BARD

FINAL REPORT

PROJECT NO. IS-1894-90

**Characterization of the Phytophagous Nematode
Surfaced Coat to Provide New Strategies for
Biocontrol**

Y. Spiegel, M.A. McClure, I. Kahane, B.M. Zuckerman

מכון ויצמן למדע
הרצליה
ישראל

1995

**CHARACTERIZATION OF THE PHYTOPHAGOUS
NEMATODE SURFACE COAT TO PROVIDE NEW
STRATEGIES FOR BIOCONTROL**

BARD project No. IS-1894-90R

Y. Spiegel - A.R.O., The Volcani Center, Israel.

M. A. McClure - University of Arizona, Tucson, AZ, USA.

**I. Kahane - Hebrew University, Hadassah Medical School,
Jerusalem , Israel.**

**B. M. Zuckerman - University of Massachusetts, Amherst,
MA, USA.**

Project's strating date: July 10, 1991

Final Scientific Report

***Special Acknowledgement:* The authors are most grateful to Edna Sharon
for her major contribution to this project.**

630.72
BAR/SP1
2nd copy

BARD

P.O. Box 6

Bet Dagan, ISRAEL

BARD Project No. IS-1894-90R

Date: 19-11-95

Title: Characterization of the Phytophagous Nematode Surface Coat to
Provide New Strategies for Biocontrol

Investigators' Names and Institutions:

Y. Spiegel - A.R.O., The Volcani Center, Israel.

M. A. McClure - University of Arizona, Tucson, AZ, USA.

I. Kahane - Hebrew University, Hadassah Medical School, Jerusalem,
Israel.

B. M. Zuckerman - University of Massachusetts, Amherst, MA, USA.

Project's starting date: July 10, 1991

Final Scientific Report

Signature

Principal Investigator

DR. DAN LEVANON
SCIENTIFIC DIRECTOR

Institution's Authorizing Official

Special Acknowledgement: The authors are most grateful to Edna Sharon
for her major contribution to this project.

TABLE OF CONTENT

	<u>Page</u>
- Abstract	3 - 4
- Summary	5 - 7
- Objectives of the original research proposal	8
- Introduction	8 - 10
- I. Surface Coat Characterization: Materials & Methods	10 - 26
- I. Surface Coat Characterization: Results	26 - 33
- Table 1	34
- Table 2	35
- Table 3	36
- Table 4	37
- Figures 1 - 11	
- II. Biological Role of the SC: Materials & Methods	38 - 42
- II. Biological Role of the SC: Results	42 - 44
- Table 5	45
- Table 6,	46
- Table 7	47
- Table 8	48
- Discussion	49 - 55
- Evaluation of the research achievement with respect to the original research proposal and objectives.	55 - 57
- Conclusion remarks	57 - 58
- Literature cited	58 - 66
- List of publications derived from this BARD project	67 - 68

Abstract

Chemical composition and biological role of the surface coat (SC) of the root-knot nematodes, *Meloidogyne* spp. are described. SC proteins of *M. incognita* race 3 infective juveniles (J2) were characterized by electrophoresis and western blotting of extracts from radioiodine and biotin-labelled nematodes. J2 labelled with radioiodine and biotin released ^{125}I and biotin-labelled molecules into water after 20 hours incubation, indicating that SC proteins may be loosely attached to the nematode. Antiserum to the principal protein reacted with the surface of live J2 and with surface proteins previously separated by electrophoresis.

Human red blood cells (HRBC) adhered to J2 of several tylenchid nematodes over the entire nematode body. HRBC adhered also to nylon fibers coated with SC extracted from *M. javanica* J2; binding was $\text{Ca}^{++}/\text{Mg}^{++}$ dependent, and decreased when the nylon fibers were coated with bovine serum albumin, or pre-incubated with fucose and mannose. These experiments support a working hypothesis that RBC adhesion involves carbohydrate moieties of HRBC and carbohydrate-recognition domain(s) (CRD) distributed on the nematode surface. To our knowledge, this is the first report of a surface CRD in the phylum Nematoda.

Gold-conjugated lectins and neoglycoproteins combined with silver enhancement have been used for the detection of carbohydrates and CRD, respectively, on the SC of *M. javanica* J2. Biotin reagents were used to trace surface proteins, specifically, on live J2.

The labile and transitory nature of the SC was demonstrated by the dynamics of HRBC adherence to detergent-treated J2, J2 at different ages

or fresh-hatched J2 held at various temperatures. SC recovery was demonstrated also by a SDS-PAGE profile.

Monoclonal antibodies developed to a cuticular protein of *M. incognita* J2 gave a slight, but significant reduction in attachment of *Pasteuria penetrans* spores. Spore attachment as affected by several enzymes was inconsistent: alcian blue, which specifically blocks sulfhydryl groups, had no effect on spore attachment. Treatment with cationized ferritin alone or cationized ferritin following monoclonal antibody caused significant decreases in spore attachment. Those results suggest a role in attachment by negatively charged groups.

Summary

Chemical composition and biological role of the surface coat (SC) of the root-knot nematodes, *Meloidogyne* spp. are described..

SC proteins of *M. incognita* race 3 infective juveniles (J2) were characterized by electrophoresis and western blotting of extracts from radioiodine and biotin-labelled nematodes. Extraction of labelled J2 with cetyltrimethylammonium bromide (CTAB) yielded a principal protein band with a molecular weight larger than 250 kDa and several faint bands of lower molecular weight ranging from 31 kDa to 179 kDa. The pattern of labelling was similar for both labelling methods. Western blots of unlabelled proteins were probed with a panel of biotin-lectin conjugates, but only Concanavalin A (Con A) bound to the principal band. Nematodes labelled with radioiodine and biotin released ^{125}I and biotin-labelled molecules into water after 20 hours incubation, indicating that SC proteins may be loosely attached to the nematode. Antiserum to the principal protein reacted with the surface of live J2 and with surface proteins previously separated by electrophoresis. Differential patterns of antibody labelling were obtained on western blots of extracts from *M. incognita* race 1, 2, and 3, *M. hapla* race 2, and *M. arenaria* race B.

Human red blood cells (HRBC) adhered to J2 of several tylenchid nematodes over the entire nematode body. Binding was conspicuously confined to the head and tail of *Longidorus cohni*, *Xiphinema brevicolle* and *X. index*. Binding was Ca^{++} or Mg^{++} dependent. *Anguina tritici*, *Aphelenchoides subtenius*, *Ditylenchus dipsaci*, *M. javanica* females and *Panagrellus redivivus*, did not bind HRBC, even in the presence of these

cations. Incubation of *M. javanica* J2 with fucose, glucose, N-acetylglucosamine, mannose or trypsin, decreased the intensity of subsequent HRBC binding, while galactose and N-acetylgalactosamine, increased binding intensity. HRBC adhered also to nylon fibers coated with surface coat extracted from *M. javanica* J2; binding was $\text{Ca}^{++}/\text{Mg}^{++}$ dependent, and decreased when the nylon fibers were coated with bovine serum albumin, or pre-incubated with fucose and mannose.

These experiments support a working hypothesis that RBC adhesion involves carbohydrate moieties of HRBC and carbohydrate-recognition domain(s) (CRD) distributed on the nematode surface. To our knowledge, this is the first report of a surface CRD in the phylum Nematoda.

Gold-conjugated lectins and neoglycoproteins combined with silver enhancement have been used for the detection of carbohydrates and CRD, respectively, on the SC of *Meloidogyne javanica* J2. The amphidial secretions as well as the transverse annulations and lateral ala over the entire nematode surface were labelled with the Con A and wheat germ agglutinin (WGA) lectins. Surface proteins were extracted with detergents and separated by denaturing polyacrylamid gel electrophoresis (SDS-PAGE). On the western blots, Con A and WGA labelled the 6, 9, 14, 31, 58 and 66 kDa bands; WGA labelled also the 22 and 26 kDa bands. The 14, 22, 26, 58 and 66 bands reacted also with the fucosylated-, mannosylated- and N-acetylgalactosaminylated-bovine serum albumin; these neoglycoprotein labellings were not inhibited by competitive carbohydrates, but partially reduced with compatible lectins. The neoglycoproteins bound to the CRD located on the intact nematode SC, in a similar pattern recorded for the lectin bindings.

Biotin reagents were used to trace surface proteins, specifically, on live J2. SDS-PAGE of biotinylated-SC extracts revealed two specific biotin-protein bands at 46 and 49 kDa.

The labile and transitory nature of the SC was demonstrated by the dynamics of HRBC adherence to detergent-treated J2, J2 at different ages or fresh-hatched J2 held at various temperatures. SC recovery was demonstrated also by a SDS-PAGE profile. Polyclonal antibodies to the 250 kDa protein labelled the J2 surface and amphids except for the head region.

Monoclonal antibodies developed to a cuticular protein of *M. incognita* J2 gave a slight, but significant reduction in attachment of *Pasteuria penetrans* spores. These results implicated an undescribed protein as one of the factors which interact in binding of the spore to the nematode. Decreased spore attachment following treatments of the nematode with the detergents SDS and CTAB are proposed as being due to non-specific blockage of spore-nematode binding sites. Results are also given on differences in attachment to live and dead nematodes, as well as attachment levels of three *P. penetrans* strain to *M. incognita* Race 3. Spore attachment as affected by neuraminidase, pronase, pepsin, trypsin and lipase was inconsistent: alcian blue, which specifically blocks sulphy groups, had no affect on spore attachment. Treatment with cationized ferritin alone or cationized ferritin following monoclonal antibody caused significant decreases in spore attachment. Those results suggest a role in attachment by negatively charged groups.

Objectives of the original research proposal:

- a. To characterize the SC of the root-knot nematodes, *Meloidogyne incognita* and *M. javanica*.
- b. To investigate the biological role of the SC by testing the relationship between the specific binding of *Pasteuria* spp. to *Meloidogyne* and the nematode's SC.

Introduction

The multilayered nematode cuticle serves as an exoskeleton and as a barrier limiting entry to the body. The epicuticle is sometimes similarly differentiated into two or more layers and in many nematodes is covered by a material visualized in electron microscopy as a fuzzy coating (Bird and Zuckerman, 1989; Luft, 1976; Wright, 1987) designated 'glycocalyx' by early investigators (Bird and Zuckerman, 1989; Luft, 1976). Wright (1987) preferred the term 'surface coat' (SC) which avoids invoking cell membranes; Foley et al. (1986) showed that nematode surfaces do not behave like cell membranes. Bird and Zuckerman (1989) use the term SC but anticipate that the term 'glycocalyx' might become more appropriate because it emphasizes the carbohydrate-rich nature of this extra-cellular peripheral material.

Little is known about the chemistry of the SC of phytonematodes. Several techniques usually required to characterize carbohydrate, lipid and protein constituents have been used by different investigators. These studies have shown that the entire surface, or sometimes only parts of it, contains various carbohydrate residues that readily bind a

number of carbohydrate-specific lectins (Robertson et al. 1989; Rudin, 1990; Spiegel et al. 1982; Spiegel et al. 1983; Zuckerman, 1983). The carbohydrates of the SC appear to be glycoprotein complexes in animal-parasitic nematodes (Jansson, 1987) and in free-living nematodes, such as *Panagrellus redivivus* (Jansson, 1987).

Several methods have been used to characterize and map SC proteins. Whole nematodes have been labelled with biotin derivatives or radioiodine, and detergent extracts have been electrophoretically analyzed to identify surface associated proteins from free-living, plant-parasitic, and animal-parasitic species (Blaxter, 1993; Devaney, 1991; Proudfoot et al. 1991). Several specific surface proteins were characterized on *M. incognita* Race 1 using ^{125}I -Bolton Hunter reagent (Robinson et al. 1989)

Recent evidence implicates involvement of the SC in interactions between nematodes and microorganisms (see below), as well as between parasitic nematodes and their hosts. In animal parasites the SC is believed to have an important role in adaptation and survival in paratenic hosts because recognition of exposed surface molecules by the host immune system can contribute to development of an effective immune response (Kennedy, 1991).

Pasteuria spp. are obligate parasites of a number of sedentary plant-parasitic nematodes (Davies et al 1988). Their development and life cycle is highly synchronized with that of their host (Sayre, 1980; Stirling, 1985). Host mortality and the ability of *Pasteuria penetrans* to survive in soil under extreme conditions, make *P. penetrans* a promising biocontrol candidate for future suppression of plant-parasitic

nematodes. The nature of its spore attachment to the nematode cuticle is not understood. A biochemical interaction between pathogen spores and nematode cuticular surfaces was assumed by Stirling et al. (1980). Studies with lectins led Stirling et al. (1980) to conclude that lectins are not involved in the attachment of *P. penetrans* spores. A different conclusion was reached by Davies and Danks (1992) who proposed that non-collagenous domains on nematode surfaces could interact with N-acetylglucosamine moieties on the spore surface. This possibility was not confirmed in a recent attempt (Davies, 1994) using a potential antigenic ladder on the nematode surface; a 190 kD glycoprotein presented in the cuticle extract was found to be involved in the attachment of *Pasteuria* spores to the nematode cuticle (Davies, 1994). In other recent studies it is reported that antibodies to proteins from *P. penetrans* can prevent attachment of spores to *M. incognita* and that antibody specificity exists among different isolates of *P. penetrans* (Davies et al, 1992). Differences in adhesiveness of *P. penetrans* to *M. incognita* and *M. javanica* compared with *M. arenaria* (Davies and Dank, 1992) may be explained by interspecific differences in the nematode SC.

I. Surface Coat Characterization

Materials & Methods

Nematodes. *Anguina tritici* J2 were dissected from seed galls of infected wheat (*Triticum aestivum* cv. Lakhish) in distilled water (McClure, 1988). The J2 were passed through a 30- μ m sieve to remove plant tissue and then washed thoroughly with distilled water and PBS

(unless otherwise mentioned, the PBS contained 0.5 mM calcium and magnesium cations). *Aphelenchoides subtenius* were extracted from infected narcissus (*Narcissus* sp.) roots. *Ditylenchus dipsaci* were propagated on garlic (*Allium sativa* cv. Lavinia) in the greenhouse or aseptically on Petri plates containing garlic callus tissue. Parasitic juveniles and adults females and males were dissected from infected plant tissue. *Heterodera avenae* J2 were hatched from brown cysts separated from natural infected soil by sieving and decanting. *H. schachtii* were reared on sugar beet (*Beta vulgaris*) in the greenhouse. Ten weeks after inoculation of beets with J2, the soil and the roots were washed, and mature females were collected on a 150- μ m-pore sieve. Following centrifugation in a solution of 47% sucrose, the females were washed 6 times with DW, and J2 were allowed to hatch in PBS. *Longidorus cohni* and *Panagrellus redivivus* were sampled from routine cultures maintained in the Department of Nematology at Bet Dagan. *Pratylenchus mediterraneus* was propagated on trefoil (*Trifolium hybridum*) aseptically grown on Petri plates. The root-knot nematode *Meloidogyne incognita* (Kofoed and White) race 3 were reared in the greenhouse on eggplant, *Solanum melogena* cv. Black Beauty, and J2 were collected from hatched eggs. *Meloidogyne javanica* was propagated on tomato cv. Hosen Eilon in the greenhouse or aseptically on Petri plates containing excised root cultures of tomato. Eggs were separated from egg-masses or infected roots, with sodium hypochlorite (0.5%, 1 min) and hatched overnight in DW or PBS to obtain infective J2; J2 from this source have had no direct contact with host tissues and therefore their secretions did not contain host residues. Adult females were dissected from aseptically-grown

infected roots 4 weeks after inoculation. The reniform nematode *Rotylenchulus reniformis* was propagated in the greenhouse on potted seedlings of upland cotton (*Gossypium hirsutum* cv. Acala 1517-C); egg-masses were dissected from infected roots and hatched to obtain J2. Males and juveniles of the citrus nematode *Tylenchulus semipenetrans* were collected from field-grown infected roots which had been placed, for 24 hour on a 25- μ m pore nylon cloth immersed in a Petri plate filled with 10 ml PBS. Females, males and juveniles of *Xiphinema brevicole* and *X. index* were extracted from soil around fig trees (*Ficus carica* L.) grown in the screenhouse at Bet Dagan.

Electrom microscopy. Approximately 1,000 J2, freshly-hatched from eggs, were incubated in distilled water at 14°C for three days to permit accumulation of SC materials. After incubation, they were washed three times with distilled water, concentrated by centrifugation at 1000 g and divided into two equal lots.

One lot was incubated in cationized ferritin, 1 mg/ml for 2 hours at 37 C, rinsed three times with distilled water and resuspended in 0.06 M phosphate buffer. The J2 were then transferred to special processing chambers (McClure and Stowell, 1978), fixed for 90 minutes at 21-24°C in 0.06 M phosphate buffer, pH 7.3 containing 3% glutaraldehyde, rinsed three times with PBS, postfixed for 1 hour at 21-24°C with 1% osmium tetroxide, dehydrated in acetone (10% increments), and infiltrated with Spurr's low viscosity resin in acetone (10% increments). Infiltrated specimens, in undiluted resin, were cast between two glass microscope slides (Reymond and Pickett-Heaps, 1983) and cured at 60°C for 18 hours. Longitudinal silver sections of embedded J2 were mounted on uncoated 200

mesh copper grids, stained for 20 minutes with 2% uranyl acetate, poststained with Reynold's lead citrate, and photographed on a Hitachi H-500 TEM at 75 kV.

The other lot of J2 was stained with ruthenium red, purified and prepared in 0.2 M cacodylate buffer containing 3.6% glutaraldehyde according to Luft (1971). Stained J2 were post-fixed for 3 hours at 25° C with 1.5% osmium tetroxide in 0.06 M cacodylate buffer also containing 500 ppm ruthenium red. They were then rinsed 3 times with distilled water, dehydrated and processed for electron microscopy as described previously.

Labelling with FITC- or TRITC-conjugated lectins and neoglycoproteins. Nematodes were washed thoroughly with PBS and incubated with either Con A and WGA, each conjugated with TRITC (for *Steinernema* spp.) or FITC (for *Heterodera* and *Meloidogyne* spp.) as described previously (Davis et al. 1988; Spiegel and McClure, 1991). The conjugated lectins were obtained from Sigma (Rehovot, Israel). Protocols for specificity of lectin binding and fluorescence microscopy were described previously (Davis et al. 1988; Spiegel and McClure, 1991).

Neoglycoproteins were used to detect carbohydrate binding sites. *M. javanica* J2 were first blocked for 30 minutes with 1% BSA in PBS, washed with 0.1% BSA in PBS and then incubated for 1 hour with one of the following 0.1 mg/ml FITC-conjugated reagents (Sigma): BSA-FITC (as control), BSA- α (or β)-D-galactopyranosyl-phenylisothiocyanate, BSA- α -L-fucopyranosyl-phenylisothiocyanate, and BSA- α -D-mannopyranosyl-phenylisothiocyanate.

Labelling with gold-conjugated lectins and neoglycoproteins.

Juveniles for these experiments were hatched in the presence of Ca^{++} ions. Nematodes were blocked and washed as described before and incubated for 30 minutes at RT with 2.5 $\mu\text{g}/\text{ml}$ PBS solutions (containing 0.1% BSA) of 10 nm gold-conjugated Con A or WGA in the presence of Ca^{++} , Mg^{++} and Mn^{++} ions. Binding specificity was confirmed by preincubation of the lectins, for 30 minutes at RT, with 0.05 M of the following haptens: mannose (Con A) and N-acetylglucosamine or chitobiose (WGA). Nematodes were then washed with PBS, 0.1% BSA and finally with distilled water before incubation for 5-15 minutes with silver enhancing solution (BioCell Cardiff, UK) until labelling could be visualized by light microscopy. Further enhancement was prevented by washing with distilled water. Nematodes treated with the enhancing solution alone served as controls.

Meloidogyne javanica J2 were incubated with 1 $\mu\text{g}/\text{ml}$ gold conjugated neoglycoproteins, fucosylated-BSA or mannosylated-BSA or glucosylated-BSA or galNAc-beta-(1,4)gal-beta-O-BSA (E.Y. Laboratories, San Mateo CA), in PBS containing 0.1% BSA and Ca^{++} and Mg^{++} ions. Preincubation with 0.1 M of fucose, α -methyl mannoside, glucose and galNAc or galactose were used to verify the specificity. Gold conjugated BSA was used as a negative control. Washed nematodes were then exposed to silver enhancing solution as described before.

Interaction of nematodes with HRBC. Human red blood cells of groups A, B, and O, type Rh(-) or Rh(+), were washed several times with PBS to remove serum proteins and used without fixation with 1% glutaraldehyde. Assays were performed in a 24-well, flat-bottomed tissue culture plate

(Becton Dickinson Labware, CA USA). Each well contained 0.4 ml of 0.25% (final concentration) HRBC in PBS which contained Ca^{++} and Mg^{++} ions and 20-30 individuals of a particular nematode. Plates were incubated at room temperature and binding was determined by examination of the nematodes by phase contrast microscopy after 30 minutes. Nematodes were fixed with 3.7% formaldehyde solution and the number of bound HRBC per nematode was counted. Ten individuals were recorded each well. Each treatment was replicated in three wells, and all experiments were repeated at least 3 times with consistent results. *M. javanica* J2 were exposed to several treatments, washed with PBS, and then incubated with HRBC. Observations were made after 30 minutes. Treatments consisted of: (I). J2 hatched in the presence of PBS or Ca^{++} - and Mg^{++} -free PBS and incubated with HRBC prepared with PBS or Ca^{++} - and Mg^{++} -free PBS. (II). 1.0 M NaCl solution, 60 min. (III). Incubation with 0.5 mg/ml trypsin (Sigma, EC 3.4.21.4.) in PBS for 150 minutes at 37°C in a water-bath shaker. (IV). Incubation with 0.025, 0.05, 0.1 and 0.2 M PBS solution of all of α -methyl-mannoside, fucose, glucose, glucose, N-acetylglucosamine, and with 0.2 M (in PBS) of galactose and N-acetylgalactosamine for 2 hours at 25°C. (V). Incubation with each of the following detergents in PBS solution: 1.5% nOG (30 minutes on ice), 1% SDS (30 minutes at 25°C), and 0.25% CTAB (90 minutes at 37°C).

SC proteins extraction. In Tucson AZ, extraction was made with J2 of *M. incognita* race 3: About 1 ml of J2 were collected as described above and washed by pelleting in distilled water several times at 1,000g for 3 minutes. Four ml of 0.25% cetylmethylammonium bromide (CTAB) containing proteinase inhibitors (5 $\mu\text{g/ml}$ aprotinin, 2 mg/ml

iodoacetamide, 0.2 mg/ml phenylmethylsulfonyl fluoride and 50 µg/ml soybean trypsin inhibitor) was added to resuspend the J2 pellet (27,28) and the suspension was incubated up to 4 hours at 37°C with agitation for 10 seconds every 30 minutes. After concentration by centrifugation, the supernatant was filtered through a 0.22 µm Millipore (Millex-GV) membrane and concentrated with a 10,000 NMWL Immersible-/CX Ultrafilter (Millipore Corp.). The concentrated extract was then diluted with 3 volumes of 2% SDS in 0.5 M Tris-HCL, pH 6.8, and concentrated to approximately 200 µl. The extract was stored at -70°C.

In the Israeli laboratory at Bet Dagan, 200 ml of *M. javanica* J2 was gently agitated with 2 ml solutions of the following detergents dissolved in PBS/2: 1. 0.25% CTAB (37°C for 1.5 hour); 2. 1.0% SDS (RT, 1 hour); 3. 1.5% n-OG (on ice, 30 minutes). The reaction mixtures contained several protease inhibitors: 1 mM Pefabloc (Boehringer Mannheim, Germany), 1 µg/ml Leupeptin (Boehringer) and 0.1 mM TPCK (Sigma Chemical Co., St. Luis, MO). Nematodes were then pelleted by centrifugation at 250g and the supernatant was filtered through 0.2 µm cellulose acetate membrane (Corning, Corning NY). Detergents were finally excluded from the supernatant by dialysis overnight against distilled water at 15°C, or by using Detergent Absorber Gel (Boehringer). Proteins were concentrated by lyophilization.

For demonstration of SC proteins recovery after detergent stripping, SDS-extracted juveniles were washed thoroughly and divided into 2 groups for two days incubation at 4°C or 25°C, and then further extraction with SDS. The extracts were then loaded on gels.

Reaction of HRBC with SC-coated nylon fibers. SC extract solution was covalently bound to fibers of nylon 66 (13- μ m approximate diameter) using the method of Inbar and Chet (1992). Total protein concentration of the SC solution, determined by Bradford (1976) method, was 25 μ g/ml. This amount (25 μ g protein) was extracted from ca. 200,000 *M. javanica* J2. The experiments were carried out by incubating HRBC in the presence or absence of Ca^{++} and Mg^{++} with SC-coated fibers or with BSA-coated fibers as a control. These incubations were at 25°C for 1 hour, after which fibers with adhering HRBC were examined by light microscopy. Ten to 15 fiber sections (0.2-0.3 mm long) of each treatment were photographed and numbers of HRBC bound per millimeter of fiber were recorded. Specificity of the binding was verified by preincubating coated fibers for 2 hours at 25°C with 0.1 M fucose, washing thoroughly with PBS, and then incubating with HRBC.

Gel Electrophoresis and western blots. In Tucson AZ, polyacrylamide gel electrophoresis in the presence of SDS (SDS-PAGE) was performed using either 4-20% (w/v) gradient precast gels (Jule Inc.), or 6% or 12% non-gradient gels (pH 8.8) prepared on a Hoefer SE 250 gel casting form, according to Laemmli (1970). A stacking gel (4% acrylamide, pH 6.8) was pipetted on top of the resolving gel prior to use. For each 8 μ l sample of extract per track, 2 μ l glycerol and 1 μ l of 0.1% tracking dye (bromophenol blue) was added and the mixture was boiled for 90 seconds to denature the protein. Gradient gels, 0.75 mm thick, were run at a constant 10 mA for 2 hours while 6% gels, 1.0 mm thick, were run overnight at 3 mA. Following electrophoresis, the gel slab was silver stained (Morrissey, 1981) or used unstained for Western blotting (Towbin

et al. 1979).

Biotinylated J2 were extracted with CTAB and the extract was electrophoretically separated and blotted as previously described. The blot was blocked by incubation with 3% albumin, bovine fraction V (BSA) in TBS buffer (10 mM Tris-HCL, pH 7.4, 0.9% NaCl, w/v) for 2 hours at 25°C. Biotin-labeled proteins were detected by incubating the blot in 1.25 µg/ml (1:2000 of stock) alkaline phosphatase avidin (Calbiochem Inc.) for 1 hour at room temperature, followed by three washes with TBS. The blot then was developed in bromochloroindolyl phosphate/nitro blue tetrazolium (NBT/BCIP) (Sigma Co.) substrate solution for 5 minutes. Radioiodinated J2 were treated in a similar fashion, and autoradiographs were prepared by exposing X-ray film to the Western blots for seven days.

Glycosylated SC proteins were detected by a modification of the method reported by Kaplan and Gottwald (1992). Western blots of extracts were blocked with BSA as described above for biotin-avidin blotting. Blots were then incubated with agitation for 1 hour at 23°C in biotinylated lectin (E-Y Laboratories, Inc. P.O. Box 1787 San Mateo, CA 94401): Con A (1.0 µg/ml *Canavalia ensiformis* agglutinin), GS-1 (3 µg/ml *Griffonia simplicifolia* agglutinin), SBA (2 µg/ml *Glycine max* agglutinin), WGA (2 µg/ml *Triticum vulgaris* agglutinin), LPA (10 µg/ml *Limulus polyphemus* agglutinin), BPA (2 µg/ml *Bauhinia purpurea* agglutinin), DBA (4 µg/ml *Dolichos biflorus* agglutinin), UEA (2 µg/ml *Ulex europaeus* agglutinin), PNA (0.4 µg/ml *Arachis hypogaea* agglutinin) in HEPES (N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)) buffered saline (10 mM HEPES, 150 mM NaCl, 0.1 mM CaCl₂·2H₂O, 0.1 mM

MgCl₂.6H₂O, 0.01 mM MnCl₂.4H₂O, pH 7.5). Biotinylated lectins that bound to the blotted proteins were visualized by incubation in 1.25 µg/ml alkaline phosphatase avidin in TBS, followed by NBT/BCIP development.

In the Israeli laboratory at Bet Dagan, analytical SDS-PAGE in 10% or 4-15% gradient gels was performed in a Mini-Protean II apparatus (Bio-Rad Laboratories, Richmond, CA), using 1.5-mm-thick vertical slab gels essentially as described by Laemmli (1970). Gels were silver-stained according to Wray et al. (1981). Molecular-weight markers for silver stain (Bio-Rad) were used. Pre-stained molecular weight standards (Sigma) were run on each gel to be electroblotted.

For western blots, proteins were transferred onto nitrocellulose sheets using a Gelman Sciences BioTrance midi-apparatus. General protein staining on blots was performed with Protogold (BioCell Cardiff, UK) according to the manufacturers' instructions.

General and specific labelling of glycoconjugates and CRDs on blots. To detect glycoproteins in the SC extract on western blots, extracts were first oxidized with sodium periodate, then treated with biotinamidocaproyl hydrazide (Sigma) according to O'Shannessy et al. (1987). Biotin-binding was detected using streptavidin-peroxidase (Pierce, Rockford, USA) and 4-chloro-1-naphthol (Sigma) as the substrate. Binding specificity was verified by running oxidized non-biotinylated extracts as controls.

Specific labelling of glycoconjugates was performed after blocking the blots overnight at 4°C with PBS containing 1% BSA and 0.05% Tween-20 (blocking solution) and washing three times with PBS containing 0.1% BSA and 0.05% Tween-20 (washing solution). Gold-conjugated Con A and WGA

lectins (E.Y. Laboratories, San Mateo, CA; 1 $\mu\text{g/ml}$) dissolved in the washing solution containing 1 mM Ca^{+2} , Mg^{+2} and Mn^{+2} ions, were incubated for 1 hour at RT with the blots. Binding specificity was verified by preincubating the lectins for 30 minutes with 0.1 M of the appropriate hapten: α -methyl mannoside for Con A, and N-acetylglucosamine or chitobiose for WGA. Blots were washed with the washing solution and distilled water and labelling was visualized with a silver-enhancing kit for blotting (BioCell).

CRDs were labelled on western blots following the blocking and washing steps described before. Gold-conjugated neoglycoproteins (E.Y. Laboratories, San Mateo, CA). Fucosylated-, mannosylated- and glucosylated-BSA diluted to a concentration of 1 $\mu\text{g/ml}$ in the washing solution containing Ca^{+2} ions, were incubated with the blots for 1 hour at 37°C. BSA-gold was used as a negative control. Specificity was verified by preincubating the blots under the same conditions, with the following competitive haptens (0.1 M): fucose, α -methyl mannoside, glucose and galNAc or galactose. As a control, the neoglycoproteins were preincubated with the appropriate lectins (20 $\mu\text{g/ml}$): UEA-I with fucosylated-BSA, and Con A with mannosylated or glucosylated-BSA.

Labelling SC proteins with biotin: localization on J2 and on western blots. Nematodes were incubated for 15 min with one of the following biotin reagents: 1. sulfo N-hydroxysuccinimide (NHS)- biotin (Pierce); 2. ϵ -aminocaproic acid NHS-biotin (Sigma). Stock solutions dissolved in 0.05 M carbonate buffer (pH 9.2) were diluted in PBS/2 to a working concentration of 500 $\mu\text{g/ml}$. Each of these biotin treatments was followed by washing the nematodes three times with PBS/2.

Biotin-labelling was localized by incubating blocked and washed nematodes for 2 hours with gold-conjugated goat anti-biotin (1-nm particles; BioCell) diluted (1/100) in the washing solution. Nonlabelled J2 which were incubated with the antibodies served as controls. Nematodes were then prepared for light microscopy as described earlier.

Biotin-labelled proteins were extracted by boiling the biotinylated and non-biotinylated nematodes in SDS-PAGE loading buffer for 15 minutes, followed by vigorous centrifugation to pellet the nematodes and collection of the supernatant.

Biotin-labelled proteins were monitored on western blots by two different methods. Blots were first blocked at 4°C, overnight, with 5% skim milk in PBS containing 0.05% Tween 20, and washed (3 x 10 min) with PBS containing 0.1% skim milk and 0.05% Tween 20. Blots were then incubated either with 1 µg/ml streptavidin HRP (Pierce) in the washing solution for 1 hour; or for 2 hours with anti-biotin HRP (Sigma) diluted 1:1000 in the washing solution. With both methods, incubation was followed by washing twice with the washing solution and finally with only PBS. Visualization of labelling was performed with 4-chloro-1-naphthol) for the streptavidin-HRP. A more sensitive ECL kit (Amersham Life Science, Buckinghamshire, UK) was used as a substrate for the anti-biotin HRP, to visualize the label on x-ray film.

Radiolabelling and release of labelled surface molecules.

Radioiodination of the SC was accomplished by exposing J2 to ¹²⁵I-Bolton Hunter reagent according to the supplier's instructions (E. I. Du Pont de Nemours & CO., Inc.). The reaction was carried out at pH 8.0 in 0.01 M PBS with 250 µCi of ¹²⁵I for one hour at 25 C. At the end of

incubation, uncombined iodine periodate was removed by reaction with excess lysine and the nematodes were washed repeatedly with PBS (Robinson et al. 1989).

Approximately 0.2 ml of ^{125}I -labelled J2 were washed on a 4 μm -pore-size filter using distilled water to remove unbound label. Eluate was assayed for radioactivity by passing it through a 0.22 μm Ultrafree filter (Millipore) in a centrifuge at 2,000g for one minute and counting gamma emission from 1.0 ml in a scintillation vial containing 20 ml of water miscible scintillation cocktail (ICN Biomedicals, EcoLite). Washing continued until the counts stabilized at approximately 21000 cpm. After washing, the volume of the solution containing the labelled nematodes was brought up to 2 ml by adding distilled water and the suspension was incubated in a 15 ml glass tube with slow agitation at 23 C. Aliquots were collected at 0, 1, 2, 4, 8, 16, and 20 hour intervals. At each interval, the nematodes were pelleted and 1 ml of supernatant was passed through a 0.22 μm filter by centrifugation at 1,000g. The filtrate was placed in a scintillation vial containing 20 ml of scintillation cocktail and assayed for gamma emission. In order to maintain a constant volume, 1.0 ml of distilled water was added to the glass tube containing the labeled nematodes, and the nematodes resuspended by agitation.

Release of SC materials also was demonstrated by incubating 0.2 ml of biotinylated, and thoroughly washed J2, 18 hours in distilled water with gentle agitation. The supernatant was collected by centrifugation and concentrated by ultrafiltration to approximately 30 μm . Biotinylated proteins in the concentrate were separated by

electrophoresis and visualized on western blots as described in this report.

Antiserum and immunoblotting. In Tucson AZ, Proteins extracted with CTAB were separated by SDS-PAGE on 6% (w/v) acrylamide slab gels. Proteins from a strip (one lane) of the gel were visualized by silver staining and the strip was aligned with the remainder of unstained gel. Regions of the unstained gel containing bands of interest were then excised and proteins were electro-eluted (BIO-RAD Model 422) with constant current of 10 mA/Tube for 8 hours. After elution, the elution buffer (25 mM Tris-HCl, 192 mM Glycine, 0.1% SDS) was removed by dialysis against PBS. The resultant partially purified SC proteins were used as antigens for immunization. Rabbits were immunized subcutaneously (st) and intramuscularly (im) with a 2-3 µg suspension of SC proteins in saline mixed 1:1 with RIBI adjuvant (RIBI Immunochem Research Inc.), followed by three injections (st and im) with 0.5 µg proteins in PBS 1:1 mixed with RIBI adjuvant at 3-4 week intervals. Serum was collected 3 weeks after the final immunization and antibody was purified by ammonium sulfate precipitation and by Protein A-DEAE Affi-Gel Blue gel chromatography for the IgG fraction, according to the protocol provided by BIO-RAD. Reactivity of the purified antibody with the nematode SC was determined by incubating live J2 in the antibody for 2 hours, washing them three times with PBS and incubating them in goat anti-rabbit antiserum-FITC (Sigma) for one hour. Preimmune serum and/or elimination of the primary antibody were used as controls. After incubation, the treated nematodes were washed to remove unbound antibody conjugates and examined by fluorescence microscopy (McClure and

Stynes, 1988).

In Israel, polyclonal antiserum was produced in mouse injected with SDS-extracted SC proteins. Antiserum from nonimmune mice was collected as well. Immunoblotting was performed on western blots blocked and washed as described previously. Membranes were incubated for 2 hours at 37°C with the antiserum (immune or nonimmune) diluted 1:1000 in the washing buffer. After washing, membranes were incubated for 1 hour at 37°C with a secondary antibody (anti-mouse IgG alkaline phosphatase) (Sigma) diluted 1:20,000. As controls, the secondary antibody alone or the nonimmune antiserum were incubated separately with the membrane. Blots were developed in bromo-chloro-indolyl phosphate and nitro blue tetrazolium (BCIP/NBT).

Labelling of nematodes with antibodies. In Tucson AZ, proteins from CTAB extracts, separated by SDS-PAGE, were transferred to a nitrocellulose membrane (Towbin et al. 1979), and then blocked in Blotto/Tween (5% nonfat dry milk (w/v) and 0.2% Tween 20) in 0.01 M pH 7.2 PBS with agitation on an orbital shaker for two hours at 23°C. The blot was washed with PBS once for 5 minutes and incubated in anti-SC IgG (1:100), containing 0.2% Tween 20, for at least two hours at 23°C. Blotted proteins labeled with the anti-SC antibodies were detected by incubation in alkaline phosphatase secondary anti-rabbit antiserum (1.1 µg/ml, Sigma) in Tris-buffered Saline (TBS) (25 mM Tris-HCl, 0.8% NaCl, 0.002% KCl, pH 8.0) for 1 hour at 23°C. The blot was washed 3 times in TBS between each incubation and then developed in NBT/BCIP (Sigma Co.). Development was stopped by rinsing with PBS containing 20 mM EDTA.

In Israel, blocked and washed J2 were incubated for 1 hour with

antiserum (immune or nonimmune) diluted 1:500 in washing solution. After washing, nematodes were incubated in a 1:200-diluted solution of gold-conjugated secondary antibody (goat anti-mouse IgG, 5 nm particles; BioCell), and then exposed to the silver-enhancement solution described previously. Preparation for light microscopy was described before.

SC lability. Temperature's effect on the shedding phenomena was tested by incubating the antibody-labelled J2 for 5 days at 25°C or 4°C, and monitoring the labelling daily under a light microscope. Parallel treatments were performed with pre-fixed J2.

To verify the effect of the silver-enhancement reaction on SC shedding, J2 were treated with the primary antibody and incubated overnight, after washing, at 25°C or 4°C. Then J2 were treated with the gold-conjugated secondary antibody and with the silver-enhancement solution. As controls, nematodes which were kept at 25°C or 4°C for 24 hours were then treated with both antibodies and the silver-enhancement solution on the following day.

Interaction of nematodes with HRBC. Preparation of HRBC and assay performance were according to previously mentioned procedure. To evaluate the effect of J2 age on HRBC adherence, J2 were collected daily for 6 days, and each harvest was kept separately at 25°C or 4°C in PBS/2. The HRBC-binding assay was performed with each harvest on day 6; J2 used in these assays were preincubated for 1 hour in PBS/2 containing Ca^{+2} ions.

SDS-treated nematodes were assayed for HRBC adherence according to previously mentioned procedure. To test the recovery phenomenon, SDS-treated and non-treated species were further incubated for 2 days, at 4°C or at 25°C, and the number of adhering HRBC was recorded. In

addition, nematodes kept at 4°C for 2 days were then transferred to 25°C for another 2 days and the number of HRBC adhered to them was also monitored.

Results

The SC of *M. incognita* J2 covered the mid-body region in a continuous layer, approximately 5 nm thick, including the incisures of the transverse annulations. It bound cationized ferritin and stained with ruthenium red. Irregular patches or clumps associated with the SC also stained with ruthenium red and similar-staining material often accumulated in the incisures (Fig. 1).

Labelling with FITC- or TRITC-conjugated lectins and neoglycoproteins. As reported previously (McClure and Stynes, 1988; Spiegel, unpublished), FITC-Con A and FITC-WGA labelled only the amphids of *M. javanica* J2. The entire cuticle of *H. avenae* was labelled with FITC-WGA but no binding was recorded with *H. latipons* (Table 1). TRITC-Con A did not bind to either species of *Steinernema* tested. TRITC-WGA bound to the head region of *S. carpocapsae* and strongly to the amphids of *S. glaseri*.

Gold-Con A labelled the amphidial secretions of *M. javanica* as well as the transverse annulations and lateral alae over the entire nematode surface though less intensively in the head region (Fig. 2). Labelling was inhibited by the appropriate hapten. Gold-WGA labelled the amphidial secretions of *M. javanica* but the entire nematode surface labelling was much weaker than that recorded with Con A (Table 1). No binding was

recorded on *H. latipons* with gold-WGA, while strong labelling was recorded on the entire cuticle of *H. avenae* with this reagent (Table 1). Binding of gold-WGA to *S. carpocapsae* and *S. glaseri* revealed clear and strong labelling of the transverse annulation and lateral alae, but not of the head region nor of the amphidial openings of these species.

No binding was recorded with any of the FITC-conjugated neoglycoproteins tested with *M. javanica* J2. Gold-conjugated fucosylated- and mannosylated-BSA labelled the nematode surface with much the same pattern observed for Con-A except that the head region was not labelled (Fig. 3). Fucose and α -methyl mannoside pretreatments reduced these labellings. The galNAC- β -(1-4)gal-O-BSA labelled J2 in a similar pattern but specificity could not be verified by pretreatment with the appropriate hapten.

Ideally, a cell surface reagent should possess the following properties: its molecules should be small but without the ability to penetrate into the cell, it should react under physiological conditions and form stable bonds, and it should be easily detectable. Fluorescent lectins do not comply perfectly with these requirements and several problems have been experienced with these reagents: penetration of the cuticle by fluorescent lectins (Spiegel, unpublished results), non-glycoconjugate specificity (Davis et al. 1988; Jansson et al. 1986), strong nematode autofluorescence at the same wavelength as the fluorescent emission of fluorescein or rhodamine (Davis et al. 1988; Jansson et al. 1986; Spiegel et al. 1982), and prevention of persistent visualization due to rapid quenching of the fluorescent components.

The gold labelling method with silver enhancement offers a

sensitive, usually specific, clear visualization of labelling. Moreover, it produces stable bindings and so enables long term observations under light microscopy.

Binding of HRBC to nematodes. Differential binding abilities of HRBC to nematodes tested are presented in Table 2. HRBC adhered to J2 of the sedentary endoparasitic nematodes *H. avenae*, *H. schachtii* and *M. javanica*, and to the sedentary semiendoparasitic forms *P. mediterraneus*, *R. reniformis*, and *T. semipenetans*. This binding was over the entire nematode body except for the head region. Binding was conspicuously confined to the head and tail of the adenophorean plant parasites *L. cohni*, *X. brevicole* and *X. index*. Binding was Ca^{++} and Mg^{++} dependent. In contrast, no binding of HRBC to the aboveground plant parasites *A. tritici*, *A. subtenius*, and *D. dipsaci*, to *M. javanica* and *P. mediterraneus* adult females, and to the free-living form *P. redivivus* was recorded, even in the presence of Ca^{++} and Mg^{++} . The binding pattern just described did not vary with HRBC group (A, B, O) or Rh type. NaCl-treated J2 became nonmobile and binding of HRBC decreased; however, 24 hr later, J2 were recovered and the binding increased to the level which had been recorded in the control. Trypsin diminished HRBC binding to *M. javanica*. HRBC failed to adhere to nematodes pretreated with detergents to remove their SC (Table 3).

α -Methyl-mannoside and fucose decreased the binding at concentrations as low as 0.05 M (Table 3), while glucose and N-acetylglucosamine were effective only at 0.1 M (Table 3). Galactose and N-acetylgalactosamine enhanced the binding to HRBC at 0.2 M (Table 3).

Binding of HRBC to nylon fibers. HRBC binding to the SC-coated fibers was Ca^{++} and Mg^{++} dependent (58.8 ± 3.0 and 0.0 HRBC per mm fiber, with and without Ca^{++} and Mg^{++} , respectively). In contrast, the HRBC binding intensity to BSA-coated fibers was 23.1 ± 1.5 HRBC per mm fiber and fucose and α -methyl-mannoside also reduced the binding compared with the SC-coated treatment (31.6 and 20.0 ± 3.0 HRBC per mm fiber, respectively).

Separation of SC extracts on SDS-PAGE. In Tucson AZ, the electrophoretic profiles of proteins from *M. incognita* J2 labelled with either Biotin-NHS or ^{125}I showed similar patterns of labelling (Fig. 4). Both labelled the same principal protein (SC_1). Labelling with water-soluble biotin resulted in a greater number of labelled bands than labelling with biotin-NHS. The principal protein in all cases had a molecular weight greater than 250 kDa based on comparison of its electrophoretic mobility with that of known proteins. Other, much fainter bands, ranged in molecular weight from 31 kDa to 179 kDa. The profiles of proteins labelled with biotin-avidin were not altered by the addition of a reducing agent, β -mercaptoethanol nor did the SC extract from non-biotinylated J2 react with avidin conjugates (data not shown).

In the Israeli laboratory, the electrophoretic separation under reducing conditions of the SC proteins extracted with various detergents was repeated several times and a typical polypeptide pattern of these proteins is presented in Fig. 5. With CTAB, the most prominent polypeptides were 14, 22, 26, 31, 38, 49, 66 and 250 kDa in size. Extraction with nOG resulted in a main band at 49 kDa and some faint bands similar to those in the CTAB extract. SDS extraction yielded bands

at 6, 9, 26, 31, 49, 58, 66, 80, 205 and 250 kDa (Fig. 6, lane 1).

Gold-conjugated lectin and neoglycoprotein labellings on nematodes.

Con A labelled mainly the amphidial secretions, the transverse annulations and lateral alae over the entire nematode surface. Labelling was less intensive in the head region and was inhibited by the appropriate hapten. WGA labelled the amphidial secretions but labelling of the nematode surface was much weaker than that recorded with Con A.

Gold-conjugated fucosylated- mannosylated- and glucosylated-BSA labelling of the nematode surface followed much the same pattern observed with Con A, except for the head region which was not labelled. Fucose and α -methyl mannoside pretreatments reduced labelling. Although galNAc- β -(1-4)gal-O-BSA labelled J2 in a similar pattern, binding specificity could not be verified by the haptens used.

General and specific labelling of glycoconjugates and CRDs on blots.

In Tucson AZ, of the nine lectins tested, only Con A bound to SC₁ protein (Fig. 4). It also bound to two smaller proteins, but because corresponding bands were not observed in blots of biotin-labelled SC proteins these two were not considered to be associated with the surface coat. In Tucson and in the Israeli laboratory at Bet Dagan, following the biotinamidocaproyl hydrazide reaction, glycoprotein-related bands were found on western blots at 31 and 6 kDa (Fig. 4, lanes 2, 3; Fig. 7, lane 5). SC extract-labelling with lectins revealed bands additional to those obtained with the general glycoprotein staining. On western blots, Con A and WGA labelled the 6-, 9-, 14-, 31-, 58- and 66-kDa bands; WGA also labelled the 22- and 26-kDa bands. The compatible haptens, α -methyl mannoside and glucNAc,

respectively, partially inhibited lectin binding (Fig. 7).

The labelling pattern of the SDS-extracted SC on western blots was the same after exposure to all neoglycoproteins tested. Labelling was observed at 14, 22, 26, 58 and 66 kDa (Fig. 8). Preincubation with the appropriate carbohydrates did not inhibit or reduce neoglycoprotein binding to the membrane; however, compatible lectins did reduce the labelling intensity of mannosylated-, fucosylated- and glucosylated-BSA (Fig. 8). When Con A was used for inhibition, a new 31-kDa band appeared, which probably resulted from the lectin binding to itself.

Biotin labelling. J2 exposed to the biotin reagents revealed the same labelling pattern reported for the lectins and neoglycoproteins. Extraction of biotinylated nematodes with CTAB and western blotting to detect a specific biotin-labelled protein revealed no bands; biotinylated nematodes were therefore boiled in electrophoresis loading buffer. When streptavidin-HRP was used to trace the biotin-labelled proteins on a western blot, the main labelling was observed at 130 kDa in both biotinylated and non-biotinylated nematodes. Using anti-biotin-HRP instead of streptavidin-HRP and visualizing with ECL reagents on x-ray film revealed nonspecific labelling at 39 (in two out of four experiments) and 100 kDa for both biotin reagents, and specific labelling at 46 and 49 kDa using the ϵ -aminocaproic acid NHS-biotin (Fig. 7).

Polyclonal antibodies labelling. Polyclonal antibodies raised to partially purified SC₁ of *M. incognita* race 3 bound to the J2 cuticular surface (Fig. 9). On western blots of CTAB extracts from *Meloidogyne* spp. J2, the purified IgG fraction of the antiserum reacted with proteins not only from *M. incognita* race 3, but also with proteins from

M. incognita races 1 and 2, *Meloidogyne hapla* race 2, and *Meloidogyne arenaria* race B (Fig. 9). A common band occurred among all *Meloidogyne* species, but because it was not labelled with either biotin or ^{125}I , it was not considered to have originated from the surface of the J2.

SC lability

a. **Dynamic of HRBC adherence.** HRBC did not adhere to freshly hatched J2 (0-24 hr). Maximum adherence of J2 kept at 25°C was observed with 24- to 48-hr-old J2 which then decreased gradually (Fig. 10). Almost no adherence was recorded with nematodes kept at 4°C (Fig. 10).

b. **SC recovery after detergent-stripping.** Less HRBC adhered to the surfaces of detergent-treated J2 than to those of non-treated J2. When SDS-treated J2 were kept at 25°C for 2 days, the intensity of HRBC adhesion was the same as that recorded for the non-treated J2, whereas no adhesion of HRBC was recorded with the SDS-treated J2 which were kept at 4°C for 2 days. However, when these J2 were then transferred to 25°C for 2 days, adhesion recurred.

The electrophoretic profile of SDS-stripped SC of nematodes kept at 25°C for 2 days was similar to the original SDS-extraction's protein pattern, whereas the profile of SDS-stripped SC of nematodes kept at 4°C for 2 days presented only two bands at 58 and 66 kDa.

c. **Release of ^{125}I -labelled surface components.** Release of ^{125}I -labelled surface components from the nematodes was demonstrated by incubating labeled J2 in water and assaying the incubation supernatant over a period of 20 hours. Radioactivity of the supernatant increased fourfold in the first hour and then decreased to the initial background level after 20 hours incubation (Table 4). The same trend was observed

in a second trial with a different experimental design in which individual lots of ^{125}I -labeled J2 were incubated in separated tubes (data not shown). Because different experimental designs were used, the results could not be combined. Release of the SC was also demonstrated by detection of SC_1 -biotin in aqueous incubates of labeled J2 (Fig 4).

d. SC components turnover after labelling with antibodies, lectins and neoglycoproteins. The polyclonal antiserum raised against the *M. javanica* J2 SC was recognized on the immunoblot as a 250-kDa antigen. When exposed to live J2, the whole J2 surface was labelled with the antibody except for the head region, where only the amphids were labelled. Antibody-labelled nematodes incubated at 25°C for 5 days exhibited a gradual shedding reaction until no label could be recorded after 5 days. Labelling was relatively stable in J2 kept at 4°C. When pre-fixed J2 were exposed to the antibody, no shedding was recorded, even though the nematode surface had been labelled.

Live nematodes which were heavily labelled due to either exposure to high concentrations of a labelling material (i.e. primary or secondary antibody, lectin, neoglycoprotein), or longer exposure to the silver-enhancing reaction, exhibited rapid shedding from the labelled SC (Fig. 11).

J2 that were treated only with the primary antibody and left overnight for incubation at 4°C or 25°C were labelled intensively after they were exposed to the secondary antibody, similar to the controls which had been treated with the two successive antibodies.

Table 1. Fluorescent- and gold-lectin binding to cuticular surfaces of *Heterodera avenae* (H.a.), *H. latipons* (H.l.), *Meloidogyne javanica* (M.j.), *Steinernema carpocapsa* (S.c.) and *S. glaseri* (S. g.).

=====					
Lectin	H. a.	H. l.	M. j.	S. c.	S. g.
<hr/>					
Fluorescent lectin					
Con A	nt	nt	+++a;+b	-	-
WGA	+++b;+a	-	++a	h;+l	+a
Gold lectin					
Con A	nt	nt	++++l;+a	-	-
WGA	++++l	-	++l;+a	+++l	++l
<hr/>					

- = no labelling.

+b; ++b; +++b = increasing intensities of labelling of entire cuticle.

+a, ++a, +++a = increasing intensities of labelling to the amphids.

h = labelling of the head area.

+l; ++l; +++l; ++++l = increasing intensities of labelling of lateral alae and transverse annulation .

nt = not tested.

Table 2. Binding of human red blood cells (HRBC) to the cuticle of different nematode species.

=====	
Nematode [#]	Percent of cuticle with HRBC
<hr/>	
<i>Anguina tritici</i> (J)	0
<i>Aphelenchoides subtenius</i>	0
<i>Ditylenchus dipsaci</i>	0
<i>Heterodera avenae</i> (J)	75-80
<i>Heterodera schachtii</i> (J)	70-80
<i>Longidorus cohnii</i>	head & tail
<i>Meloidogyne javanica</i> (ad)	0
<i>Meloidogyne javanica</i> (J)	75-85
<i>Panagrellus redivivus</i>	0
<i>Pratylenchus mediterraneus</i> (ad)	0
<i>Pratylenchus mediterraneus</i> (J)	75-85
<i>Rotylenchulus reniformis</i> (J)	50-80
<i>Tylenchulus semipenetrans</i> (J)	50-80
<i>Xiphinema brevicole</i>	head & tail
<i>Xiphinema index</i>	head & tail
=====	

'head & tail' = adhesion was limited to the head and tail areas of some specimens. 'ad, J' = adults and juveniles, respectively. [#]Otherwise mentioned, mixed generations were tested

Table 3. Binding of human red blood cells (HRBC) to the outer cuticle of *Meloidogyne javanica* second-stage juveniles.

=====	
Treatment	Binding intensity [#]
<hr/>	
Non-treated (control)	75-85
Sodium azid solution (1%, 1 hour)	25-50
Sodium chloride (1.0 M, 2 hours)	50-75
Fucose (0.1 M, 2 hours)	25-50
Galactose (0.1 M, 2 hours)	90-100
Glucose (0.1 M, 2 hours)	50-75
Mannose (0.1 M, 2 hours)	25-50
N-acetylgalactosamine (0.1 M, 2 hours)	90-100
N-acetylglucosamine (0.1 M, 2 hours)	50-75
Trypsin (0.1 mg/ml, 2.5 hours, 37°C)	60-75
Trypsin (0.5 and 1.0 mg/ml, 2.5 hours, 37°C)	25-50
Cetyl triethylammonium bromide	10-15
n-Octyl glucoside	75-85
Sodium dodecyl sulphate	75-85
=====	

Except where noted, tests were run at room temperature. Binding was recorded 1 hour after incubation with HRBC.

[#]percent of cuticle with HRBC.

Table 4. Release of ^{125}I from the surface of infective juveniles (J2) of *Meloidogyne incognita*.

Incubation time (hours)	CPM
0	23854
1	77993
2	66100
4	59905
8	57045
16	46704
20	24517

CPM: Counts per minute (gamma emission) from a scintillation vial containing 20 cc of water miscible scintillation cocktail and 1.0 cc of supernatant from ^{125}I -labeled *M. incognita* J2. Samples were drawn at intervals from a 2.0 cc pool of labeled nematodes and, after sampling, the original volume of the pool was restored by addition of 1.0 cc of sterile distilled water.

Fig 1. Electron micrographs of the surface coat (arrows) of *Meloidogyne incognita* infective juveniles. A) Nematode stained with ruthenium red. B) Nematode treated with cationized ferritin. Scale = 0.1 μm .

Fig. 2

Figs. 2 3. Anterior end of *Meloidogyne javanica* second-stage juveniles. 2) Labelling with gold-Concanavaline A. 3) Labelling with gold-conjugated fucosylated-BSA (bovine serum albumin).

Fig. 3

Fig. 4. Western blots of *Meloidogyne incognita* surface coat proteins labeled with 1) ^{125}I [autoradiograph]. 2) Biotin-NHS. 3) Biotin-X-NHS. 4) Concanavalin A. 5) prestained standard proteins. SC_1 (arrow) = principal surface protein, approximately 250 kDa.

Fig. 5. Silver-stained SDS-polyacrylamide gels of detergent-extracted surface coat proteins from *Meloidogyne javanica* second-stage juveniles. Lane 1 - extraction with cetyltrimethylammonium bromide. Lane 2 - extraction with n-octyl glucoside.

Fig. 6. Protogold-stained western blots of SDS-extracted surface coat proteins of *Meloidogyne javanica* second-stage juveniles, demonstrating the temperature-dependence of surface coat recovery. Lane 1 - SDS-extraction. Lane 2 - same as lane 1, but after 2 days at 25°C. Lane 3 - same as lane 1, but after 2 days at 4°C.

Fig. 7. Western blots of SDS-extracted surface coat proteins of biotinylated and non-biotinylated *Meloidogyne javanica* second-stage juveniles. Lane 1 - biotinylation with sulfo N-hydroxysuccinimide (NHS) - biotin. Lane 2 - biotinylation with ϵ -aminocaproic acid NHS-biotin. Lane 3 - non-biotinylated nematodes.

Fig. 8. Western blots of SDS-extracted surface coat proteins of *Meloidogyne javanica* second-stage juveniles, labelled with gold-conjugated neoglycoproteins. Lane 1 - fucosylated-bovine serum albumin. Lane 2 - same as lane 1, but pretreated with *Ulex europaeus* agglutinin.

Fig. 9. A) Infective juvenile of *Meloidogyne incognita* treated with anti-SC₁ antibodies and FITC-conjugated goat, anti-rabbit immunoglobulins. Labeling of the surface coat is indicated by UV fluorescence. Scale = 10 μ m. B) Western blot of CTAB extracts from unlabeled J2 of different *Meloidogyne* spp., probed with anti-SC antibodies against *M. incognita* race 3. (1) extract from *M. incognita* race 3; (2) extract from *M. incognita* race 2; (3) extract from *M. incognita* race 1; (4) extract from *Meloidogyne hapla* race 2; (5) extract from *Meloidogyne arenaria* race B.

Fig. 10. Binding intensity[#] of human red blood cells (HRBC) to the outer cuticle of *Meloidogyne javanica* second-stage juveniles (J2). Tests were run at room temperature. J2 were collected daily, for 6 days, and each harvest was kept separately in phosphate buffer 0.1 M saline, pH 7.2. The HRBC binding assays for each harvest were performed on day 6. Binding was recorded 30 min after incubation with the HRBC. Ten individuals were monitored per well. Experiments were repeated at least three times with consistent results. [#]Number of HRBC per nematode $\pm$ s.e.

Fig. 11. The rapid shedding observed when second-stage juveniles of *Meloidogyne javanica* were heavily labelled with anti-surface coat antibodies and gold-conjugated goat anti-mouse IgG, and then exposed to a silver-enhancement solution as described in the text.

II. Biological Role of the SC

Materials & Methods

Bacteria. *Pasteuria penetrans* (Thorne, 1940) Sayre and Starr, 1985, as amended by Starr and Sayre (1988) was mostly the Tifton isolate obtained from R. M. Sayre, Beltsville, MD, but for comparative trials were also with a Riverside, CA strain from R. Mankau and a Florida strain from D. Dickson.

Preparation of monoclonal antibodies and attachment studies:

M. incognita Race 3 were reared in the greenhouse on eggplant, *Solanum melogena* cv. Black Beauty, and J2 were collected from hatched eggs.

Freund's Complete Adjuvant, 0.01 ml, was mixed with 0.01 ml of live J2 and the mixture injected into the peritoneum of BALB/C mice. Four injections were given at 7-day intervals, and 34 days after the fourth injection, a final injection of the same preparation was administered. Three days after the final injection, the mice were sacrificed and their splenocytes fused with murine myeloma cells.

Hybridoma supernatants were screened for antibodies to the nematode's surface by incubating live J2 in the supernatant for 1 hour, rinsing the J2 three times with PBS, and incubating them for 1 hour in goat-antimouse-FITC secondary antibody. Labelled nematodes were detected by viewing the preparations with a UV-fluorescence microscope at a magnification of 200x and cell lines were selected on the basis of intensity of labelling. Selected cell lines were cloned, and culture medium supernatant from these clones was lyophilized and used for attachment assays. For attachment studies, lyophilized

antibody/hybridoma supernatant was dissolved in sterile, distilled water to a concentration of 1.7 mg antibody/supernatant per ml. Lyophilized culture medium supernatant diluted in water to 1.5 mg/ml was used as a control. Three ml of rehydrated antibody or control solution were placed in a sterile scintillation counting vial, and 6000 J2 and 6×10^6 *Pasteuria* spores/ml in 0.1 ml of water were added. Air from an aquarium pump, delivered through a 1 cc tuberculin syringe with a 26G needle, was bubbled into the solution overnight at 30° C. The solution was then poured onto a 500 mesh sieve which allowed the unattached spores to pass through but retained the larvae. The larvae were washed from the sieve and mounted live on a haemocytometer counting slide for observation. Counts were made of the number of spores attached to each of 116 nematodes in each treatment. Treatments were antibody + hybridoma medium, hybridoma medium alone, and water. Three categories were established to record the density of attachment. 0-2 spores/nematode; 3-15 spores/nematode and more than 15 spores/nematode. The experiment was replicated 4 times and data analyzed by analysis of variance.

Parallel, but different, experiments were run in Florida using the centrifugal method for attachment of bacterial spores (T.E. Hewlett & D.W. Dickson, JON. 1994 25:785-788). Two *M. incognita* race 3 second stage juveniles were placed in a 0.25 ml tube in 0.1 ml of water and an equal volume of rehydrated antibody or culture medium was added. After mixing, the preparation was incubated for either 4 or 8 hours. The antibody or culture medium was then removed by centrifugation/rinsing three time, and a suspension of 10000 endospores of *P. penetrans* was added. The tubes were centrifuged at 9,500g for 2 minutes and

the nematodes removed for observation. Numbers of attached endospores were counted. Each treatment was replicated 5 times and each experiment was run once.

Attachment of three *P. penetrans* isolates: As a first step in the current experiments, attachment of 3 isolates of *P. penetrans* to *M. incognita* race 3 was studied. Isolate 1 was a Tifton strain, Isolate 2 a Florida strain and Isolate 3 a Riverside strain. The procedures were the same as for other studies with the exception that the spore/nematode mixture was not aerated during incubation hence the lower numbers of spores attached/nematode. Attachment of spores to 20 nematodes was counted for each replicate. This experiment was replicated 3 times.

Effect of chemicals on attachment. The effect of several chemicals on attachment of *P. penetrans* had been reported (Stirling et al., 1986) but variations of these studies were carried out since the *P. penetrans* strain and the root-knot species differed. In addition, several chemicals previously not utilized were evaluated. Enzymes tested were neuraminidase Type V (Sigma 2876) 2 U/ml in buffer (Na acetate 10 mM, CaCl_2 4mM, pH 5.5); pronase (Sigma 5380) 7-15 u/mg; 50 U/ml, in PBS, pH 7.3, 3 hours at 37°C, trypsin (Sigma T-8462) 10 mg/ml; PBS, pH 7.2, 3 hours at 37°C, lipase (Sigma 3001) 10 U/mg) 2 U/ml, pepsin 5 mg/ml. Na acetate buffer, pH 4.0, 3 hours at 37°C. Each of the enzyme tests was replicated 8 times, with the exceptions of pepsin with 6 replicates spore attachment in the appropriate buffer alone serves as a control. Since alcian blue blocks sulfyl groups and these groups are present within the *Caenorhabditis elegans* cuticle (Himmelhoch & Zuckerman,

1985), nematodes were treated with 0.5% alcian blue in sodium acetate buffer, pH 5.5. Control was buffer alone. There were 3 replicate trials.

Detergents: Experimental conditions for detergents were 1% CTAB (Sigma #MTA-5), 2% SDS in PBS, and 10 mM Na periodate in 2 M Na acetate. SDS and CTAB were at room temperature for 1 hour and Na periodate in an ice bath in the dark for 1 hour. Treatments were added to newly hatched *M. incognita* J2 held in watch glasses in a laminar flow hood. After 1 hour J2 were concentrated by centrifuging in a 1.5 ml sterile Eppendorf tube, washed 3 X with sterile tap water, spore suspension added (3×10^6 spores/ml), incubated at 30°C with aeration overnight, nematodes collected on a 500 mesh screen, mounted on and observed for attachment on a hamocytometer slide. Controls were nematodes similarly exposed to spores but incubated for 1 hour in sterile tap water.

Effect of cationized ferritin: To determine the involvement in attachment by negatively charged groups on the surface of the nematode cuticle in attachment, cationized ferritin (Himmelhoch et al., 1977) was applied to 8,000 *M. incognita* J2 larvae. In the first experiment, J2 were first exposed to monoclonal antibody 1 hour as previously described. Cationized Ferritin (1 mg/ml in 0.15 NaCl) was added + sterile distilled water for 1 hour. The solution containing nematodes was concentrated by centrifugation, washed once with sterile, distilled water, brought up to 3 ml in sterile distilled water in a scintillation counting vial, *P. penetrans* (8×10^6 spores added, incubated overnight under vigorous aeration at ambient room temperature, and then spore attachment recorded as before. The second experiment with cationized

ferritin proceeded in the same way, but without treatment with the monoclonal antibody.

Attachment to live or dead nematodes: Comparisons were made between spore attachment to live nematodes as compared to dead nematodes. Conditions for spore attachment were the same as for the monoclonal antibody studies including aeration. These studies encompassed six replicate trials and observations on attachment to 240 nematodes.

Results

Attachment to *M. incognita* by 3 different *Pasteuria penetrans* strains: In each of the 3 trials, attachment to *M. incognita* J2 larvae was significantly higher ($P > 0.05$) for the Tifton strain, than for the Florida or Riverside strains. Stirling (1985) noted similar differences in attachment of different populations of *P. penetrans* to *M. incognita* and other root-knot species.

Alcian Blue and Enzymes: There was a significant reduction ($P = 0.05$) of attachment of *P. penetrans* spores in the first alcian blue trial, but in the succeeding two trials alcian blue did not reduce spore attachment. The results suggest that SO_4^{-2} radicals are not important to the attachment of *P. penetrans* spores. None of the enzymes tested had a consistent, significant effect on attachment of spores to the larvae.

Detergents: The detergent CTAB consistently brought about a significant reduction ($P > 0.05$) in attachment of *P. penetrans* spores (Table 5). In 8 of 9 trials, SDS also caused a reduction of spore

attachment. Results with Na periodate were more variable, with significant decreases or increases in attachment. The current results can be noted in context with those of Persidis et al. (1991) wherein SDS spore fragment extracts did not inhibit attachment of *P. penetrans* spores to *M. incognita*. Davis and Danks (1993) reported no effect of Na periodate on spore attachment, and the current results are in accord with those findings. Davis and Danks (1993) reported that SDS at room temperature significantly increased spore attachment, but significantly decreased spore attachment at 2 minutes at 100°C. The current results showing a significant decrease in attachment follows SDS treatment at room temperature are different from those of Davis and Danks (1993), but the race of both *Pasteuria* and *M. incognita* in the current studies were probably different. CTAB, which gave consistent reduction in spore attachment, was not, to our knowledge, previously tested.

Attachment to live or dead nematodes: Spore attachment to dead nematodes was significantly less ($P = 0.05$ or 0.01) than to live nematodes in all 6 trials. These results differ from those of Stirling et al. (1986) who reported equal attachment to live or dead nematodes.

Effect of cationized ferritin on spore attachment: The blocking of negative charges on the nematode cuticle surface caused a significant additive reduction in spore attachment when compared to treatment with antibody alone (Table 6). Treatment with cationized ferritin alone gave about the same amount of reduction of attachment, suggesting that negative charges on cuticle surface molecules have an important role in attachment of *Pasteuria* spores (Table 7). In this case, the attachment mechanism would be non-specific.

The effect of monoclonal antibody on attachment: There was a significant reduction ($P = 0.05$) in the numbers of attached *P. penetrans* spores to larvae when larvae were pretreated with monoclonal antibody plus hybridoma culture medium. Medium alone significantly reduced attachment. However, it was possible to discriminate the additive effect of the monoclonal antibody on reductions of attachment. In each case, attachment of spores to water series in was much greater than with antibody or medium-treated nematodes. Parallel tests conducted in Florida with different methods gave comparable results. Attachment of endospores following treatment of the nematodes with antibody was significantly reduced ($P=0.01$) (Table 8).

Table 5. Attachment of *P. penetrans* spores to *M. incognita* J2 as affected by detergents.

Trial #	Control	CTAB	SDS	Na periodate
1	43 ± 8	23 ± 15*	14 ± 7*	25 ± 7*
2	76 ± 17	35 ± 13*	33 ± 10*	70 ± 32
3	88 ± 18	45 ± 11*	53 ± 16*	73 ± 33
4	63 ± 32	29 ± 8*	-----	57 ± 30
5	33 ± 7	20 ± 6*	9 ± 6*	48 ± 12*
6	16 ± 5	13 ± 4*	16 ± 8*	21 ± 6*
7	44 ± 15	9 ± 5*	4 ± 4*	10 ± 8*
8	86 ± 15	32 ± 12*	11 ± 8*	81 ± 23
9	48 ± 13	36 ± 16*	35 ± 9*	81 ± 9*
10	37 ± 9	28 ± 9*	24 ± 10*	40 ± 21

* Significantly

greater or less than untreated control (P = 3D 0.05) Data shown

mean

+ standard deviation. ---- + no observation.

Table 6. Effects of the monoclonal antibody combined with treatment by cationized ferritin on attachment of *Pasteuria* species.

Treatment	0-2 spores	3-15 spores	>15 spores
	/ nematode	/ nematode	/nematode
antibody + medium	2.0 $\pm$ 0.0	31.5 $\pm$ 2.1	82.5 $\pm$ 2.1
medium	5.0 $\pm$ 1.4	12.0 $\pm$ 9.9	99.0 $\pm$ 8.5
antibody +	0.5 $\pm$ 0.7	52.5 $\pm$ 7.8	63.0 $\pm$ 7.1
med./Ferritin			
medium/Ferritin	1.0 $\pm$ 0.0	38.0 $\pm$ 2.8	77.0 $\pm$ 2.8

Each treatment significantly differs from the others at $P = 0.05$ specifically when 3 or more spores were attached/nematode.

Table 7. Effects of cationized ferritin treatment on attachment of *Pasteuria** spores.

Treatment	0-2 spores / nematode	3-15 spores / nematode	> 15 spores / nematode
0.15 M NaCl	0.5 $\pm$ 0.7	15.0 $\pm$ 1.4	100.5 $\pm$ 0.7
cationized ferritin			
in 0.15 M NaCl	2.0 $\pm$ 0.7	38.0 $\pm$ 5.7	75.5 $\pm$ 6.4
dist. water	0.5 $\pm$ 0.7	5.0 $\pm$ 2.8	110.5 $\pm$ 3.5

* Each treatment differs significantly ($P < 0.05$) from each other treatment.

Table 8. Attachment of endospore of *Pasteuria penetrans*, isolate P-110, to *Meloidogyne incognita* race 3, following treatment of the nematode with monoclonal antibody or hybridoma culture medium.

Treatment	Incubation Time	Mean No. of spores/nematode
Water	4 hours	32.6a
Water	8 hours	35.6a
Antibody	4 hours	14.6b
Antibody	8 hours	15.5b
Medium	4 hours	44.2a
Medium	8 hours	24.4a

Means followed by the same letter did not differ significantly ($P=0.01$).

Discussion

Erythrocytes have been used as a tool to trace CRD (Jungrey and Weatherall, 1986) since the preponderance of their membrane carbohydrate consists of oligosaccharides linked to three types of glycophorine molecules, also known as sialoglycoproteins (Mirelman, 1986).

Our results show different HRBC binding ability for nematode species and life stages within species (Table 2). Among the tylenchid plant-parasitic nematodes tested, HRBC bound to migratory endoparasitic (*P. mediterraneus*), sedentary endoparasitic (*H. avenae*, *H. schachtii* and *M. javanica*), and semiendoparasitic (*R. reniformis* and *T. semipenetrans*) nematodes. No binding was recorded to the three major genera that attack aerial parts of plants: *Anguina*, *Aphelenchoides* and *Ditylenchus*). With the ectoparasitic dorylaimid nematodes *Xiphinema* and *Longidorus*, HRBC binding was restricted to only the head and tail areas. Clear differences were also apparent in HRBC binding to different life stages of *M. javanica* and *P. mediterraneus*, an observation consistent with the concept of development-related changes in cell surface components (Himmelhoch and Zuckerman, 1978).

Sedentary J2 of *H. avenae*, *H. schachtii*, *M. javanica*, and of *R. reniformis* were hatched from eggs, namely, no plant material could be present on their surfaces. It is possible, however, that other species mentioned in this work which were extracted from plant materials could have their CRD blocked by previous association with plant oligosaccharides, although worms were carefully washed with PBS before any treatment.

Regulation of cell to cell adhesion is well documented (Muller,

1985; Springer, 1990) and includes nonspecific bindings mediated by surface charge (Springer, 1990) and specific adhesions, in which the activity and function of cell adhesion molecules are assumed to be modulated by enzymatic means or nonenzymatically by reversible interaction of cell adhesion molecules with lectins (Muller, 1985; Springer, 1990).

Our results show that HRBC binding to intact phytonematodes or to *M. javanica* SC is a Ca^{++} - and Mg^{++} -dependent process: these cations not only are necessary in the binding process per se, but also are prerequisite in the preincubation period of the nematodes with the buffer. Moreover, the binding to both intact *M. javanica* J2 and its SC can be inhibited by pre-incubation with several carbohydrates and trypsin (Table 3). These results demonstrate the HRBC adhesion involves carbohydrate moieties of HRBC and corresponding carbohydrate-recognition domains on the nematode surface. To our knowledge, this is the first report of surface CRD on the phylum Nematoda.

The phenomenon of increased binding of HRBC to J2 of *M. javanica* CRD following preincubation of J2 with galactose or N-acetylgalactosamine (Table 3) is statistically significant, but very difficult to explain.

A classification of animal lectins (Drickamer and Taylor, 1993) proposes several groups, one of which is Ca^{++} -dependent (C-type). Several mannose- and fucose-binding proteins belong to this group. Most of the mannose-binding proteins consist of collagen-like sequences, repeated Gly-X-Y triplets, in which X is often proline and Y is often 4-hydroxyproline. They also contain hydroxylysine to which the carbohydrate moiety is attached and, along with other C-type CRD, many

cysteine residues (Drickamer and Taylor, 1993). Therefore, the C-type CRD inferred here corresponds to proteins moieties which have been identified in the nematode cuticle (Cox et al. 1990; Pasternak, 1988, Selkirk et al. 1989). Unlike animal- parasitic nematodes such as *Ascaris lumbricoides* (Pasternak, 1988), *Brugia malayi*, *B. pahangi* (Selkirk et al. 1989) and *Haemonchus conturtus* (Cox et al. 1990), or in the free-living nematodes - *Caenorhabditis elegans* (Cox et al. 1981) and *Panagrellus silusiae* (Pasternak, 1988) in which the collagen-like molecules are located mainly in the inner cuticular layers, the plant-parasitic nematode, *Meloidogyne incognita*, has collagen-like proteins distributed throughout the entire cuticle (Hussey and Jansma, 1988).

If these proteins also appear in the SC, an explanation for differences in binding between the free-living (*P. redivivus*), and the tylenchid phytonematodes that we examined is available. Cuticles of the plant-parasitic nematodes that fail to bind HRBC may contain components with a different collagenous sequence, no collagenous domains, or CRD masked by other components.

Politz and Philipp (1992) defined surface proteins by their accessibility to non-penetrating labelling methods and their ability to be solubilized from the cuticle by mild extraction procedures. We chose the SDS extraction method for our analyses because it produced more protein fragments on a SDS-PAGE than other detergent-extracts of the SC, while having no effect on J2 viability.

Blaxter (1993) reported avidin-binding activity in a non-biotinylated adult *Caenorhabditis elegans* extract. A similar phenomenon was observed with the biotinylated and non-biotinylated *M. javanica* J2

extract using a streptavidin conjugate on the western blots. However substituting an anti-biotin conjugate for the streptavidin reagent enabled the detection of specific labelling. In this report, SC proteins of *M. incognita* pre-parasitic J2 were labelled with either biotin or radioiodine. Extraction of biotinylated nematodes with CTAB and the use of avidin to detect the biotin-labelled protein on a western blot yielded a main protein band with a molecular weight higher than 250 kDa, whereas non-biotinylated nematodes did not exhibit this band. In this work, CTAB extract of *M. javanica* SC did not reveal any biotin-labelled protein on a western blot when streptavidin was used as a detector.

Surfaces of intact juveniles reacted with lectins, neoglycoproteins and antibodies, reagents which also reacted on western blots with the SDS-extracted SC proteins, indicating that these labelled proteins are part of the nematode's surface complex.

On the western blot, the 6-, 9-, 14-, 31-, 58- and 66-kDa bands could be related to glycoproteins because some of these bands reacted with biotinamidocaproyl hydrazide and also with lectins. The labelling of *M. javanica* J2 has already been reported with fluorescent-conjugated lectins: labellings with Con A and WGA were seen mainly in the amphid region, whereas labelling on the cuticle was very faint; the appropriate haptens failed to inhibit the labelling (Davis et al. 1988; McClure and Stynes 1988). The results reported here demonstrate labelling by both Con A and WGA, which was reduced by the competitive haptens, on the J2 cuticle surface including the amphids. SDS-PAGE-separated SC proteins of *M. javanica* J2 reacted with both Con A and WGA, but only partial inhibition was recorded with the appropriate haptens.

J2 exposed to neoglycoproteins revealed a binding pattern similar to that recorded for HRBC. These neoglycoproteins also reacted with the SC protein extract on a western blot. However, whereas the neoglycoprotein labelling on live nematodes could be inhibited by competitive carbohydrates, labelling on western blots could not. Although partial inhibition of the neoglycoprotein labelling was achieved with compatible lectins, lectins such as Con A are also capable of binding to SC extracts as shown in this work. The western blot of J2 SC extract yielded three bands at 14, 58 and 66 kDa which reacted with both lectins and neoglycoproteins, thereby putting into question the efficacy of neoglycoprotein-binding inhibition by lectins; moreover, while attempting to inhibit mannosylated-or glucosylated- BSA binding to the western blot with Con A, a new 31-kDa band appeared on the blot.

SC lability is well-documented in several animal parasites and free-living nematodes and it is generally accepted that the biological function of this feature is to avoid detection by the host's immune system (Blaxter *et al.* 1992; Page *et al.* 1992; Politz and Philipp 1992). In plant-parasitic nematodes evidence supporting this phenomenon is documented in this report: 1. J2 of *M. incognita* race 3 labelled with radioiodine and incubated in water for 20 hours, released ^{125}I into the water suggesting the transitory nature of the SC proteins. 2. Using monoclonal antibodies produced in McClure's laboratory, British workers have found SC from *M. incognita* bound to the middle lamellae of infected tomato root cells along the path of J2 migration (Von Mende, personal communication). The role this material plays in the host-parasite relationship is unknown, as is what, if any, changes occur in the SC in

post-parasitic forms.

This report explores a few aspects of SC lability in *M. javanica* pre-parasitic J2. The dynamics of HRBC adherence to pre-parasitic J2 surfaces indicates changes occurring in the SC during the life cycle. Maizels *et al.* (1987) reported that antibodies do not bind to freshly hatched *Toxocara canis* larvae. HRBC barely adhered to freshly-hatched 0- to 24-hours-old J2, although they were assumed to adhere to the nematode's surface CRDs. It was therefore concluded that the CRDs are probably absent at this early life stage and produced only later on. Consequently, nematode age and SC lability should always be considered when SC-labelling or extraction are performed.

The ability to be replaced after external removal reflects another aspect of the SC's transitory nature. Detergent-treated *M. javanica* J2 are capable of re-adhering HRBC after a recovery period at an optimal temperature (25°C). Nematodes which were metabolically arrested due to incubation at low temperature (4°C) did not re-produce all the SC components, as indicated by the absence of HRBC binding and by the electrophoretic profile of surface proteins extracted before and after incubation of J2 at different temperatures. This protein profile demonstrates the ability of the SC components to recover under conditions where the nematode is metabolically active. Similar SC recovery after detergent stripping has been demonstrated for *Nippostrongylus brasiliensis* by Preston-Meek and Pritchard (1991). Slow shedding of the gold-coated antibodies was observed at 25°C, whereas it barely occurred at 4°C. The rapid shedding observed when nematodes were heavily labelled is attributed to a mechanical effect of the silver-enhancing reaction

and nematode movement. Unlike other nematodes (Page *et al.* 1992; Smith *et al.* 1981), treatment with the primary antibody did not result in surface shedding. The sloughing off process is therefore an active, dynamic, metabolic event on the phytonematode surface, a property which may enable the nematode to avoid 'unnecessary' recognition events that might lead to resistance reactions by the plant.

Evaluation of the research achievement with respect to the original research proposal and objectives.

Our original goal was to characterize the SC of two root-knot nematode species and to investigate its role in the specific binding of *Pasteuria penetrans*, a nematode-parasitic bacterium that shows great promise as a biological tool for nematode management. We have achieved these goals and feel that the foundation of knowledge resulting from this study will form the basis for continuing investigations that will lead to real progress in reducing nematode depredations.

With regard to these objectives, four discoveries deserve special mention because of their far-reaching implications:

- 1) The surface coat of root-knot nematodes (and probably all Tylenchid plant parasites) can be shed and replaced by the living nematode. This phenomenon has been observed in animal parasitic nematodes where it has been implicated in their ability to avoid active defense mechanisms initiated by the host. A similar function could be postulated for plant parasitic nematodes.

- 2) Carbohydrate Recognition Domains (CRD) were demonstrated for the first time on the surface of nematodes. The ramifications of this

discovery are yet to be determined, but because CRD form the bases for recognition systems in a broad spectrum of biological interactions, the possibility that they play a similar role in nematode biology and host-parasite relations seems likely. An understanding of how nematodes and host plants recognize each other will be essential in modern molecular approaches to developing resistant hosts.

3) Using monoclonal antibodies we developed during this investigation, British workers have found that not only is exfoliated nematode SC distributed within the host, along the path of nematode migration, but can be found WITHIN cells adjacent to the nematode. This is the first documentation of nematode products within host cells, and the occurrence of this material in cells at the site of infection may change our concept of how nematodes affect plants and bring about the remarkable changes in plant structure and function that characterize the root-knot nematodes.

4) We have provided positive evidence that the SC is involved in the adhesion of *Pasteuria penetrans* to the nematode cuticle. Furthermore, we have shown that it is possible to block adhesion by application of SC specific antibody or other haptens that bind to the SC. Such knowledge could lead to identification of specific receptor sequences that would permit the engineering of even more effective strains of this promising biocontrol parasite.

One aspect of the original proposal that has not been fully realized is the determination of the origin of the SC. This investigation is on-going, and the availability of a monoclonal antibody that is specific to a SC protein insures that this objective

will be completed shortly.

Conclusion remarks

We found that the surface coat of *Meloidogyne* species consists of one or more glycoproteins that cover the surface of root-knot nematodes in a layer approximately 5 nm thick. The two species of root-knot nematodes examined in this study, *Meloidogyne incognita* and *M. javanica*, differed in the number and kinds of constitutive SC proteins. The SC of both species contained a protein with a putative molecular weight of 250 kDa. In *M. incognita*, it was glycosylated with mannose whereas, in *M. javanica*, it existed as an aglycone. Antibodies we raised against SC proteins were capable of blocking adhesion of one such parasite, *Pasteuria penetrans*, to the nematode and to simulated nematodes (nylon fibers) coated with SC proteins. We also found that the SC is shed and replaced continuously by living nematodes. Components of the shed SC were localized in host tissues by electron microscopy. It was also shown that *M. javanica* and other plant parasitic nematodes possess carbohydrate recognition domains (CRD) on their surfaces, probably as constituents of the SC.

From the above, we conclude that:

1. Protein profiles of the SC differed, qualitatively and quantitatively, between the two nematode species and these differences may be exploited for the development of advanced diagnostic tools. Differences in SC composition could also figure prominently in several important aspects of nematode biology and parasitism as described below.
2. The SC plays an important role in the process of infection of

nematodes by certain nematode parasites and that further examination of this interaction will provide additional clues to the role of the SC in adhesion of parasitic biocontrol organisms (bacteria, fungi, etc.) that depend upon adhesion for infection of the nematode, ultimately leading to more efficient biocontrol agents.

3. The SC is involved in host-recognition phenomena through CRD-target systems and through deposition of SC materials in host tissues. Further study of SC materials in host plants will lead to a greater understanding of nematode-host interactions and to the development of new strategies for breeding nematode resistant crop cultivars.

In summary, we conclude that the SC of root-knot nematodes is essential to nematode survival and is a vital component of manifold biological functions and interactions essential to host infection and parasite invasion. Continued investigation of the nematode SC will yield important discoveries in the areas of biocontrol and host plant resistance.

Literature citation

Bird, A. F. and B. M. Zuckerman. 1989. Studies on the surface coat (glycocalyx) of the dauer larva of *Anguina agrostis*. *International Journal of Parasitology* 19:235-240.

Blaxter, M. L. 1993. Cuticle surface proteins of wild-type and mutant *Caenorhabditis elegans*. *The Journal of Biological Chemistry* 268:6600-6609.

Blaxter, M. L., Page, A. P., Rudin, W. and Maizels, R. M. 1992.

Nematode surface coats: actively evading immunity. *Parasitology Today* 8:243-247.

Bradford, M. 1976. A rapid and ensitive method for the quantitation of microgram quantities of proten utilizing the principle of protein dye binding. *Analytical Biochemistry* 72:248-254.

Cox, G. N., Kusch, M. and Edgar, R. S. 1981. Cuticle of *Caenorhabditis elegans*: Its isolation and partial characterization. *Journal of Cell Biology* 90:7-17.

Cox, G. N., Shamansky, L. M., and Boisvenue, R. J. 1990. *Haemonchus conturtus*: Evidence that the 3A3 collagen gene is a member of an evolutionarily conserved family of nematode cuticle collagens. *Experimental Parasitology* 70:175-185.

Davies, K. G. 1995. In vitro recognition of a 190 kD putative attachment receptor from the cuticle of *Meloidogyne javanica* by *Pasteuria penetrans* spore extract. *Biocontrol Science and Technology*, in press.

Davies, K. G., and C. Danks. 1992. Interspecific diffeences in the nematode surface coat between *Meloidogyne incognita* and *M. arenaria* related to the adhesion of the bacterium *Pasteuria penetrans*. *Parasitology* 105:475-480.

Davies, K.G., B. R. Kerry and C.A. Flynn. 1988. Observations on the pathogenicity of *Pasteuria penetrans* a parasite of root knot nematodes. *Annals of Applied Biology* 112:491-501.

Davies, K. G., M. P. Robinson and V. Laird. 1992. Proteins involved in the attachment of a hyperparasite, *Pasteuria penetrans*, to its plant-parasitic nematode host, *Meloidogyne incognita*. *Journal of*

Invertebrate Pathology 59:18-23.

Davis, E. L., D. T. Kaplan, T. A. Permar, D. W. Dickson and D. J. Mitchell. 1988. Characterization of carbohydrates on the surface of second-stage juveniles of *Meloidogyne* spp. *Journal of Nematology* 20:609-619.

Devaney, E. 1991. The surface antigens of the filarial nematode *Brugia*, and the characterization of the major 30 kDa component. Pp. 46-65 in M.W. Kennedy ed. Parasitic nematodes - antigens, membranes and genes. London: Taylor and Francis.

Drickamer, K. 1988. Two distinct classes of carbohydrate-recognition domains in animal lectins. *Journal of Biological Chemistry* 263:9657-9560.

Foley, M., J. P. Kusel and P. B. Garland. 1986. Fluorescence recovery after photobleaching: Application to parasite surface membranes. *Parasitology Today* 2:318-320.

Himmelhoch, S. and Zuckerman, B. M. 1978. *Caenorhabditis briggsae*: Aging and the structure turnover of the outer cuticle surface and the intestine. *Experimental Parasitology* 45:208-224.

Hussey, R. S. and Jansma, P. 1988. Immunogold localization of collagen in the cuticle of different life stages of *Meloidogyne incognita*. *Journal of Nematology* 20:641 (Abstr.).

Inbar, J. and Chet, I. 1992. Biomimics of fungal cell-cell recognition by use of lectin-coated nylon fibers. *Journal of Bacteriology* 147:1055-1059.

Jansson, H. -B. 1987. Receptors and recognition in nematodes. Pp. 153-158 in J.A. Veech and D. W. Dickson, eds. *Vistas on nematology*,

Hyattsville: Society of Nematologists.

Jansson, J.-B., A. Jeyaprakash, G. C. Coles, N. Marban-Mendoza, and B. M. Zuckerman. 1986. Fluorescent and ferritin labelling of cuticle surface carbohydrates of *Caenorhabditis elegans* and *Panagrellus redivivus*. *Journal of Nematology* 18:570-574.

Jungrey, M. and Weatherall, D. J. 1986. Carbohydrate recognitions mediate attachment of *Plasmodium falciparum* malaria to erythrocytes. Pp. 335-357 in D. Mirelman ed. Microbial lectins and agglutinins John Wiley & Sons, New York.

Kaplan, D. T. and T. R. Gottwald. 1992. Lectin binding to *Radopholus citrophilus* and *R. similis* proteins. *Journal of Nematology* 24:281-288.

Kennedy, M. W. 1991. Parasitic nematodes - antigens, membranes and genes. Taylor and Francis, London. 243 p.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680-685.

Luft, J. H., 1971. Ruthenium red and violet. II. Fine structural localization in animal tissues. *Anatomical Record* 171: 369-416.

Luft, J. H. 1976. The structure and properties of the cell surface coat. *International Review of Cytology* 45:291-382.

Maizels, R.M., M.L. Blaxter, B.O. Robertson and M.E. Selkirk. 1991. Parasite antigens, parasite genes - A laboratory manual for molecular parasitology. Cambridge: Cambridge University Press.

Maizels, R. M., Kennedy, M. W., Meghji, M., Robertson, B. O. and Smith, H. V. 1987. Shared carbohydrate epitopes on distinct surface and secreted antigens of the parasitic nematode *Toxocara canis*.

Journal of Immunology 139:207-214.

McClure, M. A., and L. J. Stowell. 1978. A simple method of processing nematodes for electron microscopy. *Journal of Nematology* 10:376-377.

McClure, M. A. 1988. First molt in *Anguina tritici*. *Journal of Nematology* 20:167-169.

McClure, M. A. and B. A. Stynes. 1988. Lectin binding sites on the amphidial exudates of *Meloidogyne*. *Journal of Nematology* 20:321-326.

Mirelman, D. 1986. Microbial Lectins and Agglutinins. John Wiley and Sons, New York.

Morrissey, J. H.. 1981. Silver stain for proteins in polyacrylamide gels: a modified procedure with enhanced uniform sensitivity. *Analytical Biochemistry* 117:307-310.

Muller, W. E. 1985. Cell-cell recognition in lower multicellular eukaryotes. Models supporting the modulation theory of adhesion. Pp. 156-175 in: F. E. Hahn, D. J. Kopecko, and W. E. G. Mullere eds. Progress in molecular and subcellular biology Vol. 9, Springer-Verlag Press, Berlin, Heidelberg, New York and Tokyo.

O'Shannessy, D. J., Voorstad, P. J. and Quarles, R. H. 1987. Quantitation of glycoproteins on electroblots using the biotin-streptavidin complex. *Analytical Biochemistry* 163:204-209.

Page, A. P., Rudin, W., Flur, E., Blaxter, M. L. and Maizels, R. M. 1992. *Toxocara canis*: A labile antigenic surface coat overlying the epicuticle of infective larvae. *Experimental Parasitology* 75:72-86.

Pasternak, J. J. 1988. Molecular biology of nematodes: some

recent studies on *Panagrellus* and *Ascaris*. *Canadian Journal of Zoology* 66:2591-2599.

Politz, S. M. and Philipp, M. 1992. *Caenorhabditis elegans* as a model for parasitic nematodes: a focus on the cuticle. *Parasitology Today* 8:6-12.

Preston-Meek, C. M. and Pritchard, D. I. 1991. Synthesis and replacement of nematode cuticle components. Pp. 84-94, in: M. W. Kennedy, Ed, Parasitic nematodes-antigens, membranes and genes, Taylor & Francis, London, New York, Philadelphia.

Pritchard, D. I., C. R. Crawford, I. R. Duce and J. M. Behnke. 1985. Antigen stripping from the nematode epicuticle using the cationic detergent cetyltrimethylammonium bromide (CTAB). *Parasite Immunology* 7:575-585.

Pritchard, D. I., P. G. McKean and M. T. Rogan. 1988. Cuticle preparations from *Necator americanus* and their immunogenicity in the infected host. *Molecular and Biochemical Parasitology* 28:275-284.

Proudfoot, L., J. R. Kusel, H.V. Smith and M.W. Kennedy. 1991. Biophysical properties of the nematode surface. Pp. 1-26. in M. W. Kennedy, ed. Parasitic nematodes - antigens, membranes and genes. London: Taylor and Francis.

Reymond, O. L., and J. D. Pickett-Heaps. 1983. A routine flat embedding method for electron microscopy of microorganisms allowing selection and precisely oriented sectioning of single cells by light microscopy. *Journal of Microscopy* 130:79-84.

Robinson, M. P., J. Delgado and R. M. E. Parkhouse. 1989. Characterization of stage-specific cuticular proteins of *Meloidogyne*

incognita by radio-iodination. *Physiological and Molecular Plant Pathology* 35:135-140.

Robertson, W. M., Y. Spiegel, H.-B. Jansson, N. Marban-Mendoza, and B.M. Zuckerman. 1989. Surface carbohydrates of plant parasitic nematodes. *Nematologica* 35:180-186.

Rudin, W. 1990. Comparison of the cuticular structure of parasitic nematodes recognized by immunocytochemical and lectin binding studies. *Acta Tropica* 47:255-268.

Sayre, R. M. 1980. Promising organisms for biocontrol of nematodes. *Plant Disease* 64:527-532.

Selkirk, M. E., Nielsen, L., Kelly, C., Partono, F., Sayers, G. and Maizels, R. M. 1989. Identification, synthesis and immunogenicity of cuticular collagens from the filarial nematodes *Brugia malayi* and *Brugia pahangi*. *Molecular and Biochemical Parasitology* 32:229-246.

Smith, H. V., Quinn, R., Kusel, J. R. and Girdwood, R. W. A. 1981. The effect of temperature and antimetabolites on antibody-binding to the outer surface of second-stage *Toxocara canis* larvae. *Molecular and Biochemical Parasitology* 4:183-193.

Spiegel, Y., E. Cohn, and S. Spiegel. 1982. Characterization of sialyl and galactosyl residues on the body wall of different plant parasitic nematodes. *Journal of Nematology* 14:33-39.

Spiegel, Y., and M. A. McClure. 1991. Stage specific differences in lectin binding to the surface of *Anguina tritici* and *Meloidogyne incognita*. *Journal of Nematology* 23:259-263.

Spiegel, Y., McClure, M. A., Kahane, I., Robertson, W. M. and Salomon, R. 1991. Wheat germ agglutinin associated with the outer

cuticle of the seed-gall nematodes, *Anguina agrostis* and *A. tritici*. *Journal of Nematology* 23:451-456.

Spiegel, Y., W. M. Robertson, S. Himmelhoch, and B. M. Zuckerman. 1983. Electron microscope characterization of carbohydrate residues on the body wall of *Xiphinema index*. *Journal of Nematology* 15:528-534.

Spiegel, Y., Sharon, E., Kahane, I., and M. A. McClure. 1991. Surface binding of red blood cells to different nematodes. *Journal of Nematology* 23:551.

Springer, T. A. 1990. The sensation and regulation of interactions with the extracellular environment: the cell biology of lymphocyte adhesion receptors. *Annual Review of Cell Biology* 6:359-402.

Stirling, G. R. 1985. Host specificity of *Pastueria penetrans* within the genus *Meloidogyne*. *Nematologica* 31:203-209.

Stirling, G. R., A. F. Bird, and A. B. Cakurs. 1986. Attachment of *Pasteuria penetrans* spores to the cuticle of root-knot nematodes. *Revue de Nematology* 9:251-260.

Towbin, H., T. Staehelin and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Science of the United States of America*. 76:4350-4354.

Wray, W., Boulikas, T., Wray, V. P. and Hancock, R. 1981. Silver staining of proteins on polyacrylamide gels. *Analytical Biochemistry* 118:197-203.

Wright, K. A. 1987. The nematode cuticle-its surface and the epidermis: Function, homology, analogy - a current consensus. *The*

Journal of Parasitology 73:1077-1083.

Zuckerman, B. M. 1983. Hypotheses and possibilities of intervention in nematode chemoresponses. *Journal of Nematology* 15:173-182.

Zuckerman, B. M. and Jansson, H.-B. 1984. Nematode chemotaxis and mechanisms of host/prey recognition. *Annual Review of Phytopathology* 22:95-113.

List of publications derived from this BARD grant

Lin, H.-J. and M. A. McClure. 1995. The surface coat of *Meloidogyne incognita*. *Journal of Nematology*, accepted for publication.

Sharon, E., L. Cohen, I. Kahane, J. Inbar and Y. Spiegel. 1994. Surface coat of the root-knot nematode *Meloidogyne javanica*: characterization and biological role. *Phytoparasitica* 22, 77.

Sharon, E., L. Cohen, I. Kahane and Y. Spiegel. 1995. Attachment of *Pasteuria penetrans* spores to second-stage juveniles of the root-knot nematode *Meloidogyne javanica*. *Phytoparasitica* 23, 269-70.

Sharon, E., I. Kahane, L. Cohen, and Y. Spiegel. *Meloidogyne javanica* surface coat: lability and characterization of proteins and carbohydrate-recognition proteins. *Experimental Parasitology*, submitted.

Sharon, E. and Y. Spiegel. 1996. Gold-conjugated reagents for the labelling of carbohydrate-recognition domains and glycoconjugates on nematode surfaces. *Journal of Nematology* 28, (1) in press.

Spiegel, Y., J. Inbar, I. Kahane and Edna Sharon. 1995. Carbohydrate-recognition domains on the surface of phytophagous nematodes. *Experimental Parasitology* 80, 220-227.

Spiegel, Y. and M. A. McClure. 1995. The surface coat of plant-parasitic nematodes: chemical composition, origin and biological role - a review. *Journal of Nematology* 27, 127-134.

Spiegel, Y., M. Mor and E. Sharon. Attachment of *Pasteuria penetrans* spores to the surface of *Meloidogyne javanica* second-stage juveniles. *Journal of Nematology*. submitted.

Zuckerman, B. M., J. Esnard, M. A. McClure, D. W. Dickson, and T. E. Hewlett. 1995. Studies on adhesion of the bacterium *Pasteuria penetrans* to the root-knot nematode, *Meloidogyne incognita*. *Journal of Nematology*, submitted.