Isolation and characterization of secretions from the plant-parasitic nematode *Globodera pallida*

L. H. DUNCAN[1]*, L. ROBERTSON[1], W. M. ROBERTSON[1] *and* J. R. KUSEL[2]

[1] *Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA*
[2] *Division of Biochemistry and Molecular Biology, IBLS, University of Glasgow, Glasgow G12 8QQ*

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SUMMARY

Electrophoresis of secretions collected from *Globodera pallida* revealed a smeared region between 25 and 50 kDa, and a single band of < 20 kDa. The secretions were used to raise an antiserum (LW1). Immunoblotting of parasite homogenates with LW1 differentiated *G. pallida* from its sibling species *G. rostochiensis* and revealed differences between different populations of *G. pallida* and *G. rostochiensis*. Indirect immunofluorescence studies with LW1 indicated that at least some of the secretions were surface localized and that antibody binding to the nematode surface was periodate sensitive. Periodate sensitivity indicated that these differences could be due to glycosylation differences. Glycosylation differences were also detected by blotting nematode homogenates with the lectin wheat germ agglutinin (WGA). WGA was also able to differentiate between *G. rostochiensis* which gave 2 bands at 130 kDa and 110 kDa, and *G. pallida* which produced 2 bands present at 120 kDa and 110 kDa. Further localization studies using immunoelectron microscopy demonstrated that antibody binding could be seen to secretions found in the pump chamber of the metacorpal bulb at the base of the stylet. From further specimens it could be observed that the contents of the subventral glands were heavily labelled, indicating that the material seen in the metacorpal bulb had originated from the subventral glands.

Key words: *Globodera pallida*, *Globodera rostochiensis*, nematode, secretions, amphids, surface, subventral glands.

INTRODUCTION

The potato cyst nematodes (PCN) *Globodera pallida* and *G. rostochiensis* are major pathogens of the commercial potato *Solanum tuberosum*. The toxicity of many of the nematicides routinely used to control plant-nematodes has caused problems, and alternative control strategies based on disruption of the nematode life-cycle are therefore being sought. One subject that has received attention is the role of nematode secretions in the infection process, with particular interest being focused on the role of oesophageal gland and amphidial secretions.

It is generally accepted from ultrastructural and neuro-anatomical studies that the paired amphids situated at the anterior end of the nematode are the main chemosensory organs of these animals (Ward, 1973; Lewis & Hodgkin, 1975; Bargmann, Thomas & Horowitz, 1990). More recently Riga *et al.* (1995*a*) used direct electrophysiological recordings to demonstrate the chemosensory nature of the amphids. Various roles have been postulated for the secretions which are found in the amphidial pores of plant-parasitic nematodes. These include protection of the sensory dendrites at the base of the pore (Altner, 1977), capture and transport of chemotactic stimuli to the sensilla membranes (Zuckerman, 1983) and osmoregulation (Nelson & Riddle, 1984). Amphidial secretions are also thought to induce the phytoalexin response in resistant hosts (Forrest & Robertson, 1986). Several amphidial proteins have been identified to date (Stewart *et al.* 1993; Stewart, Perry & Wright, 1993; Riga *et al.* 1995*b*; Curtis, Segers & Evans, 1996) although the functions of such proteins are unknown.

Other nematode secretions that may participate in the host–parasite interaction include those originating from the oesophageal glands, the excretory/secretory system and other body pores (Jones & Robertson, 1997). Oesophageal gland secretions are widely considered to be involved in the onset of parasitism, in particular the establishment of the feeding site (Jones & Northcote, 1972; Wyss & Zunke, 1986; Wyss, Grundler & Munch, 1992). Completion of the plant-parasitic nematode life-cycle is dependent upon the formation of a feeding site. Following invasion of the root the juvenile nematodes move towards the vascular cylinder where each selects a single cell. Oesophageal secretions are injected into the cell through the nematode stylet, whereupon the cell undergoes a series of changes leading to the formation of an enlarged, multinucleate and cytoplasmically dense syncytium (Jones, 1981; Hussey, 1989*a*). The syncytium remains active and provides nutrients required by the nematode for the duration of its development. Induction and maintenance of the syncytium involve significant changes in plant gene expression which

* Corresponding author: Scottish Crop Research Institute, Invergowrie, Dundee DD2 5DA. Tel: 01382 562731. Fax: 01382 562724. E-mail: ldunca@scri.sari.ac.uk

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are induced as a result of nematode infection (Gheysen & Van Montagu, 1995). Only 1 subventral oesophageal gland secretory protein, a 212 kDa glycoprotein from the root-knot nematode *Meloidogyne incognita* has been isolated (Hussey, Paguio & Seabury, 1990). Despite much work in this area, the function of this and other oesophageal gland proteins is still unknown. Similarly, the functions of the excretory and other body pore secretions are open to question, although it has been suggested that they may be a source of the surface coat seen in some nematodes (Spiegel & Robertson, 1988; Spiegel *et al*. 1991).

Using a modification of the staining procedure originally described by Premachandran *et al*. (1988) to stain nematode structures and secretions, it has been possible to collect a mixture of secretions from *G. pallida* for the production of antiserum. This antiserum was subsequently used for Western blotting and immunolocalization studies.

MATERIALS AND METHODS

All materials were obtained from Sigma unless otherwise stated.

Parasite material

Cysts of *G. pallida* were grown on potato cv. Maris Piper, and *G. rostochiensis* on potato cv. Désirée. In this study cysts produced from *G. pallida* pathotype 2/3 (populations E, Luffness, Loc, Newton and Gourdie) and *G. rostochiensis* pathotypes Ro1, Ro2, Ro3 and Ro5 were used. Infective 2nd-stage juveniles (J2) of *G. pallida* and *G. rostochiensis* were obtained by pre-soaking cysts containing unhatched eggs for 7 days in tap water. Eggs were then stimulated to hatch by soaking cysts in potato root diffusate (PRD) from the potato cv. Désirée (Forrest & Farrer, 1983). Hatched juveniles were collected daily and used immediately for experiments requiring live nematodes, or were washed in phosphate-buffered saline (PBS) and stored as a pellet at −20 °C for use in other experiments.

Collection of secretions

Approximately 50000 freshly hatched J2 were washed in sterile distilled water (SDW) and the pellet suspended in a final volume of 0·5 ml of SDW. An equal volume of 40% methanol containing 0·1% Coomassie Blue R250 was added to the nematode suspension, giving a final concentration of 0·05% Coomassie Blue R250 in 20% methanol. The solution was placed in a shallow dish, covered with a large cover-slip with a smear of nail varnish on it and left overnight at room temperature. Secretions were separated from juveniles by passing the suspension through a modified syringe which had polyester mesh (Lockertex, Walsall, UK) of pore size 15 μm welded across the syringe barrel (Forrest, 1987). The collected secretions were concentrated and washed by centrifugation (bench centrifuge, 30 min) in 20% methanol to remove excess Coomassie Blue, with a final wash in distilled water. Secretions were used either for electrophoresis, or for the production of antiserum.

Homogenate preparation

Nematode homogenates were prepared by sonication (sonicator type 7532A, Dawe Instruments Limited) at 50 W for 4 periods of 15 sec, with 30 sec intervals between sonications to allow cooling, followed by centrifugation. All steps were performed in the presence of the protease inhibitors phenylmethylsulfonyl fluoride (PMSF: 1 μM), Nα-*p*-tosyl-L-lysine chloromethyl ketone (TLCK: 0·5 μM) and N-tosyl-L-phenylalanine chloromethyl ketone (TPCK: 0·5 μM). The supernatant fraction was used as homogenate for electrophoresis and immunoblotting studies. Protein concentrations were determined using the method of Bradford (1976).

Production of antiserum LW1

Secretions collected from *G. pallida* Luffness population as described earlier were used to immunize a rabbit. Secretions were collected from approximately 50000 nematodes in 500 μl of phosphate-buffered saline (PBS), and this was made up to 1 ml with Freund's Complete Adjuvant (FCA). Subcutaneous injections were administered to a rabbit over multiple sites and the rabbit boosted in a similar manner (using Freund's Incomplete Adjuvant instead of FCA) 6 weeks later. The rabbit was bled 4 weeks after the final injection, the antiserum isolated by centrifugation and stored in aliquots at −20 °C.

SDS–PAGE and immunoblotting

Electrophoresis was performed on pre-cast 10–20% gradient gels (Bio-Rad, UK) using the Mini-Protean II system (Bio-Rad, UK) according to the method of Laemmli (1970). It should be noted that due to the method used in obtaining the secretions it was not possible to quantify the amount of protein loaded onto the gel. Proteins were either silver-stained (Tsai & Frasch, 1982) or transferred onto nitrocellulose paper for immunoblotting using the method of Towbin, Staehelin & Gordon (1979). For immunoblotting, 25 μg of nematode homogenate per lane was loaded. Proteins were detected by incubation of the nitrocellulose paper with the primary antibody (antiserum LW1, 1:400) followed by horse-radish peroxidase-conjugated anti-rabbit IgG (1:400) as secondary antibody. All dilutions and washings were performed in PBS containing 0·1% Tween 20. Detection of bound antibody was carried out using 3,3-diaminobenzidine (30 mg dissolved in 100 ml of

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PBS plus 20 μl of 6% hydrogen peroxide). Molecular weights of the separated proteins were calculated by comparison with Sigma molecular weight markers (low and high range) for the silver-stained gel, and Kaleidoscope markers (Bio-Rad, UK) for the immunoblots.

Lectin blotting

For lectin blotting with wheat germ agglutinin (WGA), 25 μg of nematode homogenate were electrophoresed and transferred onto nitrocellulose paper as described above. Lectin blotting was performed using the Boehringer Mannheim Glycan Differentiation Kit according to the manufacturer's instructions. Briefly, after transfer, nitrocellulose paper was blocked in 0·5% (w/v) of the blocking reagent provided with the kit, either at room temperature for 1 h, or overnight at 4 °C. After extensive washing the nitrocellulose paper was incubated with digoxigeninylated wheat germ agglutinin (WGA, 1:10) for 1 h at room temperature, then washed as before. Bound lectin was detected using anti-digoxigenin serum conjugated to alkaline phosphatase (1:1000, Boehringer Mannheim), for 1 h at room temperature. Detection of bound antibody was performed using BCIP/NBT FAST tablets.

Indirect immunofluorescent antibody test (IFAT)

Indirect immunofluorescence was carried out on live *G. pallida* with antiserum LW1 as primary antibody (1:100) for 1 h. Detection of bound antibody was performed by incubating nematodes with goat anti-rabbit IgG conjugated to tetramethylrhodamine B isothiocyanate (TRITC) (1:300) for 30 min. All washings and dilutions were carried out in PBS. After IFAT nematodes were fixed in 1% formaldehyde for 15 min before viewing using a Leitz Ortholux microscope.

Periodate treatment

Periodate treatment was carried out on fixed nematodes prior to IFAT. Nematodes were fixed in 1% formaldehyde for 15 min then washed in 50 mM sodium acetate buffer, pH 5·8. Nematodes were exposed to 10 mM sodium metaperiodate in the same buffer for 1 h, with exposure to buffer alone as control.

Electron microscopy

Pre-embedding immunogold labelling of specimens. Living nematodes were surface labelled with LW1 using the same procedure as described for indirect immunofluorescence studies with all washings and dilutions performed in PBS. Immunogold labelling of bound antibody was performed by incubating specimens with goat anti-rabbit IgG conjugated to colloidal gold (1:50, 10 nm gold) for 1 h at room temperature. Specimens were then fixed in 3% (v/v) glutaraldehyde for 1 h, rinsed 3 times and placed in 1% osmium tetroxide for 30 min with all washings and dilutions performed in 0·1 M phosphate buffer, pH 6·8. Samples were washed again and embedded in 1% Water Agar (Oxoid). Blocks containing individual nematodes were cut out and processed through a graded ethanol series with final changes in 100% propylene oxide. Finally, samples were placed in Emix resin (Agar Scientific) at 35 °C for a minimum of 2 h, before polymerization at 70 °C overnight.

Post-embedding immunogold labelling of cut sections. Samples were fixed in 2% (w/v) paraformaldehyde for 1 h then rinsed 3 times and embedded in 1% (w/v) water agar (Oxoid) with all washings and dilutions carried out using 0·1 M phosphate buffer, pH 7·2. Blocks containing individual specimens were cut and dehydrated in a graded ethanol series with 2 final washes in 100% propylene oxide. Blocks were then placed in LR White resin (Fisons) overnight at room temperature, before polymerization at 55 °C overnight. In an alternative fixation protocol which was used to obtain better preservation of oesophageal gland secretory granules, samples were fixed in 2% (w/v) paraformaldehyde plus 3% (v/v) glutaraldehyde overnight and processed as before except that samples were placed in Emix resin (Agar Scientific) at 35 °C for at least 2 h before polymerization at 70 °C overnight.

Silver-grey sections cut on a Reichert Ultracut microtome and collected on plastic-coated copper grids were incubated in PBS containing 1 mg/ml BSA and 5% goat serum before incubation with antiserum LW1 (1:10000) in PBS containing 0·1% BSA, 0·05% Tween 20, 1% goat serum and 2·08% NaCl for 2 h at room temperature. Grids were then washed in TBS containing 0·2% (w/v) BSA then again with TBS containing 1% BSA, before incubation with goat anti-rabbit IgG conjugated to colloidal gold (1:50, 10 nm gold) in TBS containing 1% BSA for 2 h at room temperature.

All sections were counterstained with a saturated solution of uranyl acetate in 50% (v/v) ethanol for 2 min followed by staining with lead citrate for 7 min. Sections were viewed on a JEOL 1200EX electron microscope at 80 kV. Photographs of sections were taken using Agfa Scientia EM Film.

RESULTS

Production of secretions

The staining procedure resulted in the production of copious amounts of stained secretions from the nematode (Fig. 1A). The production of secretions using this method was also observed using time-lapse photography which showed that secretions

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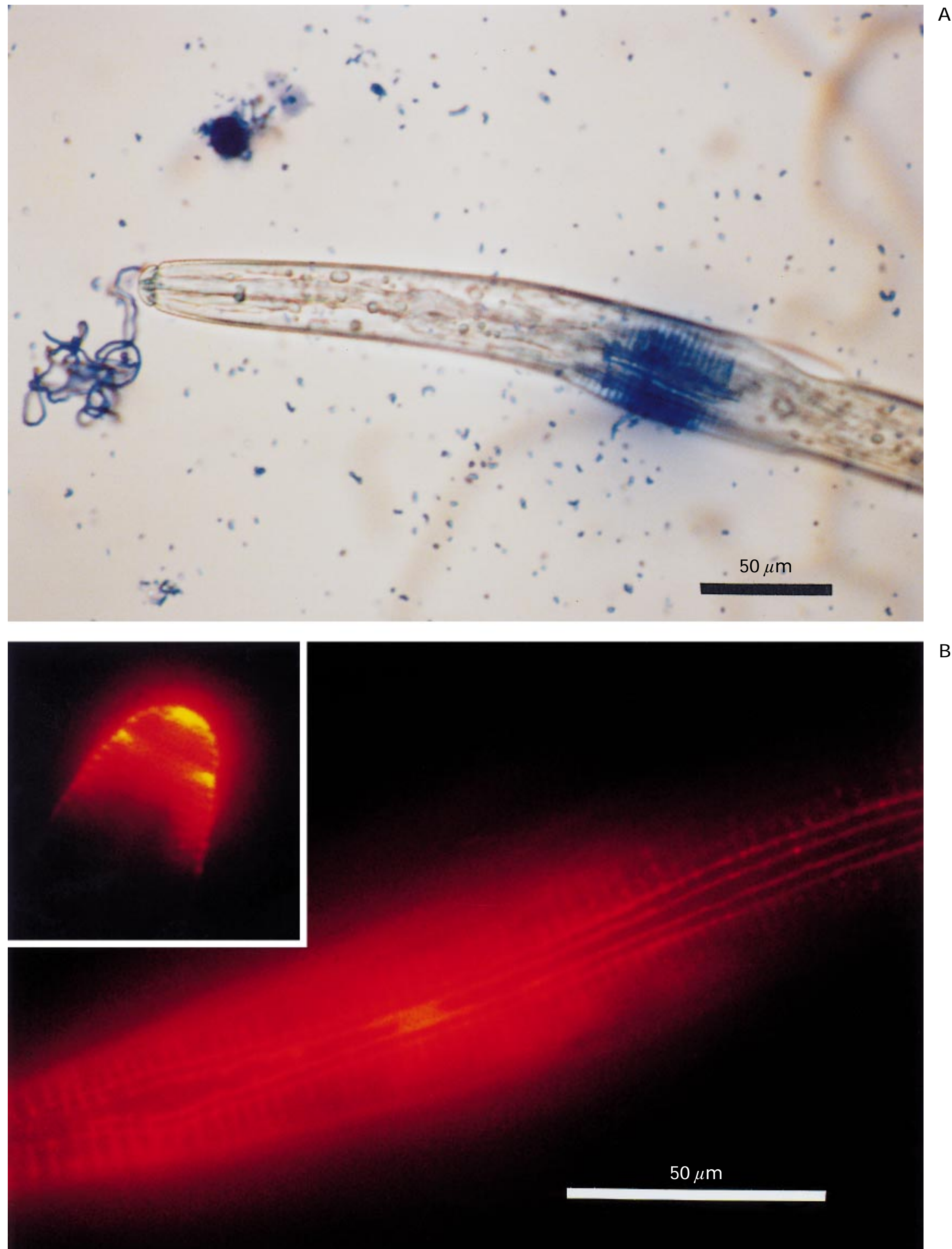

Fig. 1. (A) Photomicrograph showing the production of stained secretions from the anterior of the nematode and the excretory pore. (B) Indirect immunofluorescence of *Globodera pallida* Luffness population. Nematodes probed with antiserum LW1 show binding to the head region (insert), and the body.

appeared from the excretory pore after approximately 6 h incubation, followed by secretions from the anterior end of the nematode about 4 h later.

Indirect immunofluorescence

Indirect immunofluorescence of juveniles from Luffness population using antiserum LW1 revealed that secretions were present on the surface of the nematode. Binding occurred as a distinctive 'cap' at the head of the nematode (Fig. 1B–insert), and also along the body of the nematode (Fig. 1B).

Indirect immunofluorescence of other populations of *G. pallida* and *G. rostochiensis* was also performed. Some differences were apparent. For example, with *G. pallida* Loc population binding occurred over a greater extent of the body whereas with pathotype Ro3 of *G. rostochiensis* binding was restricted to a

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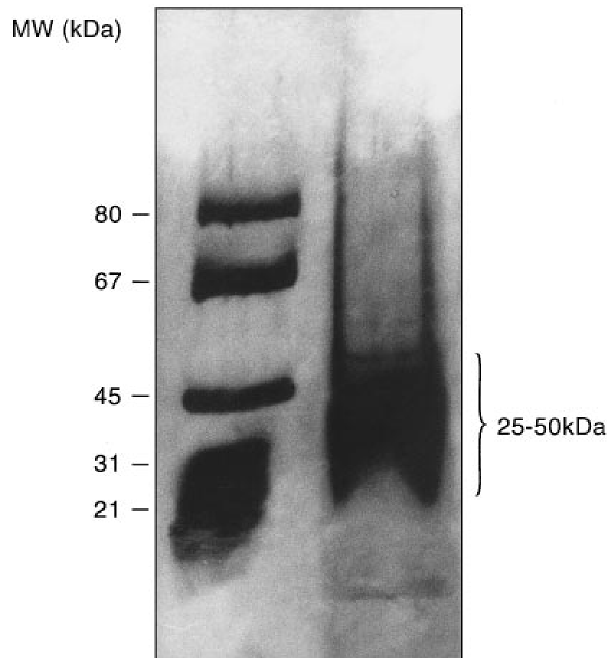

Fig. 2. SDS–PAGE of secretions collected from *Globodera pallida* Luffness population. Secretions were collected from approximately 50000 nematodes and electrophoresed on a 10–20% gradient gel. Lanes were loaded as follows: lane 1 – molecular weight markers, lane 2 – secretions from *G. pallida*.

small region of the head. These results show that at least some of the secretions produced by PCN are surface localized.

Control nematodes were probed with normal rabbit serum (NRS) and the TRITC conjugate to ensure that the results were not due to non-specific binding. Although NRS did not bind to the nematode surface, fluorescence could be seen in the region of the amphids. Recent experiments with 2 other normal rabbit sera (collected from 2 different rabbits) gave the same result, indicating that a specific interaction was occurring. Pre-incubation of nematodes with 100 mM *N*-acetyl-D-galactosamine eliminated binding of NRS to the amphids. No surface fluorescence could be seen with the TRITC conjugate alone.

SDS–PAGE of collected secretions

Electrophoresis of collected secretions revealed a smeared region between 25 and 50 kDa, which is perhaps indicative of a high carbohydrate content and a band at < 20 kDa (Fig. 2).

Periodate treatment

Incubation of nematodes with 10 mM sodium metaperiodate abolished antibody binding to the nematode surface, indicating that the epitopes recognized by antiserum LW1 were carbohydrate in nature although binding to the amphids was still observed. Periodate treatment was also performed on nematodes probed with NRS. In these nematodes, binding of NRS to the amphids could still clearly be seen indicating that the amphidial components involved in the interaction with NRS were not carbohydrate in nature.

Immunoblotting

Homogenates prepared from several populations of *G. pallida* and *G. rostochiensis* were separated by electrophoresis and immunoblotted with antiserum LW1 (Fig. 3). Differences were observed between the two species. In *G. pallida* a band at 30 kDa (arrowed) was absent from *G. rostochiensis*, whereas a major band (arrowed) of approximately 40 kDa present in *G. rostochiensis* was absent from *G. pallida*. As well as inter-species differences, intra-specific differences were detected with population (pathotype) Ro5 lacking a band of 65 kDa (asterisk) which is present in all the other *G. rostochiensis* pathotypes.

Lectin blotting with WGA

Homogenates prepared with populations of *G. pallida* and *G. rostochiensis* were separated by electrophoresis and the proteins blotted with WGA (Fig. 4). Differences were apparent between *G. pallida* and *G. rostochiensis*, and between different populations of *G. pallida*, but not of *G. rostochiensis*. All populations of *G. rostochiensis* had 2 bands of molecular weight 130 and 110 kDa respectively. In contrast, *G. pallida* Loc, Newton and Gourdie populations had 2 bands at 130 and 120 kDa. *G. pallida* Luffness and E populations also differed from the other *G. pallida* populations tested, Luffness having a major band at 135 kDa (asterisk) and a smaller band of 110 kDa, whereas E had a single band at 110 kDa. Binding of WGA was abolished by pre-incubation of WGA with 200 mM *N*-acetyl-D-glucosamine.

Electron microscopy studies

Pre-embedding immunogold labelling of the nematode surface. Sections were cut through the anterior end of the nematode to examine binding of LW1. When sections were examined, however, only very sparse labelling of the area around the head was seen. It was noticed, however, that clumps of labelled material were seen lying away from the surface of the nematode. This was in conflict with the results obtained using indirect immunofluorescence techniques as fluorescence microscopy results indicated that this region would be heavily labelled.

Post-embedding immunogold labelling of cut sections

Sections were cut through different regions of the anterior ends of the specimen. Sections cut through

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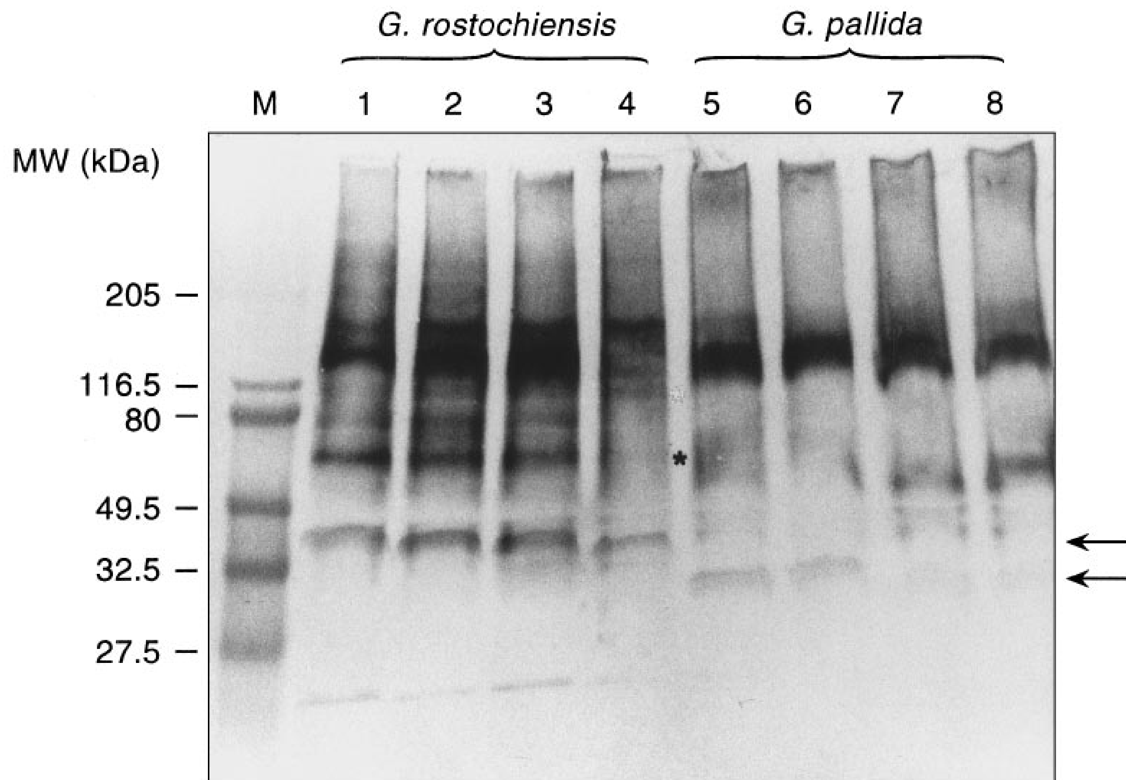

Fig. 3. Western blot analysis of homogenates of populations of *Globodera pallida* and *G. rostochiensis*. Parasite homogenate (25 μg loaded/lane) was probed with antiserum LW1 as described in the Materials and Methods section. Lanes 1–4 show *G. rostochiensis* pathotypes Ro1, Ro3, Ro4 and Ro5 respectively. Lanes 5–8 show *G. pallida* Pa2/3 populations E, Luffness, Loc and Newton respectively.

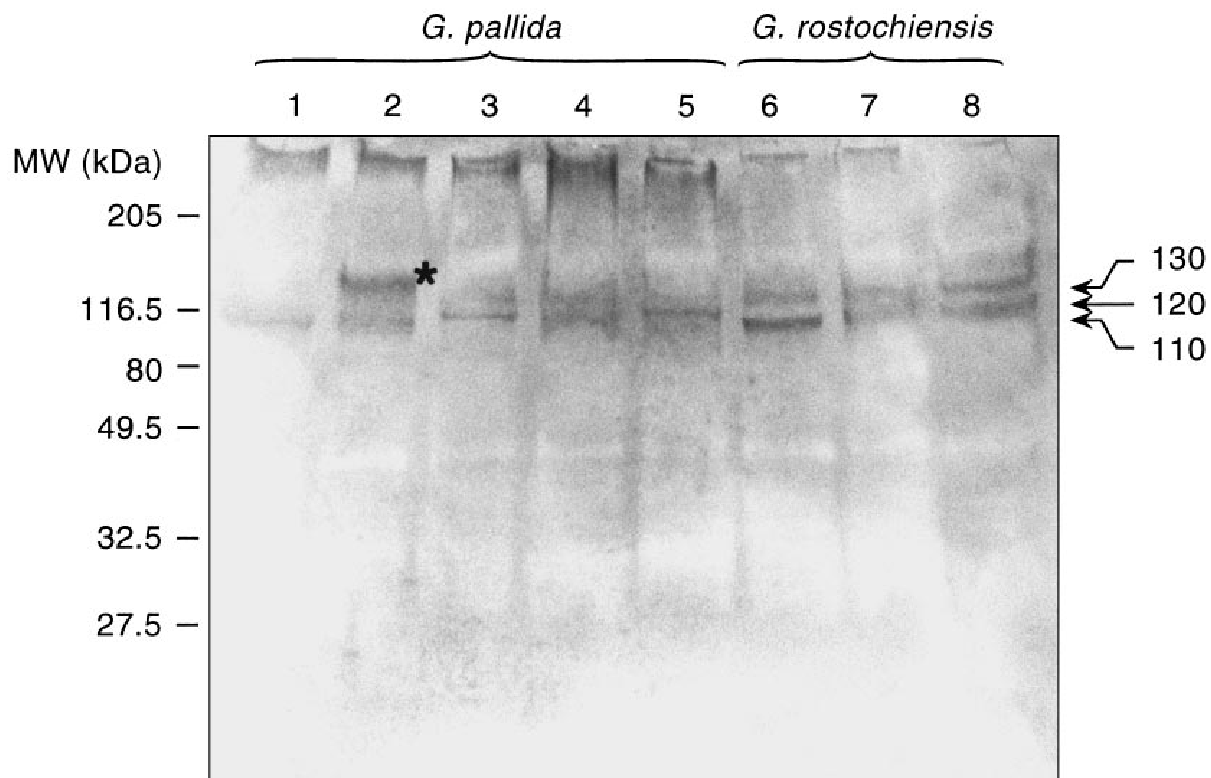

Fig. 4. Lectin blot analysis of homogenates of populations of *Globodera pallida* and *G. rostochiensis*. Parasite homogenate (25 μg loaded/lane) was probed with wheat germ agglutinin (WGA) as described in the Materials and Methods section. Lanes 1–5 show *G. pallida* Pa2/3 populations E, Luffness, Loc, Newton and Gourdie respectively. Lanes 6–8 show *G. rostochiensis* pathotypes Ro1, Ro3 and Ro4 respectively.

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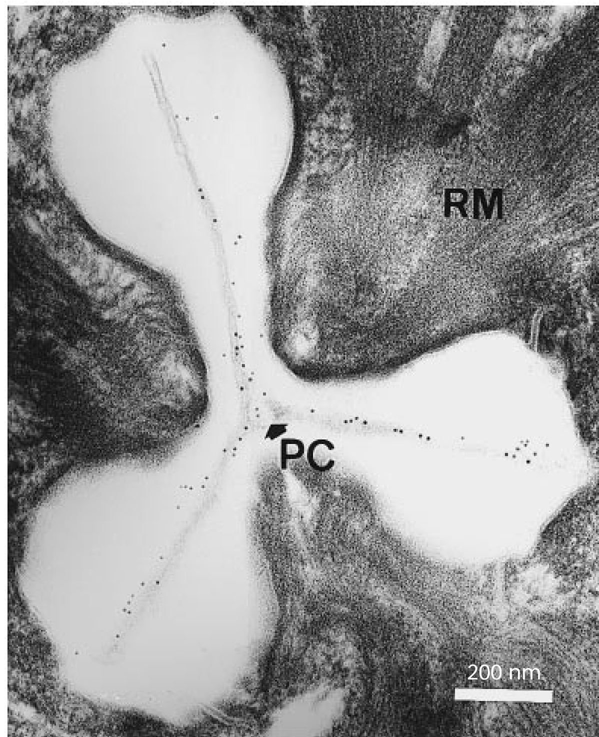

Fig. 5. Transverse section of *Globodera pallida* through the metacorpal bulb. Sections were probed with antiserum LW1 followed by goat anti-rabbit serum conjugated to 10 nm gold particles. RM, radial muscle; PC, pump chamber of the tri-radiate metacorpal bulb. Labelled secretions are arrowed.

the head of the nematode showed no significant labelling to amphidial structures. Sections cut further down the specimen, however, showed labelling of the metacorpal bulb contents (Fig. 5). When sections were cut through the oesophageal glands at the base of the metacorpal bulb, antibody binding could be seen to localize to the subventral glands (Fig. 6), specifically to granules present in the gland. The material present in the metacorpal bulb therefore appears to be derived from the subventral oesophageal glands.

DISCUSSION

A mixture of secretions from *G. pallida* was isolated using the method of Premachandran *et al.* (1988). The method used resulted in the production of secretions from the amphidial and excretory pores, although it is likely that stylet and other nematode secretions were also collected. The secretions were shown to be at least partly proteinaceous in nature, corroborating previous work on the nature of these secretions in other plant-parasitic nematodes (Spiegel & Robertson, 1988; Forrest, Robertson & Milne, 1988; Forrest, Spiegel & Robertson, 1988; Aumann, 1989).

Indirect immunofluorescence studies with LW1, an antiserum raised against the collected secretions, confirmed that some of the secretory material was surface associated. Immunogold labelling, however, using LW1 resulted in very sparse surface labelling, although results obtained with fluorescence microscopy indicated intense staining would be expected. This anomaly may be due to the glutaraldehyde fixation procedure used for pre-embedding protocols. When viewing specimens it was often possible to observe clumps of heavily labelled material lying away from the surface of the nematode, indicating that glutaraldehyde fixation may have failed to preserve most of the surface-associated epitopes recognized by LW1.

Although it is tempting to speculate that the material associated with the body wall posterior to the head originated from the excretory pore, the origin of material located at the head of the nematode is uncertain. Material found here may have originated from the amphids. Electron microscopy studies, however, show that there is no significant labelling of the amphids. In contrast, the subventral gland contents and material found in the metacorpal pump chamber at the base of the stylet is labelled. Various studies have shown that stylet secretions can be derived from both the dorsal and subventral glands (Davis *et al*. 1994; Goverse, Davis & Hussey, 1994). The material labelling the head may therefore be derived from oesophageal gland contents, or be a mixture of amphidial and oesophageal gland contents.

Previous studies have indicated the presence of glycoprotein plant-parasitic nematode secretions as determined by lectin binding (Forrest & Robertson, 1986; Aumann & Wyss, 1987; Aumann, 1994). We have shown that the secretions recognized by antiserum LW1 are glycosylated since periodate treatment completely abolished binding of antiserum LW1 to the nematode surface. Interestingly, the pattern of lectin binding reported by other workers was usually confined to the entrance of the amphidial and excretory pores which differs markedly from binding of antiserum LW1. This indicates that the sugars recognized by antiserum LW1 differ from those recognized by lectins in previous reports. Alternatively, antibody binding may require the presence of both protein and carbohydrate residues for epitope recognition.

Binding of antiserum LW1 differed between the populations tested. When antiserum LW1 was used to probe Western blots of homogenates of different populations of *G. pallida* and *G. rostochiensis* differences were observed, both between and within species. This supports the findings of Forrest & Robertson (1986) that lectin binding of glycoproteins could differ between populations and it has been postulated that such differences may determine whether the nematode is successful in establishing infection. Although differences in binding observed

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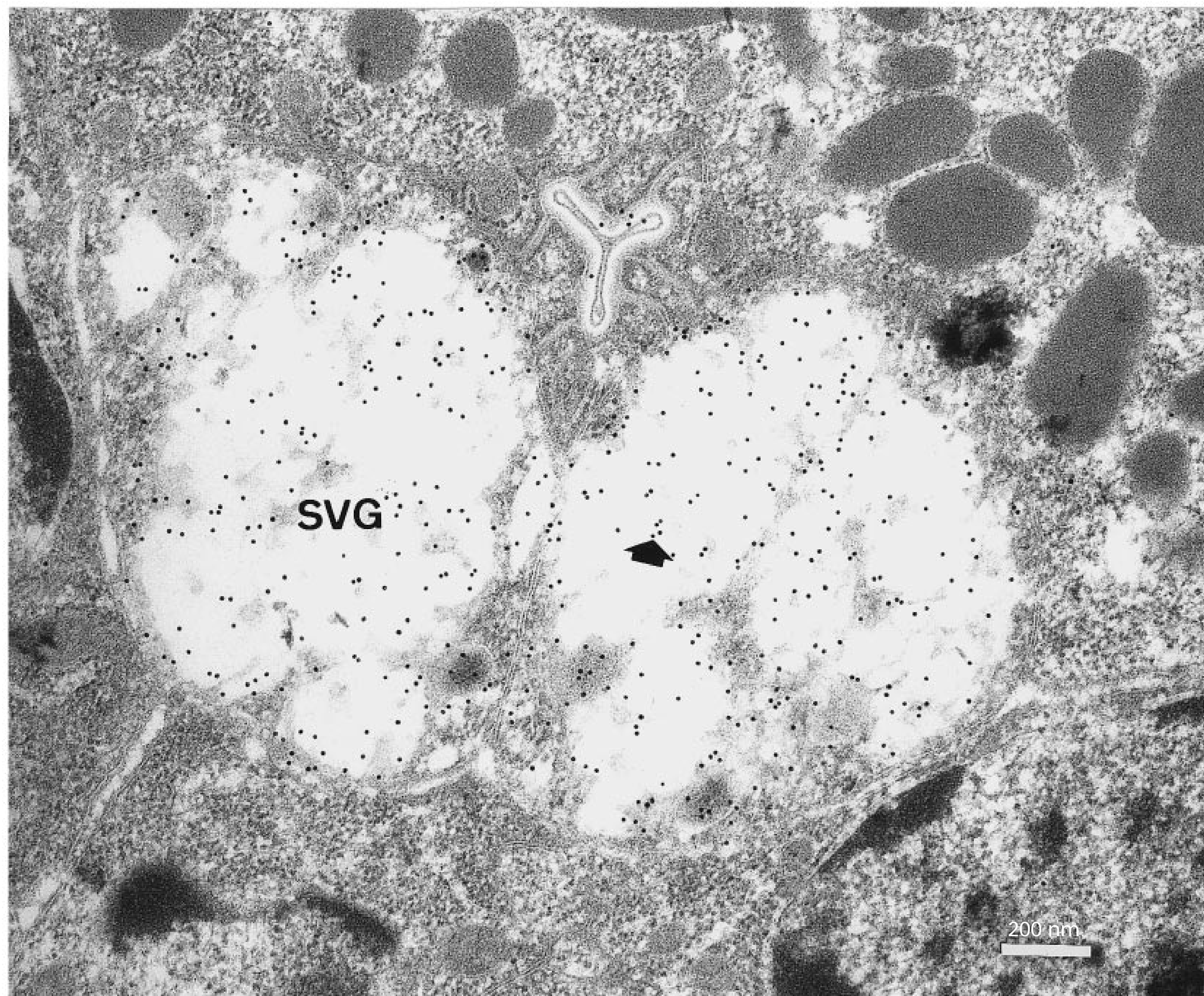

Fig. 6. Transverse secretion of *Globodera pallida* through the paired subventral oesophageal glands at the posterior of the metacorpal bulb. Sections were probed with antiserum LW1 followed by goat anti-rabbit serum conjugated to 10 nm gold particles. SVG, subventral gland. The labelled contents of the glands are arrowed. Antibody labelling can also be seen in the tri-radiate lumen of the food canal.

with antiserum LW1 between populations have not been quantified it is reasonable to postulate from the data presented that they are related to differences in glycosylation, especially since the lectin WGA recognizes differences between species. WGA was chosen to probe homogenates since it is known that this lectin binds specifically to the amphids of *G. pallida* and *G. rostochiensis*. Evidence that glycoproteins rich in mannose and galactose can elicit phytoalexin production (de Wit & Roseboom, 1980) suggests that such differences may influence the outcome of infection.

Indirect immunofluorescence studies also revealed binding of control NRS to the amphids. Similar phenomena have been described by Forrest (1995), and Hussey (1989*b*) has also reported that NRS from Balb/c mice binds strongly with secretory granules from the subventral glands of 3 species of *Meloidogyne*. In this study we demonstrate that the interaction between NRS and the amphids could be blocked by pre-incubation of nematodes with *N*-acetyl-D-galactosamine. The interaction therefore appears likely to be mediated via *N*-acetylgalactosamine residues found on immunoglobulin molecules (Taniguchi *et al*. 1986) binding to a lectin-like component of the amphidial secretions. The existence of such a lectin, however, remains open to speculation.

Research has indicated that the secretions of plant-parasitic nematodes may have diverse functions involved in the initiation and maintenance of parasitism (Jones & Northcote, 1972; Jones, 1981; Hussey, 1989*b*). The results described in this paper indicate that subtle differences in glycosylation of these secretions exist between the two sibling species of PCN, and also between populations within these two species. Such differences may be responsible for the variation in virulence that has often been described between such populations. Further work in this area will concentrate upon the identification of compounds which induce these secretions and the role of secretions in the infection process.

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