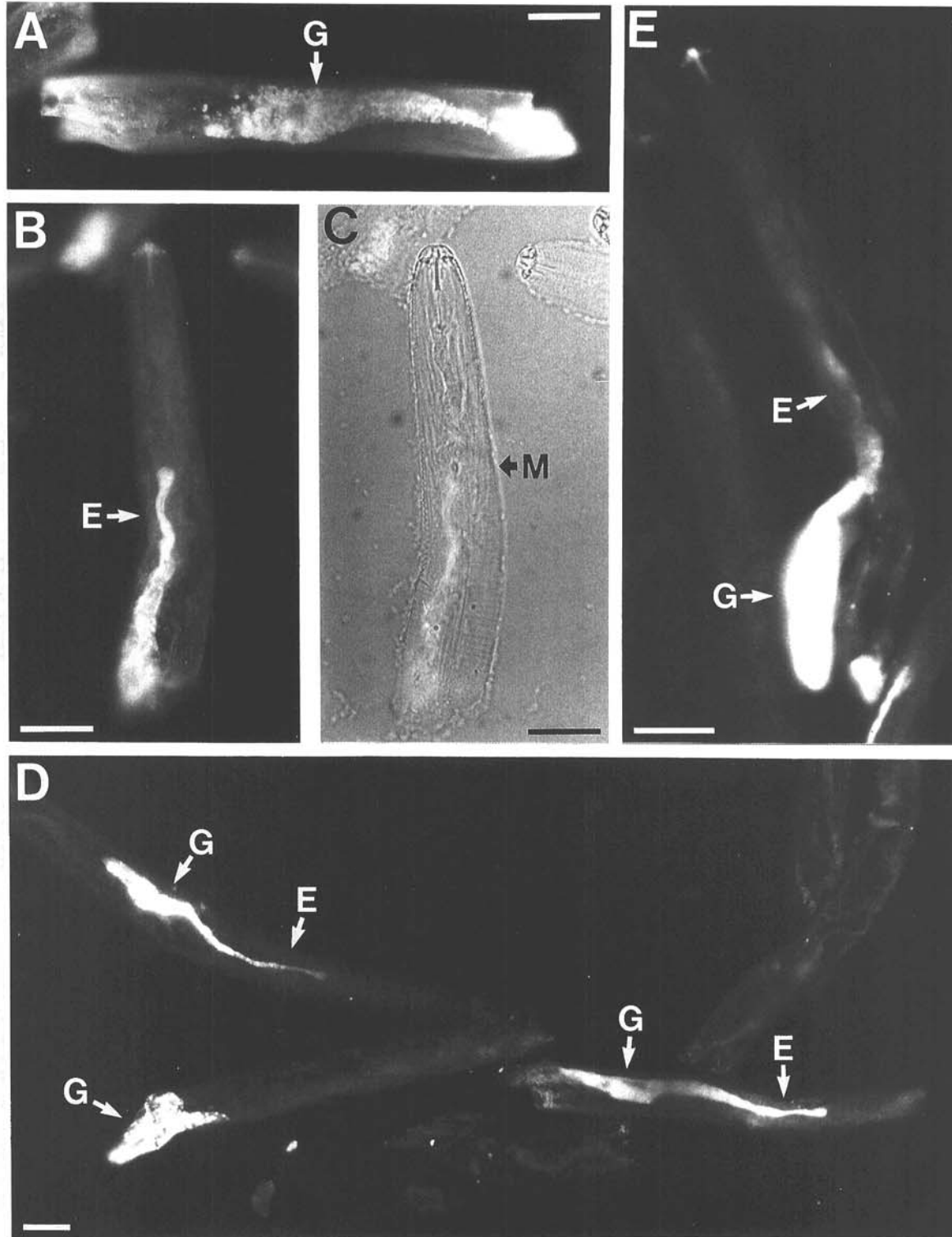
Fig. 2. Immunofluorescence labeling of the subventral esophageal glands (G) and their extensions (E) in second-stage juveniles of potato cyst-nematodes. (Because both glands lie in close apposition, their cell bodies and their extensions appear as single structures in the micrographs). **A,** *Globodera rostochiensis* stained with mouse antiserum raised to a 38.5- to 40-kDa protein fraction derived from J_2 of *G. rostochiensis*. Individual secretory granules can be distinguished within the gland cells. **B, C, and D,** *G. rostochiensis* labeled with monoclonal antibody MGR 48. Additional bright field illumination (C) shows that the gland extensions terminate in the metacorpus (M) of the esophagus. **E,** *G. pallida* labeled with monoclonal antibody MGR 48. (Due to the cutting of the J_2 prior to immunolabeling, the subventral glands have come to lie outside this juvenile, with only their extensions still in place). Bars: 20 μm.

Immunofluorescence microscopy was also used to test the cross reactivity of the MAbs with J_2 of other species of plant parasitic nematodes (Table 1). It was found that nearly all MAbs also bound to the subventral glands of *G. pallida* (Fig. 2E) and *G. tabacum*. However, except for MGR 48, which reacted with *H. glycines*, none of the MAbs showed binding to the esophageal glands of the other species examined.

Western blotting.

The MAbs were tested for reactivity with protein homogenates of J_2 which were separated by SDS-PAGE and electroblotted onto PVDF membrane. Eleven of the 12 MAbs stained protein bands in the electrophoresis pattern (Fig. 3). Together these MAbs identified four different polypeptides with apparent molecular masses of 30 (svp30), 31 (svp31), 39 (svp39), and 49 kDa (svp49), respectively. All reactive MAbs bound to the svp39 band, with 10 showing additional binding to one or more of the other bands. MGR 47, 53, 54, 55, and 59 all reacted strongly with the svp30, svp31, and svp39 bands, while MGR 46, 57, and 60 showed moderate binding to svp39 and svp49. Reactivity with all four protein bands was found only with MGR 48. In addition, MGR 48 stained several minor bands in the gel region below svp30. Staining of minor bands was also observed with MGR 56, both below svp30 and above svp39.

Electron microscopy.

Because ultrastructural information on the subventral glands in potato cyst nematodes was completely lacking and could only be inferred from studies with related plant-parasitic nematode species (Endo 1984, 1988, 1993), we have first examined subventral gland morphology in hatched J_2 of *G. rostochiensis*. Thin sections showed that both the cell body (Fig. 4) and the cell extension are packed with secretory granules. It was only in the immediate vicinity of the nucleus that parts of the cytoplasm were free of granules. The secretory granules invariably possessed an electron translucent halo which surrounded an electron dense core. This morphology made it easy to identify the subventral glands in the low-contrast formaldehyde fixed specimens that were used for immunolabeling.

Immunogold labeling of formaldehyde-fixed J_2 with MAb MGR 48 was localized to the subventral esophageal glands. Binding of the gold particles occurred both to the electron dense granule matrix and to the patches of cytoplasm surrounding the granules (Fig. 5).

Table 1. List of monoclonal antibodies to the subventral esophageal glands of *Globodera rostochiensis* and their immunofluorescence cross–reactivity with other species of sedentary plant parasitic nematodes. "+" reaction with subventral glands, "–" no reaction with subventral glands

Antibody[a]	Isotype		*Globodera pallida*	*Globodera tabacum*	*Heterodera schachtii*	*Heterodera glycines*	*Meloidogyne incognita*	*Meloidogyne hapla*
	Heavy	Light						
MGR 46	IgG2a	λ	+	+	–	–	–	–
MGR 47,50,53, 55. 56. 59	IgG1	κ	+	+	–	–	–	–
MGR 48	IgG1	κ	+	+[b]	–	+	–	–[b]
MGR 49	IgG3	κ	+	–	–	–	–	–
MGR 54	IgG2a	κ	+	+	–	–	–	–
MGR 57, 60	IgG1	λ	+	+	–	–	–	–

[a] Affinity–purified antibody (MGR 48) or hybridoma culture fluid (all other MAbs).
[b] Background staining.
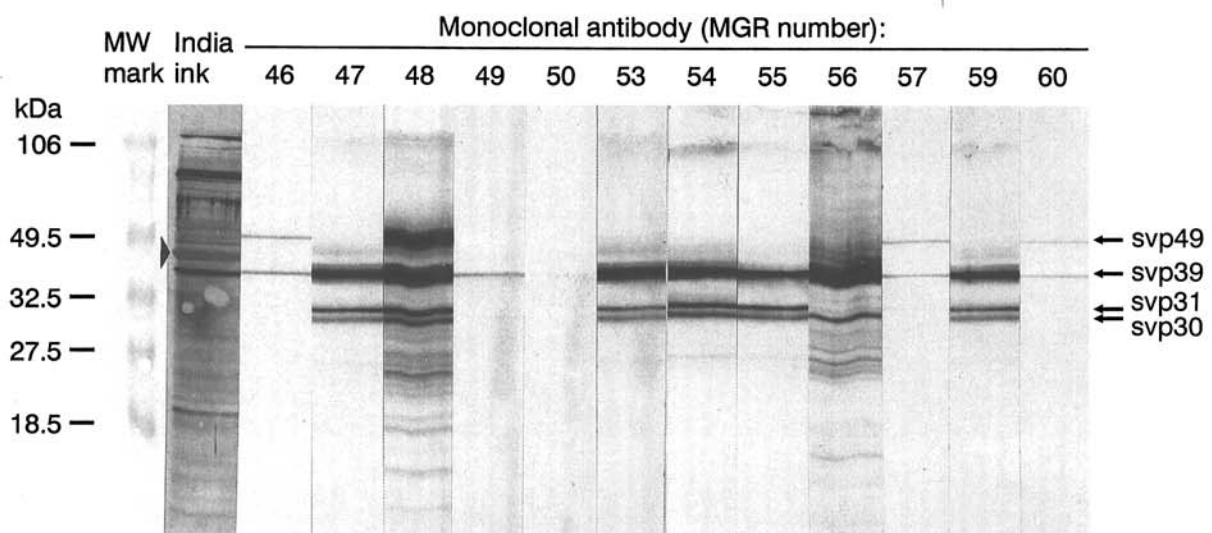
Fig. 3. Western blot of second-stage juveniles (J_2) of *Globodera rostochiensis* stained with a panel of monoclonal antibodies (MGR 46 to 60) specific to the subventral esophageal glands. Four major protein bands are identified, labeled svp30, svp31, svp39, and svp49. MW Mark = prestained molecular weight markers. India ink staining shows the complete protein pattern of J_2. The arrow indicates a characteristic major protein band (presumably actin) which can be used to compare Fig. 3 with Fig. 1.

DISCUSSION

In this paper we used a novel method of immunogen preparation to produce monoclonal antibodies specific to esophageal gland antigens of a sedentary plant parasitic nematode. Mice were immunized with protein fractions which were produced by preparative continuous flow SDS-electrophoresis of homogenized J_2. Following immunofluorescence screening of hybridomas we identified a panel of 12 MAbs specific to the subventral glands of J_2 of *G. rostochiensis*. Most of these antibodies differed from MAbs which we obtained previously to the subventral glands of *G. rostochiensis* (J. M. de Boer et al., 1996) in that they bound to SDS-denatured epitopes. This enabled the identification of subventral gland specific proteins on Western blots of J_2 of *G. rostochiensis*.

The panel of MAbs presented here identified four major protein bands in the electrophoresis pattern of J_2 of *G. rostochiensis*. All the MAbs that gave a positive reaction on Western blot identified a protein band of 39 kDa (svp39). This molecular mass corresponds with the protein fraction that was used for immunization. In addition three other proteins with smaller and larger molecular weights (svp31, svp32, and svp 49) were identified. It is likely that these additional polypeptides are structurally related to the svp39 band. Possibly these four proteins represent variants of a single gene product, which differ in posttranslational modification or in the length of the polypeptide chain. The different reactivities with our panel of MAbs would then give information about the epitopes that are available on these related polypeptides. Thus MGR 47, 53, 54, 55, and 59 may be directed to an epitope that is present only on the svp30, svp31, and svp39 bands, whereas MGR 46, 57, and 60 may bind to an epitope specific for svp39 and svp49.

Monoclonal MGR 48, which showed a reaction with all four protein bands on Western blots of J_2, was used to determine the ultrastructural location of these polypeptides within the subventral gland. Immunogold labeling showed an intense binding of MGR 48 to the electron dense core of the secretory granules. It is therefore concluded that one or more of the proteins identified by MGR 48 are localized within the secretory granules of the subventral gland. Some labeling was also found in patches of cytoplasm between the granules. This could be the result of a release of antigen from the secretory granules due to the combined effects of mild tissue fixation and ethanol dehydration during specimen preparation. Alternatively, this may indicate that one or more of the polypeptides (also) reside outside the granules, for instance in the endoplasmic reticulum.

Preliminary in vitro experiments have demonstrated that the proteins identified by MAb MGR 48 can be retrieved in stylet exudates of preparasitic J_2 of *G. rostochiensis* (G. Smant et al., unpublished). It is therefore likely that the antigens recognized by MGR 48 are secretory products of the subventral esophageal glands. Their release via the stylet indicates that these proteins may play a role in the initial stages of root infection by J_2. In that case they could be cell wall degrading enzymes that assist in root penetration (Steinbach 1972), or signal molecules involved in feeding site induction (Steinbach 1973).

The possibility of expressing genes coding for monoclonal antibodies in plants (Hiatt et al. 1989) has raised the idea that

antibodies may be suitable for introducing resistance to phytopathogens into crops (Benvenuto et al. 1991; Schots et al. 1992a). Binding of antibody to antigen by itself may exert an inhibiting effect on the antigen (Schots et al. 1992a), and by choosing suitable target proteins in a plant pathogen, monoclonal antibodies ('plantibodies') to these targets could interfere with the host-parasite interaction. An example of plant
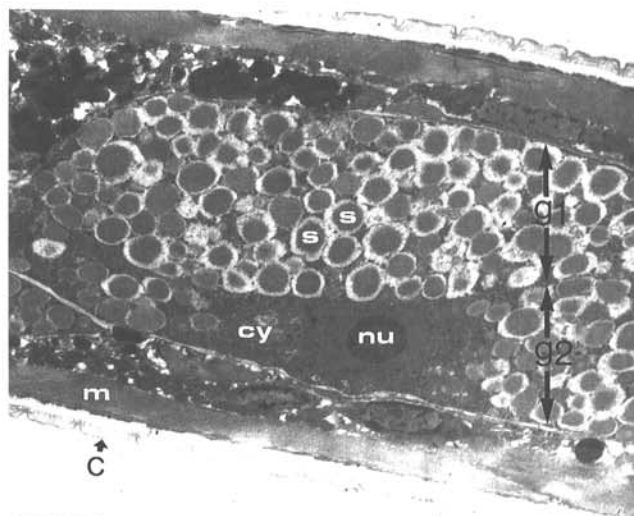

Fig. 4. Longitudinal section through the subventral gland region of a second stage juvenile of *Globodera rostochiensis*. The two subventral gland cells (g1 and g2) lie adjacent to each other, and they are both packed with secretory granules (s). Cytoplasm free of secretory granules (cy) could only be found near the subventral gland nucleus (nu). m = body wall muscle filaments. C = cuticle. Bar = 2 µm.
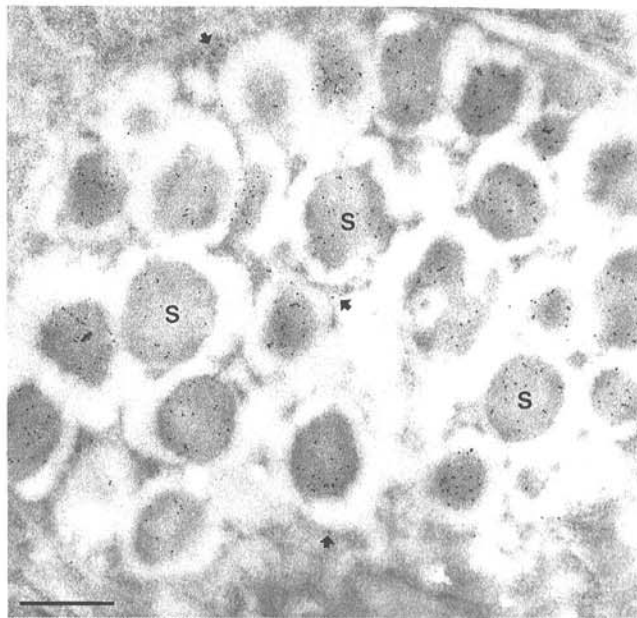

Fig. 5. Immunogold labeling of a subventral esophageal gland of a J_2 of *Globodera rostochiensis* with monoclonal antibody MGR 48. Fixation with formaldehyde, and embedding in LR-White resin. The gold particles localize to the relatively electron dense core of the secretory granules (S). Labelling is also found outside the granules (e.g. arrows). Bar = 0.5 µm.

pathogen inhibition by antibodies has recently been obtained for artichoke mottled crinkle virus infection in transgenic *Nicotiana benthamiana* (Tavladoraki et al. 1993). Also in vertebrate cells this principle of in situ blockage of a biological function by intracellular expression of antibodies has been demonstrated (Biocca et al. 1994; Werge et al. 1994). In planta expression of antibodies to esophageal gland secretions may offer new possibilities for introducing resistance to sedentary nematodes in plants. By inhibiting the function of these secretions, either following their release in the apoplasmic space, or following their injection into the cytoplasm of the host cell, the juvenile nematodes may be deprived of their food source and thus be arrested in their growth and development. The MAbs to subventral gland antigens of *G. rostochiensis* which have been presented here may prove to be useful for the control cyst-nematodes in potato. Transformation experiments are under way in our laboratories to test this hypothesis.

In conclusion, we have identified subventral gland specific proteins of J_2 of *G. rostochiensis* using monoclonal antibodies which were raised against a highly purified SDS-denatured immunogen. These antibodies will be valuable tools in future studies relating to the nature of these antigens and their possible function in the host-parasite interaction. We have shown that immunization with electrofractionated total protein homogenates can be a useful method for obtaining MAbs against esophageal gland antigens of plant parasitic nematodes. It is expected that by using fractionated proteins from other molecular weight ranges or from other developmental stages, a systematic search for additional esophageal gland antigens of *G. rostochiensis* will be possible.

MATERIALS AND METHODS

Nematodes.

Second-stage juveniles (J_2) of *Globodera rostochiensis* pathotype Ro1, and of *G. pallida* pathotype Pa2 were hatched by soaking cysts on a 100-µm sieve in potato root diffusate (Clarke and Perry 1977). J_2 of *Meloidogyne hapla* were a gift from E. Jansen, DLO Research Institute for Plant Protection, Wageningen, The Netherlands. The J_2 suspensions were mixed with an equal volume of 70% (w/v) sucrose in a centrifuge tube, covered with a layer of tap water, and centrifuged briefly at 1,000 × g. Purified juveniles were collected from the sucrose-water interface with a Pasteur pipette, washed with tap water and used for experiments. J_2 of *G. tabacum, H. glycines, H. schachtii,* and *M. incognita* were obtained as described by Goverse et al. (1994).

Preparative electrophoresis.

In total 2.75 million J_2 of *G. rostochiensis* were homogenized in 208 mM Tris-HCl, pH 6.8, supplemented with 8.33% (v/v) 2-mercaptoethanol at 5°C using a small glass mortar and pestle. The J_2 were homogenized in aliquots of approximately 300,000 individuals. Following homogenization the samples were pooled and mixed in a ratio of 3:2 (v/v) with a solution of 5% SDS/25% glycerol/0.1% bromophenol blue, thus producing standard SDS-sample buffer (O'Farrell 1975). The homogenate was heated for 5 min in boiling water, centrifuged for 5 min at 10,000 × *g*, and the supernatant was stored at −80°C until used. The nematode sample (2 ml, approxi-

mately 13 mg of protein) was fractionated by preparative SDS-PAGE using a Model 491 Prep Cell apparatus (Bio-Rad, Richmond, CA), essentially according to the instruction manual, with the correction that 0.1% SDS was added to the buffers of the acrylamide gels. The cylindrical separating gel was 50 × 28 mm (height × diameter) and contained 10% acrylamide. The stacking gel contained 4% acrylamide and was 15 mm in height. The proteins were separated with a current of 40 mA, and 1.5 ml fractions were collected using an elution buffer flow rate of 1 ml/min. The fractions were concentrated by freeze drying, washed three times in phosphate buffered saline pH 7.4 (PBS) using 1.5 ml microcentrifuge filter concentration units with molecular mass cutoff of 5 kDa (Ultrafree-MC, Millipore Corp., Bedford, MA), taken up in 50 µl PBS and stored at −20°C. Groups of 2 to 5 fractions were pooled, and each pooled sample was diluted in PBS to obtain three 200-µl aliquots for successive immunizations.

Immunizations and cell fusions.

Sixteen mice were immunized intraperitoneally with the isolated protein fractions, which were mixed 1:1 with Freund's incomplete adjuvant. After 4 weeks a second immunization was given, also with incomplete adjuvant. Two weeks later antiserum samples were collected for immunofluorescence microscopy. One mouse that was selected for monoclonal antibody production received a final booster injection 12 weeks after the first immunization. Three days later the mouse was sacrificed, and MAb-producing hybridoma cell lines were obtained by fusing spleen cells with SP 2/0 myeloma cells (Goding 1983; Schots et al. 1992b).

Immunofluorescence microscopy.

Mouse antisera and hybridoma cell line culture fluids were screened by indirect immunofluorescence microscopy for reaction with J_2 of *G. rostochiensis* as described in detail elsewhere (J. M. de Boer et al. 1996). Briefly, J_2 were fixed in 2% paraformaldehyde for 3 days, dried, and cut into sections with a razor blade. Following treatment with proteinase-K, methanol, and acetone, the nematode sections were incubated with the primary antibody and stained with rat-anti-mouse FITC-conjugated immunoglobulins. The mouse antisera were tested in a 1:400 dilution. Hybridoma cell lines producing antibodies to the subventral esophageal glands of *G. rostochiensis* were subcloned to stability and stored in liquid nitrogen. Isotyping of the light and heavy chains of the MAbs was as described by Schots et al. (1992b). Immunofluorescence testing of J_2 from *G. pallida* and *M. hapla* followed the same procedure as for *G. rostochiensis*, with the exception that the initial fixations in paraformaldehyde were different: 2 days for *G. pallida*, and 1 day for *M. hapla*. Immunofluorescence testing of J_2 from *G. tabacum, M. incognita, H. glycines,* and *H. schachtii* was as described by Goverse et al. (1994).

SDS-PAGE and Western blotting.

Analytical mini SDS-PAGE was performed essentially as described by De Boer et al. (1992). For the examination of the protein fractions that were obtained with preparative electrophoresis, 4 µl of SDS-sample buffer was added to 2 µl from the concentrated fractions in PBS, and these samples were applied to 20-µl slots in the stacking gel. Following

electrophoresis, the gels were stained with colloidal Coomassie Blue G-250 (Neuhoff et al. 1988). For Western blot testing of MAbs, J_2 of *G. rostochiensis* were homogenized as described above, and per minigel approximately 10,000 J_2 were added to a single 73-mm-wide slot in the stacking gel. An adjacent reference well (3 mm wide) was filled with prestained molecular weight markers (Bio-Rad, Richmond, CA). Following electrophoresis (13% separating gel) the proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, MA) using a semi-dry blotting apparatus. A continuous transfer buffer system was used containing 39 mM glycine, 48 mM Tris, and 20% (v/v) methanol. Transfer was carried out with 0.8 mA/cm^2 for 1 h. The blots were cut into strips, which were blocked overnight in PBS pH 7.4/0.1% (v/v) Tween-20 (PBST) supplemented with 5% (w/v) defatted milk powder. Following a wash in PBST/1% milk powder, the strips were incubated for 2 h in hybridoma culture fluid, diluted 1:6 in PBST/1% milk powder. For MAb MGR 48 affinity-purified antibody (1 µg/ml) was used instead of culture fluid. After washing three times in PBST/1% milk powder, the strips were next incubated individually in alkaline phosphatase conjugated rat-anti-mouse IgG (H+L) (Jackson Immuno Research Laboratories Inc, West Grove, USA) diluted 1:5,000 in PBST/1% milk powder for 1 h. After washing in PBST/0.1% milk powder (1×) and PBST (3×) the strips were stained individually in 0.1 M ethanolamine-HCl pH 9.6, supplemented with 4 mM MgCl$_2$, 0.06 mg/ml 5-bromo-4-chloro-3-indolyl phosphate and 0.1 mg/ml nitro blue tetrazolium.

Electron microscopy.

For ultrastructural examination, J_2 of *G. rostochiensis* were fixed at 4°C with 4% paraformaldehyde in 0.1 M HEPES-buffer pH 7.5 for 2 days. The suspension of fixed J_2 was spread on a microscope slide and the nematodes were chopped into pieces with a razor blade. The nematode sections were then sequentially fixed with 2% glutaraldehyde and 1% osmium tetroxide (both in 0.1 M HEPES, pH 7.5), and stained with 1% aqueous uranyl acetate. Following stepwise dehydration in ethanol, the J_2 were infiltrated for 1 day with Spurr epoxy resin (Spurr 1969). The nematode fragments were then transferred to fresh epoxy resin in a BEEM capsule, centrifuged to the bottom, and polymerized at 60°C. Thin sections were cut with an LKB ultramicrotome, collected on Formvar-coated 100 mesh copper grids, and poststained with 2% uranyl acetate in 50% methanol.

For immunolabeling, J_2 of *G. rostochiensis* were fixed for 2 days at 4°C in 2% paraformaldehyde in PBS, pH 7.4. After chopping them into pieces, the J_2 were washed 2 times in distilled water, dehydrated in 30-50-70-96% ethanol, infiltrated at room temperature for 1 h in a 1:1 mixture of 96% ethanol and LR-White acrylic resin (London Resin Co. Ltd., Basingstoke, England), and subsequently in pure LR-White resin for 4 h. The nematode fragments were transferred to fresh resin in a gelatin capsule, centrifuged to the bottom, and polymerized at 60°C. Following ultramicrotomy, thin sections were collected on Formvar-coated copper grids and immunolabeled with colloidal gold according to the following protocol (Aurion Immuno Gold Reagents, Wageningen, The Netherlands): 10 min PBS pH 7.6/0.05 M glycine, 2 h affinity purified MAb MGR 48 diluted to 1 µg/ml in incubation buffer

(PBS pH 7.6/0.2% BSA-C/20 mM NaN$_3$), 6× 5 min wash with incubation buffer, 2 h colloidal gold solution (10 nm particle size, conjugated with goat-anti-mouse-IgG immunoglobulins; Aurion Immuno Gold Reagents, Wageningen, The Netherlands) diluted 1:10 in incubation buffer, 6× 5 min wash with incubation buffer, 3× wash with PBS, and 3× wash with distilled water. The sections were stained with 1% aqueous uranyl acetate. Control labeling experiments were performed with a mouse monoclonal antibody specific to the body wall musculature of J_2 of *G. rostochiensis*.

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