Production and characterization of monoclonal antibodies to antigens from second stage juveniles of the potato cyst nematode, *Globodera rostochiensis*

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Summary – Following immunization of mice with different antigens from *Globodera rostochiensis*, monoclonal antibodies (MAbs) were screened by fluorescence microscopy for reaction with specific structures in second stage juveniles (J2) of *G. rostochiensis*. MAbs were obtained which bound to the subventral oesophageal glands, the body-wall muscle filaments, the genital primordium, the intestinal lumen, cell nuclei, and the cuticle surface. The MAbs to the subventral glands also bound to the subventral glands in J2 of *G. pallida* and *G. tabacum*, but not in J2 of *Heterodera glycines, H. schachtii, Meloidogyne hapla* or *M. incognita*. Three subventral gland MAbs reacted with a water soluble epitope in native protein extracts from J2 of *G. rostochiensis*. The MAbs to the body-wall muscle filaments also bound to the body-wall musculature in J2 of *G. pallida, H. schachtii, M. hapla,* and *M. incognita*. On Western blots of J2 of *G. rostochiensis* these MAbs reacted with two proteins of 39 kDa and > 106 kDa respectively.

Résumé – ***Production et caractérisation d'anticorps monoclonaux contre les antigènes de juvéniles de deuxième stade du nématode à kyste de la pomme de terre,* Globodera rostochiensis** – Après immunisation de souris avec différents antigènes de *Globodera rostochiensis*, les anticorps monoclonaux (MAbs) ont été testés par microscopie en fluorescence pour leur réaction avec des structures particulières de juvéniles de deuxième stade (J2) de *G. rostochiensis*. Il a été obtenu des MAbs qui se lient avec les glandes oesophagiennes subventrales, les filaments musculaires de la paroi du corps, le primordium génital, la lumière intestinale, les noyaux des cellules et la surface de la cuticule. Les MAbs des glandes subventrales se lient également aux glandes subventrales des J2 de *G. pallida* et *G. tabacum*, mais non à celles des J2 d'*Heterodera glycines, H. schachtii, Meloidogyne hapla* ou *M. incognita*. Des MAbs des glandes subventrales réagissent avec un épitope hydrosoluble des extraits protéiques de J2 de *G. rostochiensis*. Les MAbs des filaments musculaires de la paroi du corps se lient également à la musculature de la paroi du corps des J2 de *G. pallida, H. schachtii, M. hapla* et *M. incognita*. Par immunotransfert de J2 de *G. rostochiensis*, ces MAbs réagissent avec deux protéines de 39 et plus de 106 kDa, respectivement.

Key-words : *Globodera*, monoclonal antibodies, oesophageal glands, body-wall muscles.

Secretory products from the oesophageal glands are considered to play an important role in the formation and exploitation of feeding cells that endoparasitic nematodes induce in the roots of their host plant (Hussey, 1989 *a*). Identification of these secretory products will provide insight into the host-parasite interaction, and may also open new possibilities for endoparasitic nematode control through genetic modification of the host plant (Schots *et al.*, 1992 *a*).

Monoclonal antibodies (MAbs) have been raised against epitopes in the dorsal and subventral oesophageal glands of both the soya bean cyst nematode *Heterodera glycines* (Atkinson *et al.*, 1988; Goverse *et al.*, 1994) and the root-knot nematode *Meloidogyne incognita* (Hussey, 1989 *b*; Hussey *et al.*, 1990; Davis *et al.*, 1992). Various immunogens have been used in these studies, such as hatched J2, unhatched J2, adult females, microdissected anterior parts of females, a subcellular granule fraction from J2, and stylet secretions of females. These MAbs have provided information about the developmental expression of oesophageal gland antigens in *H. glycines* and *M. incognita* (Atkinson & Harris, 1989; Davis *et al.*, 1994; Goverse *et al.*, 1994) and they have been used for the identification of secretory granule proteins in *M. incognita* (Hussey *et al.*, 1990; Ray *et al.*, 1994).

In this paper we have used immunofluorescence microscopy to identify MAbs that bind to specific structures in the potato cyst nematode, *Globodera rostochiensis*. In the selection of immunogens, emphasis was put on putative antigens from the dorsal and subventral oesophageal glands. Five MAbs were identified which re-

acted with epitopes within the subventral oesophageal glands. In addition 13 MAbs were obtained which bound to various other structures in J2. The MAbs were characterized by immunofluorescence microscopy for cross-reactivity with J2 of other plant parasitic nematode species, and with ELISA and blotting techniques for reactivity with protein extracts from J2 of *G. rostochiensis*.

Materials and methods

Nematodes

Preparasitic second stage juveniles (J2) of *Globodera rostochiensis* pathotype Ro1, and of *G. pallida* pathotype Pa2 were hatched by soaking cysts in potato root diffusate on a 100 μm sieve (Clarke & Perry, 1977). J2 of *Meloidogyne hapla* and *Heterodera schachtii* were gifts from respectively Mrs. E. Jansen, DLO Research Institute for Plant Protection, and H. Lubberts, DLO Centre for Plant Breeding and Reproduction Research, both in Wageningen, The Netherlands. The J2 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 1000 *g*. Purified juveniles were then collected from the sucrose-water interface with a Pasteur pipette, washed with tap water, and used for experiments. J2 of *G. tabacum, H. glycines* and *M. incognita* were obtained as described by Goverse *et al.* (1994). Parasitic juveniles of *G. rostochiensis* were isolated from roots of infected potato plants as previously described (De Boer *et al.,* 1992 *a*).

Antigen preparation

Monoclonal antibodies were produced by immunizing BALB/C mice with antigen samples from *G. rostochiensis* juveniles using four different protocols :

(1) Hatched J2 were homogenized in phosphate buffered saline pH 7.4 (PBS) with a small glass mortar and pestle at 4 °C. The homogenate was stored at − 80 °C until used. The thawed sample was injected intraperitoneally (20 000 J2, 100 μg protein) with two intraperitoneal booster injections (27 000 J2, 135 μg protein) after 4 weeks and 17 weeks.

(2) The first and second immunization were as described for protocol (1); the final booster injection, however, was a crude pellet fraction derived from 200 000 J2. These J2 were taken up in homogenization buffer containing 0.20 M mannitol, 0.07 M sucrose, 0.05 M HEPES-NaOH pH 7.5, and 0.01 M EDTA. Portions of the suspension were spread on a large microscope slide and the nematodes were chopped into small pieces with a razor blade attached to a vibrating (50 Hz) aquarium air pump. The homogenate was filtered through a 10 μm sieve at 4 °C, and the filtrate was centrifuged for 5 min at 8000 *g*. The pellet was frozen in homogenization buffer. For immunization, the thawed pellet was suspended in 50 μl PBS and injected intraperitoneally.

(3) J4 females were fixed for 3 days in 0.2 % paraformaldehyde in PBS at 4 °C. Then their anterior portions were cut off with a razor blade at about 1/4 of the juvenile's body length. Forty-nine anterior sections were thus collected in PBS. The sections were pelleted by centrifugation in a microcentrifuge tube. The supernatant was removed, and the sample was frozen at − 20 °C until used. The thawed sample was homogenized in 30 μl PBS and used for intrasplenic immunization.

(4) A sodium dodecyl sulphate (SDS) extracted protein homogenate of 200 000 J2 was separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using a 9 % acrylamide separating gel (size 160 × 135 × 1.5 mm) and 4 % acrylamide stacking gel (De Boer *et al.,* 1992 *a*). After electrophoresis, a narrow zone of high molecular weight proteins was excised for electroelution, starting at the border of the separating gel and the stacking gel, and ending 1.5 mm below in the separating gel. The apparent molecular masses of the proteins in this gel segment were > 200 kDa. The proteins were eluted from the gel pieces in a Model 422 Electro Eluter (Bio-Rad, Hercules, USA). The gel pieces were placed in an elution buffer containing 25 mM Tris, 192 mM glycine, 0.1 % SDS, and a current of 50 mA was applied for 18 h at 4 °C. The eluted proteins were trapped in membrane caps with a molecular mass cut-off 3.5 kDa. After elution, the sample was concentrated using a 1.5 ml microcentrifuge filtration unit with a molecular mass cut-off of 5 kDa (Ultrafree MC, Millipore Corp., Bedford, USA). After washing twice with PBS in the same filtration unit, the sample was taken up in 50 μl PBS and stored at − 20 °C. The thawed sample was used for intrasplenic immunization.

Immunizations and cell fusions

For the intraperitoneal immunizations (protocols 1 and 2) the antigen for the first two immunizations was mixed 1:1 with Freund's incomplete adjuvant, while the final booster immunizations were done without adjuvant. Intrasplenic immunizations (protocols 3 and 4) were given only one, and performed according to Spitz *et al.* (1984). In all cases the mice were sacrificed 3 days after the final immunization and MAb-producing hybridoma cell lines were obtained by fusing spleen cells with SP 2/0 myeloma cells (Goding, 1983; Schots *et al.,* 1992 *b*).

Immunofluorescence microscopy

Preparation of the J2 of *G. rostochiensis* for indirect immunofluorescence testing of MAbs was essentially according to Atkinson *et al.* (1988) and Hussey (1989 *b*). The J2 were fixed in 2 % paraformaldehyde in PBS for 2 or 3 days. The nematodes were then washed

in distilled water, and drops of concentrated suspension were spread evenly onto aluminium dishes (diameter 2 cm), which were glued to microscope slides for easy manipulation. The drops were allowed to dry at room temperature in a box with silica gel, after which the dishes with nematodes were stored dry at – 20 °C until used. After thawing, the dried J2 were cut into small pieces on their aluminium dish using a razor blade. By cutting parallel lines in three different directions most of the nematodes were cut in two or more pieces. Then the nematode sections were taken up in 1 ml of PBS containing 1 mg/ml proteinase K (Merck, Darmstadt, Germany) and incubated for 20 min with agitation at room temperature. After this, the nematodes were pelleted (2 min 2000 *g*, swing out rotor) and subsequently taken up in cold methanol (1 min; – 20 °C) and cold acetone (2 min; – 20 °C). After removal of the acetone, the nematode sections were resuspended in blocking buffer containing PBS, 10 % horse serum, and 1 mM phenylmethylsulfonyl fluoride. Labelling of the J2 was done in 96 well filtration plates with a pore size of 0.45 μm (MultiScreen-HV, Millipore, Bedford, U.S.A.). To each well 20 μl of nematode suspension (containing approximately 200 sections) was added, followed by 80 μl of hybridoma culture supernatant. After incubation overnight in a moist atmosphere, the nematode sections were washed three times with PBS/0.1 % Tween-20 by applying vacuum to the filtration plates, and they were next incubated in the dark for 2 h with FITC-conjugated rat-anti-mouse IgG (Jackson Immuno Research Laboratories Inc, West Grove, USA), diluted to 1 μg/ml in PBS containing 0.1 % BSA and 0.1 % Tween-20. After three washes with PBS/0.1 % Tween-20, the nematode sections were taken up in 20 μl of distilled water, and transferred to 24 well microscope slides (Cel-Line Associates Inc., New Field, U.S.A.) precoated with 0.1 % poly-L-lysine (2 μl/well). After drying in the dark, 2 μl of anti-quenching agent (0.5 M sodium carbonate buffer pH 8.6 with 0.2 mM p-phenylene-diamine, mixed 1:1 with glycerol) was applied to the wells and a large coverslip was fixed to the slide with dots of nail polish. Specimens were viewed with a 50 × water immersion objective using a Leitz epifluorescence microscope with an L 2.1 or I filter block. The MAbs were scored for specific reactions with structures of the J2, and cell lines producing antibodies of interest were retained. Heavy and light chain isotyping of the MAbs was performed with hybridoma culture supernatants in a DAS ELISA (Schots *et al.*, 1992 *b*).

Immunofluorescence labelling of J2 from *G. pallida*, *H. schachtii* and *M. hapla* with MAbs followed the same procedure as described for *G. rostochiensis*, with the exception that the initial fixations in paraformaldehyde were different : two days for *G. pallida*, and one day for *H. schachtii* and *M. hapla*. Immunofluorescence labelling of J2 from *G. tabacum*, *H. glycines* and *M. incognita* was as described by Goverse *et al.* (1994).

ELISA AND DOT BLOTS

J2 of *G. rostochiensis* were homogenized at 4 °C in 20 mM sodium phosphate buffer pH 8.0 using a small glass mortar and pestle, and the homogenate was stored at – 80 °C until used. After thawing, the sample was centrifuged for 10 min at 10 000 *g* and the supernatant was used. The ELISA was performed according to Schots *et al.* (1992 *b*) with the modification that the blocking buffer was PBS/0.1 % Tween-20/0.5 % BSA, and the incubation buffer was PBS/0.1 % Tween-20/ 0.1 % BSA. For testing of the supernatant fraction, the wells were coated with aliquots of supernatant equivalent to 20 J2, diluted in coating buffer. Assuming a total protein content of 5 ng per J2 (De Boer *et al.*, 1992 *a*) this corresponds to approximately 100 ng of protein per well. The MAbs were tested as hybridoma culture fluids diluted 1:10 in incubation buffer. For the dot blot assay of the supernatant fraction, aliquots of protein equivalent to 20 J2 were diluted in coating buffer and transferred to nitrocellulose membrane using a 96-well dot-blotting apparatus (Schleicher & Schuell, Dassel, Germany). Further labelling of the blots followed the same procedure as the ELISA, with the exception that the alkaline phosphatase activity was detected using NBT/ BCIP (see below).

SDS-PAGE AND WESTERN BLOTTING

Mini SDS-PAGE was performed essentially as described by De Boer *et al.* (1992 *b*). J2 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. Following homogenization the samples were 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. Per minigel approximately 10 000 J2 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, Hercules, U.S.A.). Following electrophoresis in a 13 % acrylamide separating gel, the proteins were transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore Corp., Bedford, USA) using a semi-dry electroblotting 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/0.1 % 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. After washing three times in PBST/1 % milk powder, the strips

Table 1. *Immunofluorescence reaction of monoclonal antibodies raised against antigens from second stage juveniles (J2) of* Globodera rostochiensis. *The immunofluorescence cross-reactivity with other species of sedentary plant parasitic nematodes is listed as "+" if labelling of identical structures was observed, and as "–" if not; (additional) reactions with other structures are indicated between brackets. The following immunizations were used : (1) homogenized whole J2, intraperitoneal immunization; (2) a crude pellet fraction from homogenized J2, intraperitoneal immunization; (3) anterior portions of J4 females, intrasplenic immunization; (4) a high molecular weight protein fraction from J2, intrasplenic immunization. The blank table entries were not determined.*

Antibody	Isotype			Specificity of antibody	Immunofluorescence cross-reactivity					
	Heavy	Light	Immunization	*Globodera rostochiensis*	*Globodera pallida*	*Globodera tabacum*	*Heterodera glycines*	*Heterodera schachtii*	*Meloidogyne hapla*	*Meloidogyne incognita*
MGR 14	IgG1	*K*	1	Subventral oesophageal glands	+	+ (c)	– (c,l)	– (c,l)	–	– (c)
MGR 17	IgG1	*K*	1	Subventral oesophageal glands	+	+ (l)	– (c,l)	– (c,l)	–	– (c)
MGR 19	IgG1	*K*	1	Subventral oesophageal glands	+	+ (c)	–	–	–	–
MGR 21	IgG1	*K*	1	Subventral oesophageal glands	+	+ (c)	– (c,l)	– (c,l)	–	– (c,l)
MGR 31	IgG1	*K*	2	Subventral oesophageal glands	+	+	– (c,l)	– (l)	–	– (c)
MGR 33	IgM	*K*	3	Intestinal lumen	– (gr)	– (ss)	– (ss, sk,l)	– (ss, sk,l)	– (gr)	–
MGR 37	IgM	*K*	4	Intestinal lumen	– (gr)	– (c,m)	– (c,m)	– (c,m)	–	– (c,m)
MGR 3, 7, 20	IgG1	*K*	1	Body-wall muscles (striated)	+			+	+	+
MGR 13, 16	IgG1	*K*	1	Body-wall muscles (uniformly)	+			+	+	+
MGR 24, 25, 26	IgM	*K*	2	Cuticle surface	+			–	–	–
MGR 29	IgM	*K*	2	Cuticle surface	+			– (s)	–	–
MGR 18	IgG1	*K*	1	Genital primordium	+			–	–	–
MGR 34	IgM	*K*	3	Cell nuclei	+			+	+	+

c = cuticle; gr = fine granules; l = oesophageal lumen from stylet base through metacorporal pump chamber; m = fine muscles; s = fine specks; ss = stylet shaft; sk = stylet knobs.

were next incubated individually in alkaline phosphatase conjugated rat-anti-mouse IgG (H + L) (Jackson Immuno Research Laboratories Inc, West Grove, USA) diluted 1:5000 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 $MgCl_2$, 5-bromo-4-chloro-3-indolyl phosphate (0.06 mg/ml) and nitro blue tetrazolium (0.1 mg/ml).

Results

Immunofluorescence screening

The reactivities of the monoclonal antibodies which were obtained with the different immunization protocols are shown in Table 1. Four MAbs (MGR 14, 17, 19, 21) that bound specifically to the subventral oesophageal glands were selected after immunization with a total protein homogenate of J2 (protocol 1). These MAbs reacted with the entire contents of the gland cells, including their extensions and their terminal ampullae (Fig. 1 A). While in the gland extensions it was sometimes possible to distinguish individual secretory granules, the labelling of the gland cell body was usually uniform. The subventral gland nucleus was not labelled by these MAbs. Immunization protocol 1 resulted in 47 MAbs to the body-wall myofilaments, and among these two types of binding patterns were observed. A few representative MAbs of each type were retained. Binding of MGR 3, 7 and 20 invariably followed a clear pattern of fine oblique striations within the muscle cells (Fig. 1 B). This differed from the binding pattern of MGR 13 and 16, which usually showed a more uniform labelling of the myofilament lattice (Fig. 2 A). With the latter MAbs, muscle striations could be seen only in zones of less intense labelling, and these striations appeared to be wider than those observed with MGR 3, 7, or 20. Finally, protocol 1 yielded a MAb (MGR 18) specific for the genital primordium (Fig. 2 B). Labelling predominated at the surface of the primordial cells and usually two large cells with two to four adjacent small cells could be discerned.

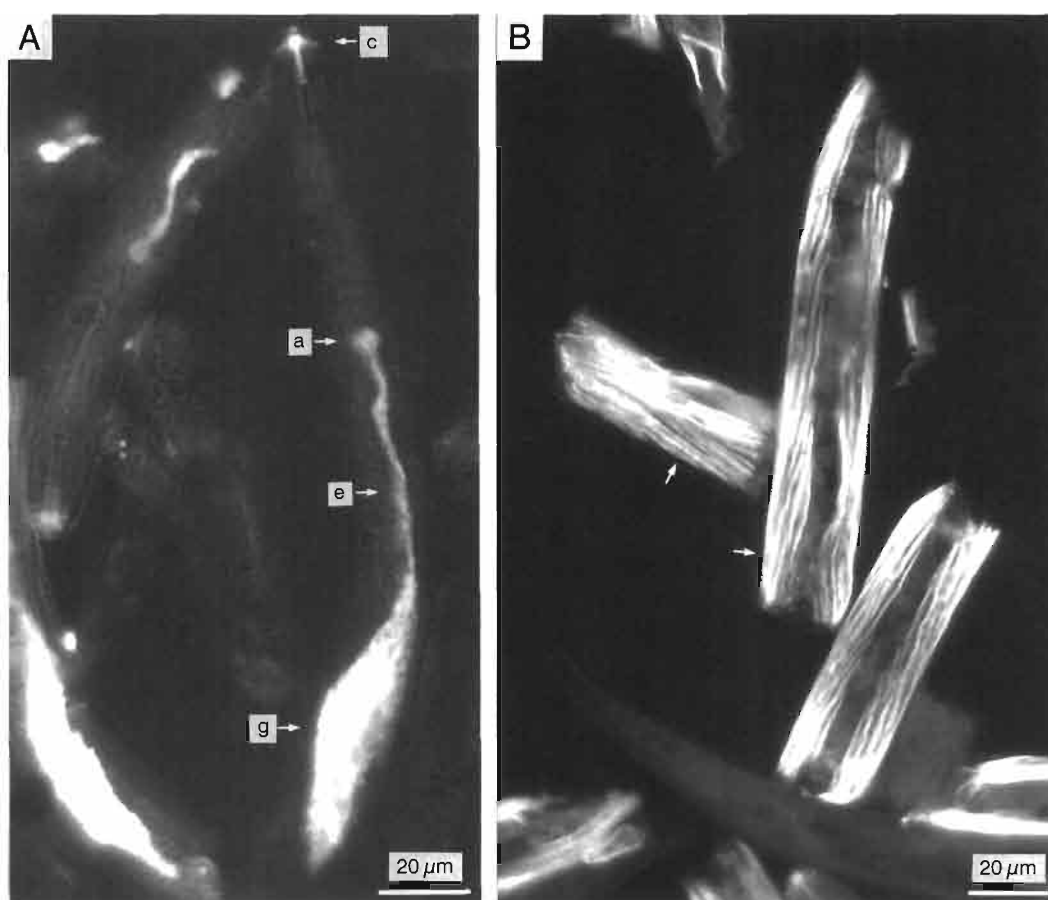

***Fig. 1.** Immunofluorescence labelling of second stage juveniles (J2) of* Globodera rostochiensis *with monoclonal antibodies (MAbs). A : Labelling of the subventral oesophageal glands (g), their extensions (e) and ampullae (a) by MAb MGR 14. At the anterior end of the J2 the cephalic framework (c) is visible due to autofluorescence; B : Labelling of myofilaments in body-wall muscle cells by MAb MGR 7 reveals a fine pattern of oblique striations (arrows).*

Immunization with a crude pellet fraction of J2 (protocol 2) produced one additional MAb against the subventral glands (MGR 31) which showed a staining pattern similar to the MAbs MGR 14, 17, 19 and 21 produced with protocol 1. From this immunization we also obtained four MAbs (MGR 24, 25, 26, 29) that bound to the cuticle surface (Fig. 3 A, B). Although these antibodies showed an even labelling of the entire cuticle surface, differences in staining intensity could be observed between individual J2.

Immunization with anterior portions of fourth stage females (protocol 3) and eluted high molecular weight proteins (protocol 4) yielded very few hybridomas that showed specific labelling of structures within the J2. From these immunizations two MAbs were obtained (MGR 33 and 37) which stained a single thread-like structure in the centre of the J2, starting at the level of the subventral glands (presumably at the position of the oesophageal-intestinal valve) and ending in the tail (Fig. 3 C, 4 A). From this staining pattern it is concluded that these antibodies bind to the intestinal lumen. MGR 34 specifically labelled cell nuclei (Fig. 4 B), which were distributed over the entire length of the J2. Reaction with the large nuclei in the oesophageal glands or in the genital primordium was not observed with this antibody.

Cross-reactivity with other species

The MAbs raised against *G. rostochiensis* were tested with immunofluorescence microscopy for cross-reactivity with J2 of other sedentary plant parasitic nematodes (Table 1). All five MAbs specific to the subventral glands in *G. rostochiensis* also reacted with epitopes in the subventral glands of *G. pallida* and *G. tabacum*. In *G. tabacum* the binding pattern was often granular as opposed to a uniform staining in *G. rostochiensis* and

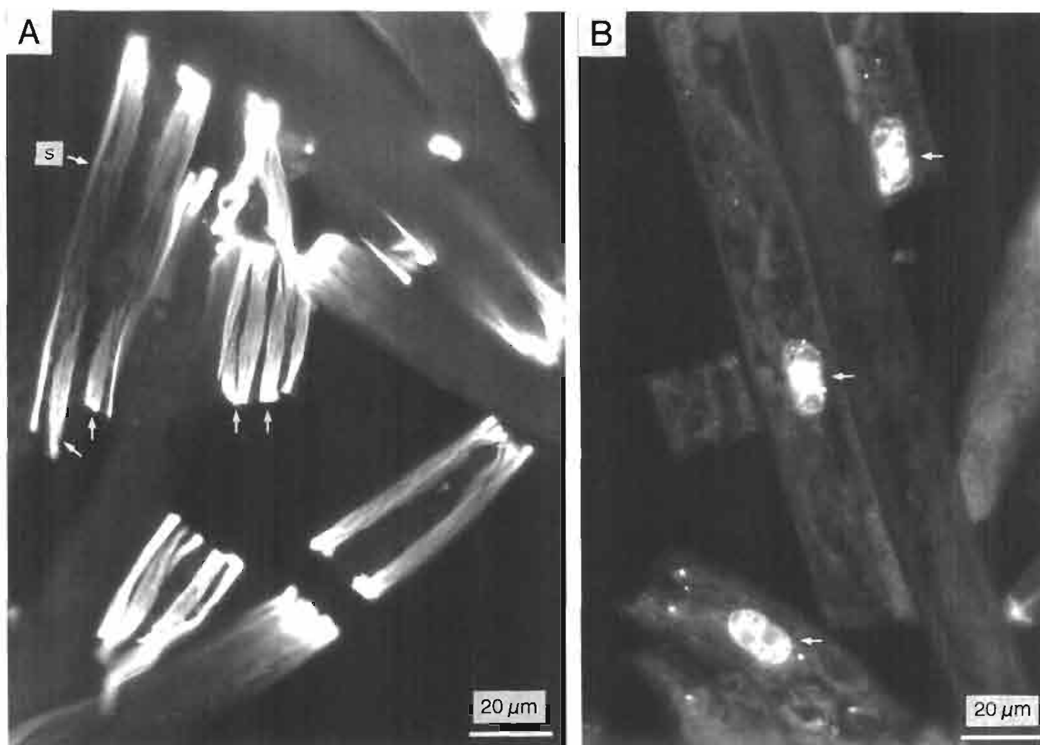

Fig. 2. *Immunofluorescence labelling of J2 of* Globodera rostochiensis *with MAbs. A : Labelling of myofilaments in body-wall muscle cells (arrows) by MAb MGR 13. The myofilament lattice is usually stained more or less uniformly, and striations(s) are seen only occasionally in zones of less intense staining; B : Labelling of genital primordia (arrows) by MAb MGR 18.*

G. pallida. In the *Heterodera* and *Meloidogyne* species tested, no binding to the subventral glands was observed, although reactions with the cuticle surface or the oesophageal lumen often occurred. The MAbs specific to the intestinal lumen of *G. rostochiensis* (MGR 33 and 37) did not react with the intestinal lumen in J2 from any of the other species. Instead, binding to various other structures occurred such as the stylet, the cuticle or the body-wall muscles. The MAbs that bound to the body-wall musculature and to cell nuclei bound to the same structures in the other species tested. MGR 24, 25, 26, and 29 all showed cross reactivity with the cuticle surface of *G. pallida* but did not bind to the cuticle surface of *H. schachtii, M. incognita* or *M. hapla.* MAb MGR 18 bound only weakly to the genital primordium in *G. pallida,* and did not bind to the genital primordia of the other species tested.

WESTERN BLOTTING

The MAbs presented in Table 1 were tested for reactivity with proteins from preparasitic J2 of *G. rostochiensis* which were separated by SDS-PAGE and blotted onto PVDF membrane. None of the MAbs to the subventral glands or to the intestinal lumen gave a positive reaction. Also MGR 18, 24, 25, 29 and 34 showed no reaction on Western blots. MGR 3 and 7 (Fig. 5) and MGR 20 (not shown) reacted with a major protein band with an apparent molecular mass of approximately 39 kDa. MGR 13 and 16 (Fig. 5) both reacted intensely with a protein band positioned above the 106 kDa molecular weight marker. In addition, MGR 13 stained several minor bands below 106 kDa. MGR 26 identified a series of protein bands with molecular masses of 39 kDa and more (Fig. 5).

ELISA AND DOT-BLOT

Because the subventral gland MAbs did not bind to SDS-denatured proteins on Western blots of *G. rostochiensis,* their reactivity with native protein homogenates from preparasitic J2 was tested using an ELISA and a dot-blot assay (Table 2). With three subventral gland MAbs (MGR 14, 17 and 31) antigen could now be detected in the protein homogenate. Control tests performed with four MAbs to body-wall muscle proteins (MGR 7, 13, 16, and 20) all gave positive reactions. Similar tests with MGR 18, 24, 25, 26, 29, 33, 34 and 37 (not in table) were negative, both in the ELISA and dot-blot assay. The subventral gland MAbs were tested

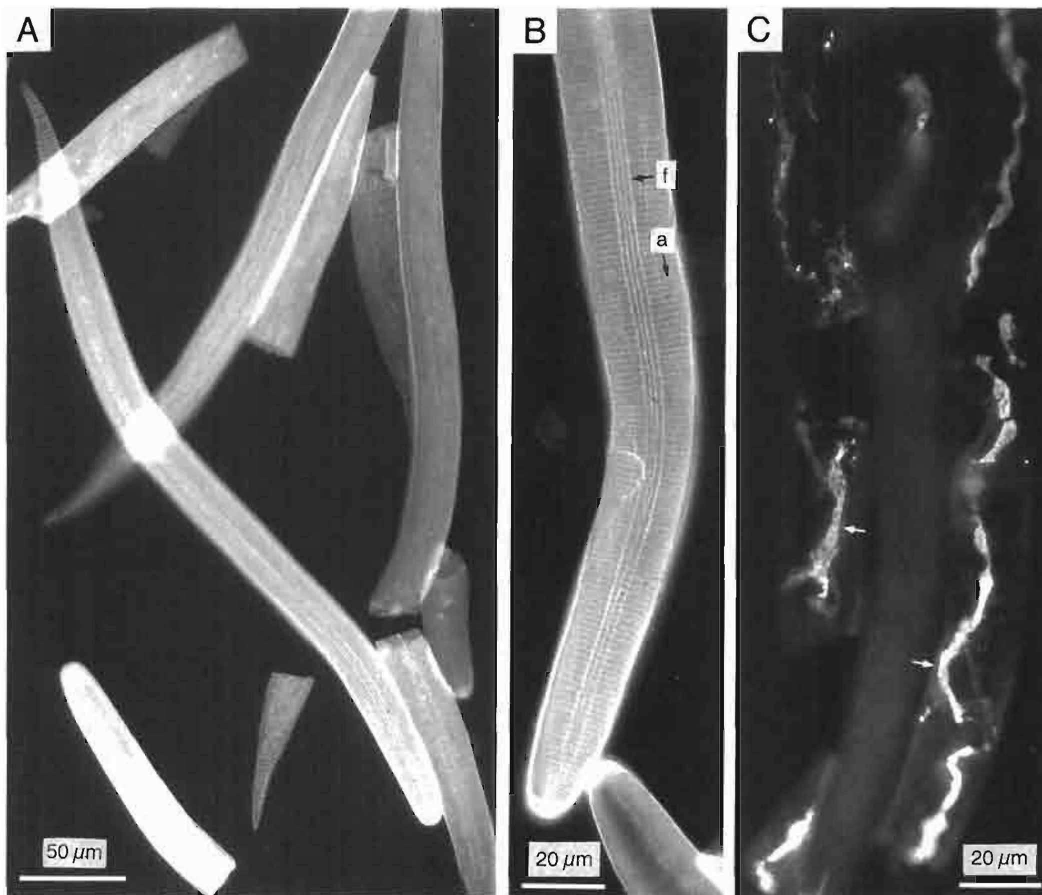

Fig. 3. *Immunofluorescence labelling of J2 of* Globodera rostochiensis *with MAbs. A : Labelling of the cuticle surface by MAb MGR 29. Note the difference in labelling intensity between individual J2; B : Detail of cuticle surface labelling by MAb MGR 29. Both the cuticle annulations (a) and the lateral field lines (f) have become visible; C : Staining of the intestinal lumen (arrows) by MAb MGR 33.*

Table 2. *Reactivity of monoclonal antibodies with native protein homogenates from J2 of* Globodera rostochiensis; *(+) positive reaction; (–) negative reaction.*

Antibody	Specificity	ELISA	Dot-blot
MGR 14, 17	Subventral glands	–	+
MGR 31	Subventral glands	+	+
MGR 19, 21	Subventral glands	–	–
MGR 7, 13, 16, 20	Body-wall muscles	+	+

also on dot blots of native homogenates of parasitic juveniles of *G. rostochiensis.* It was found that the reactivity of MGR 14, 17, and 31 had disappeared in the parasitic J2 stage, and did not reappear in later (J3, J4) parasitic stages. Control tests with muscle antibody MGR 7 remained positive in these parasitic stages.

Discussion

Following immunization with a total protein homogenate from J2 (protocol 1) MAbs were raised against the subventral oesophageal glands, the body-wall muscle fibres, and the genital primordium of *G. rostochiensis.* Similar antibodies were produced by Atkinson *et al.* (1988) when they immunized mice with a total protein homogenate from J2 of *H. glycines.* While Atkinson *et al.* (1988) also identified a MAb specific for the dorsal oesophageal gland following their immunization with

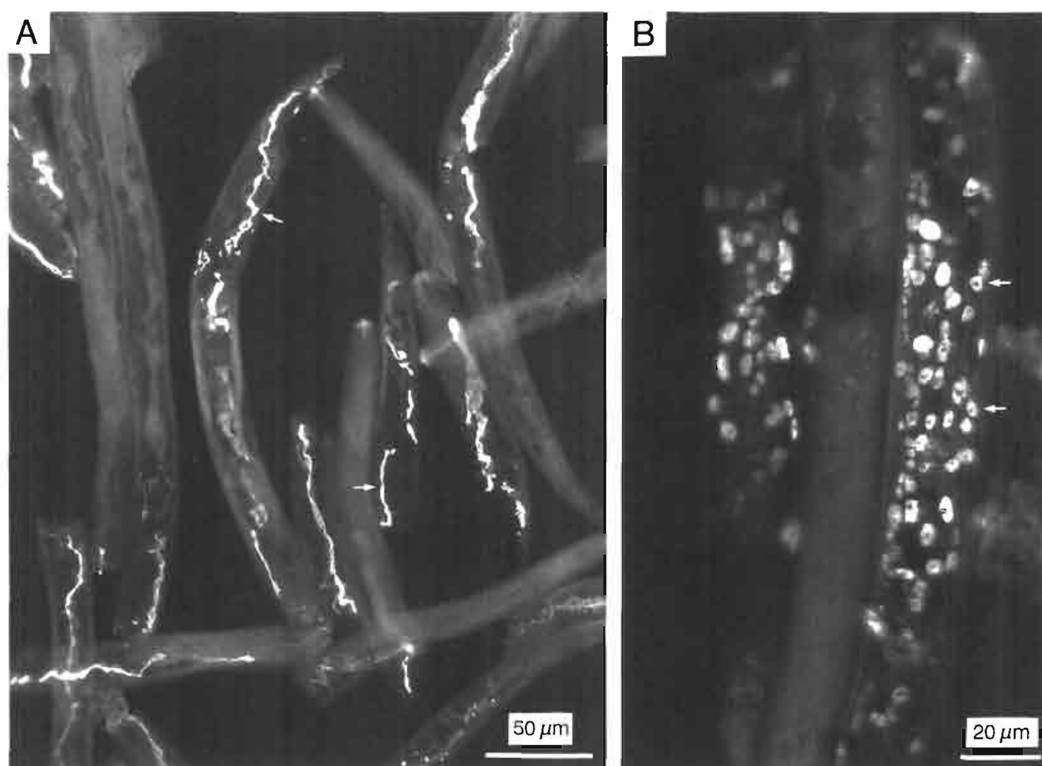

Fig. 4. *Immunofluorescence labelling of J2 with MAbs. A : Staining of the intestinal lumen (arrows) by MAb MGR 33 in* Globodera rostochiensis; *B : Staining of cell nuclei (arrows) with MAb MGR 34 in* Heterodera schachtii. *The nucleoli remain unstained and are visible as a dark spots within the nuclei.*

homogenized J2, we did not observe this binding activity during the screening of the hybridomas.

Because the immunization with homogenized J2 had not produced MAbs specific for the dorsal oesophageal gland of *G. rostochiensis,* mice were also immunized with three samples that were expected to be enriched in antigens from the dorsal gland cell. The crude pellet fraction derived from J2 (protocol 2) was expected to contain secretory granules from both the dorsal and the subventral glands (Reddigari *et al.,* 1985). The intrasplenic immunization with anterior portions of J4 females (protocol 3) followed an immunization schedule which was successful in generating MAbs against the dorsal gland in *M. incognita* (Davis *et al.,* 1992). Finally, the high molecular weight protein sample eluted from an SDS-PAGE gel of J2 (protocol 4) was expected to contain secretory components of the oesophageal glands (Hussey *et al.,* 1990). However, none of these immunizations produced MAbs to the dorsal gland of *G. rostochiensis.* Because autofluorescence of the cuticle prevented immunofluorescence testing of antibodies with J4 females, the MAbs from protocol 3 were screened with J2. It is therefore possible that a difference in the expression of dorsal gland antigens between J4 and J2 may have prevented the detection of dorsal gland MAbs in this experiment.

The MAbs to the subventral oesophageal glands (MGR 14, 17, 19, 21 and 31) reacted with the entire contents of the gland cells, including the gland extensions to the median bulbus. This binding pattern is similar to that observed for MAbs to the subventral glands of J2 of *H. glycines* (Atkinson *et al.,* 1988; Goverse *et al.,* 1994). The MAbs raised by Goverse *et al.* (1994) bound specifically to secretory granules within the gland cell, and several of these MAbs were shown to react with induced stylet secretions released by J2 of *H. glycines.* With our MAbs to the subventral glands of *G. rostochiensis* binding to individual secretory granules could sometimes be observed in the gland extensions. It is therefore possible that one or more of the subventral gland MAbs presented here react with a secretory product in the gland cells. In the cross-reactivity tests the subventral gland MAbs of *G. rostochiensis* only showed binding to the subventral glands of other *Globodera* spe-

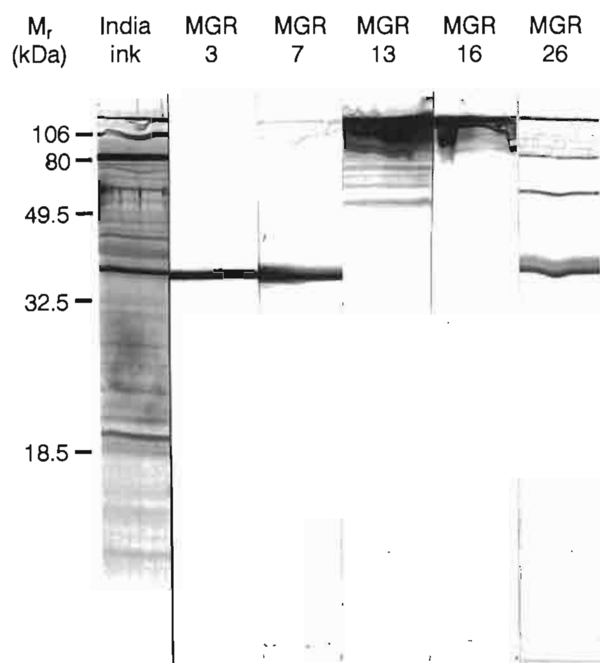

Fig. 5. *Western blot of proteins from second stage juveniles (J2) of* Globodera rostochiensis *stained with monoclonal antibodies to the body-wall muscle filaments (MGR 3, 7, 13 and 16) and to the cuticle surface (MGR 26).*

cies, and therefore the epitope that they recognize can be considered genus specific.

None of the subventral gland MAbs reacted with protein bands on Western blots of J2 of *G. rostochiensis.* Since MGR 14, 17 and 31 did react with supernatants of native protein extracts of J2, it can be concluded that their corresponding antigens are water soluble, and that their epitopes are susceptible to denaturation by SDS or mercaptoethanol. MGR 14, 17 and 31 did not bind to native protein homogenates of parasitic stages of *G. rostochiensis.* This may indicate that in these stages the subventral glands have switched to producing other secretory products (Atkinson & Harris, 1989; Davis *et al.,* 1994)or that the subventral glands are no longer active in parasitic juveniles (Endo, 1987).

Two types of MAbs to the body-wall muscle filaments were identified. MGR 3, 7, and 20 stained thin oblique striations within the myofilament lattice (see also : Francis & Waterston, 1985), while MGR 13 and 16 showed a more even staining of the muscle filaments. This difference in immunofluorescence staining reaction corresponded with a different reaction on Western blots of J2 : the former MAbs all bound to a 39 kDa protein, while the latter MAbs stained a major protein band with a molecular mass of > 106 kDa. These molecular weight values indicate that MGR 3, 7 and 20 may bind to tropomyosin and that the antigen identified by MGR 13 and 16 may be the myosin heavy chain subunit (De Boer *et al.,* 1992 *a*). The staining of several additional thin protein bands by MGR 13 possibly reflects a susceptibility of this antigen to proteolysis during sample homogenization. An acute proteolytic susceptibility during homogenization has been reported for myosin of *Caenorhabditis elegans* (Harris & Epstein, 1977).

Genetic transformation with genes coding for antibodies (or fragments thereof) may offer a new route for introducing resistance to phytopathogens in plants (Benvenuto *et al.,* 1991; Schots *et al.,* 1992 *a*). Binding of *in planta* expressed antibodies to target molecules of phytopathogens can inhibit the function of these molecules and thus disturb the host-parasite interaction (Tavladoraki *et al.,* 1993). Suitable target molecules of endoparasitic nematodes are the secretions from the oesophageal glands (Hussey, 1989 *a*). The MAbs to the subventral glands of *G. rostochiensis* which have been presented here form a starting point for engineering resistance to cyst-nematodes in potato. They can be used for the purification and identification of antigens from the subventral gland cells. In addition, it may be possible that some of these MAbs are suitable for *in planta* inhibition of subventral gland secretions that are released by preparasitic J2 in the roots.

In conclusion, we have generated a panel of MAbs reacting with a variety of antigens of *G. rostochiensis.* These antibodies will be used in future molecular and structural studies concerning *G. rostochiensis* and its development in the host plant. Several of these MAbs showed cross-reactivity with other sedentary nematodes in immunofluorescence assays, and it can therefore be expected that these MAbs will be useful also for the study of similar antigens in these related nematode species.

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