Meloidogyne javanica surface proteins: characterization and lability

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SUMMARY

Characterization of surface coat (SC) proteins including carbohydrate-binding proteins and glycoproteins of the plant-parasitic nematode *Meloidogyne javanica* 2nd-stage juvenile (J2) is reported. Extraction of surface proteins with sodium dodecyl sulfate (SDS) and separation by denaturing polyacrylamide gel electrophoresis (SDS–PAGE) results with bands at 6, 9, 14, 22, 26, 31, 46, 49, 58, 66, 80, 205 and 250 kDa. On Western blots, the neoglycoprotein, fucosylated-, mannosylated- and glucosylated-bovine serum albumin, reacted with the 14, 22, 26, 58 and 66 kDa bands. The lectins, Concanavalin A and wheat-germ agglutinin (WGA) labelled surface protein bands of 6, 9, 14, 31, 58 and 66 kDa; WGA also labelled the 22 and 26 kDa bands. Biotin reagents were used to specifically trace surface proteins on live J2. SDS–PAGE of biotinylated J2 extracts revealed only 2 specific biotin-protein bands at 46 and 49 kDa. The labile and transitory nature of *Meloidogyne javanica* SC was demonstrated by the dynamics of human red blood cells (HRBC) adherence to J2 of different ages. HRBC adherence was also used to demonstrate the SC recovery of detergent-treated J2, which was further exhibited in the SDS-PAGE profiles.

Key words: carbohydrate-binding proteins, cuticle, glycoproteins, *Meloidogyne javanica*, nematode, surface coat.

INTRODUCTION

The presence and characterization of carbohydrate-binding proteins (CBPs) in animals has been investigated intensively over the last few years. The 'animal lectins' are classified into several groups, one of which is Ca^{2+}-dependent (C-type). Several mannose-binding proteins belong to this group, which can also bind fucose, glucose and *N*-acetylglucosamine (glucNAc) (Drickamer & Taylor, 1993).

Animal lectins have scarcely been reported on nematode surfaces. Human red blood cells (HRBC) have been reported to bind to the surface of several plant-parasitic nematodes, and preincubation of *Meloidogyne javanica* pre-parasitic 2nd-stage juvenile (J2) with a series of carbohydrates inhibited this binding, which was Ca^{2+}- and Mg^{2+}-dependent (Spiegel *et al*. 1995). HRBC also adhered to nylon fibres coated with SC extracted from *M. javanica* J2: binding decreased when the nylon fibres were pre-incubated with fucose or mannose (Spiegel *et al*. 1995). These experiments support a working hypothesis that HRBC adhesion involves surface carbohydrate residues of HRBC and CBPs on the nematode surface. A direct labelling of *M. javanica* J2 surfaces with neoglycoproteins further supported this hypothesis (Sharon & Spiegel, 1996).

The epicuticle of many nematodes is covered by a thin layer, characterized by a net negative charge and recognized by electron microscopy as a fuzzy coating. This layer has been termed 'surface coat' (Bird & Bird, 1991; Wright, 1987). Surface coat (SC) investigations have focused mainly on animal and free-living nematodes, and the labile nature of the SC has been explored (Proudfoot *et al*. 1991). In plant-parasitic species, the labile and transitory nature of the SC was demonstrated for *M. incognita* using radio-iodinated and biotinylated nematodes (Lin & McClure, 1996), and with *Anguina agrostis* where surface proteins were replaced after treatment with proteolytic enzymes (Bird & Zuckerman, 1989). 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). In plant-parasitic nematodes, studies of the interactions with their parasites (nematophagous fungi or *Pasteuria* spp., for example) or other microorganisms (e.g. *Clavibacter*) demonstrated the biological role of the nematode SC in those interactions (Bird, Bonig & Bacic, 1989; Davies & Danks, 1993; Spiegel & McClure, 1995). Recently it has been shown that the SC is released during the infection process; the SC was detected in infected host tissues

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with antibodies raised to *M. incognita* SC (Gravato Nobre *et al.* 1995; McClure & Gravato Nobre, personal communication).

In this paper we describe (1) the characterization of CBPs and other surface proteins of a plant-parasitic species, the root-knot nematode *M. javanica* and (2) the dynamic properties attributed to the plant-parasitic nematode SC.

MATERIALS AND METHODS

Nematodes

The root-knot nematode *M. javanica* was propagated on tomato (*Lycopersicon esculentum* Mill) cv. Hosen Eilon in the greenhouse, or aseptically in Petri dishes containing excised root cultures of tomato. Eggs were separated from egg masses with sodium hypochlorite (0·5 %, 1 min) and hatched in 2-fold-diluted phosphate buffered 0·1 M saline, pH 7·2 (PBS/2) to obtain infective J2 that had no direct contact with host tissues. Unless otherwise specified, 2- to 3-day-old J2 were used.

SC protein extraction

Proteins were extracted with sodium dodecyl sulfate (SDS) according to a method described previously (Spiegel, Mor & Sharon, 1996).

Gel electrophoresis and Western blots

Analytical SDS–polyacrylamide gel electrophoresis (SDS–PAGE) through 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). Pre-stained molecular weight standards (Sigma) were run on gels destined for electro-blotting.

For Western blots, proteins were transferred onto nitrocellulose sheets using a Gelman Sciences Bio-Trance midi-apparatus (Ann Arbor, MI, USA). General protein-staining on blots was performed with Protogold (BioCell, Cardiff, UK) according to the manufacturer's instructions.

Labelling of glycoproteins on blots

Labelling of glycoproteins was performed using gold-conjugated lectins. Blots were blocked overnight at 4 °C with PBS containing 1 % bovine serum albumin (BSA) and 0·05 % Tween–20 (blocking solution) and then washed 3 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 μg/ml) dissolved in the washing solution containing 1 mM Ca^{2+}, Mg^{2+} and Mn^{2+} ions, were incubated for 1 h at room temperature with the blots. Binding specificity was verified by pre-incubating the lectins for 30 min with 0·1 M of the appropriate hapten: α-methyl mannoside for Con A, and gluc*N*Ac or chitobiose for WGA. Blots were washed with the washing solution and then distilled water and labelling was visualized with a silver-enhancing kit for blots (BioCell).

Labelling of CBPs on blots

CBPs were labelled on Western blots, following the aforementioned blocking and washing steps. Gold-conjugated neoglycoproteins (E. Y. Laboratories), fucosylated-, mannosylated- and glucosylated-BSA, were diluted to a concentration of 1 μg/ml in washing solution containing Ca^{2+} ions and incubated with the blots for 1 h at 37 °C. BSA-gold was used as a negative control. Specificity was verified by (1) pre-incubating the blots under the same conditions, with the following competitive haptens (0·1 M): fucose, α-methyl mannoside, glucose and *N*-acetylgalactosamine (gal*N*Ac) or galactose, and (2) pre-incubating the neoglycoproteins with the appropriate lectins (20 μg/ml): *Ulex europaeus* agglutinin (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

Meloidogyne javanica J2 were incubated for 15 min with both of the following biotin reagents: (1) sulfo-*N*-hydroxysuccinimide (NHS)–biotin (Pierce) and (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 μg/ml. Each of these biotin treatments was followed by washing the nematodes 3 times with PBS/2 containing 0·1 % BSA.

The biotin label was localized by incubating nematodes for 2 h with gold-conjugated goat anti-biotin (1 nm particles; BioCell) diluted (1/100) in the washing solution. Non-labelled J2 which were incubated with the antibodies served as controls. Nematodes were then prepared for light microscopy, using the silver enhancement method, as described elsewhere (Sharon & Spiegel, 1996).

Biotin-labelled proteins were extracted by boiling the biotinylated and non-biotinylated nematodes in SDS–PAGE loading buffer for 15 min (Maizels *et al.* 1991), followed by vigorous centrifugation to pellet the nematodes and collection of the supernatant.

Biotin-labelled proteins were monitored on Western blots by the following methods. Blots were first blocked at 4 °C, overnight, with 5 % skimmed milk in PBS containing 0·05 % Tween–20, and washed (3 × 10 min) with PBS containing 0·1 % skimmed milk and 0·05 % Tween–20. Blots were then incubated either with 1 μg/ml streptavidin horseradish peroxidase (HRP) (Pierce) in the washing solution for 1 h or for 2 h with anti-biotin HRP (Sigma)

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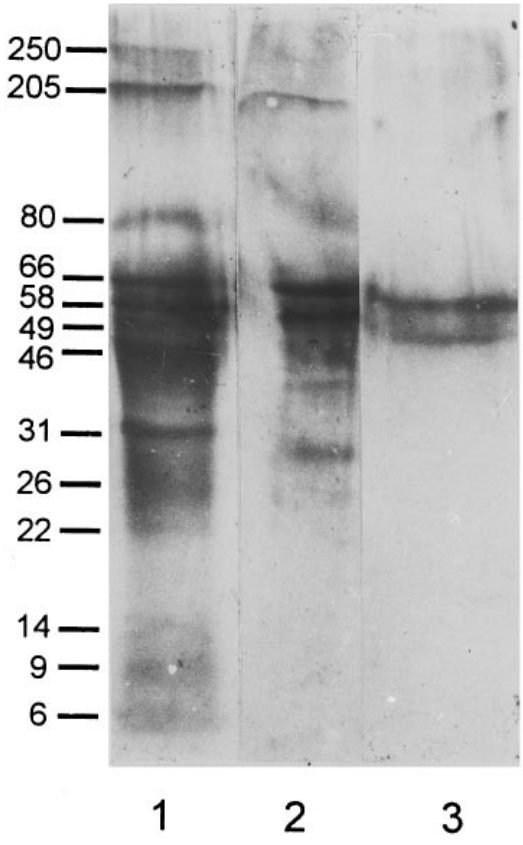


Fig. 1. Protogold-stained Western blots of SDS-extracted surface coat proteins of *Meloidogyne javanica* 2nd-stage juveniles, demonstrating the temperature dependence of surface coat recovery. Lane 1, SDS extraction; Lane 2, further SDS extraction after 2 days at 25 °C; Lane 3, further SDS extraction after 2 days at 4 °C. Molecular weight markers (kDa) are indicated.

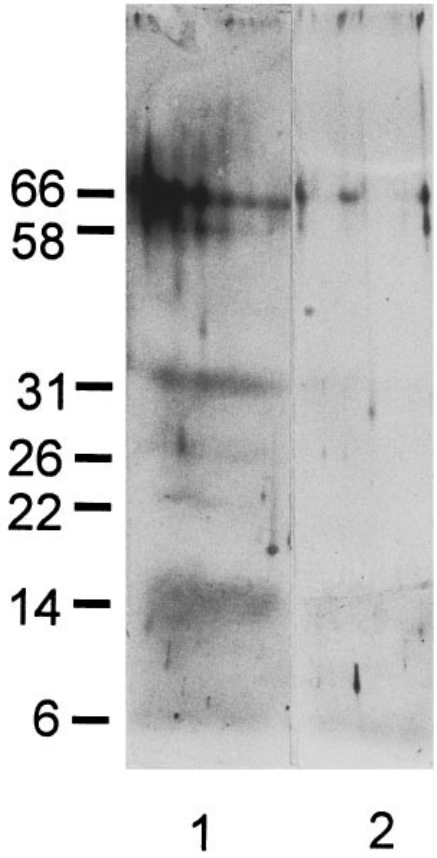


Fig. 2. Western blots of SDS-extracted surface coat proteins of *Meloidogyne javanica* 2nd-stage juveniles. The blots were labelled with: Lane 1, gold-conjugated wheat-germ agglutinin (WGA); Lane 2, pre-treatment with 0·1 M *N*-acetylglucosamine, and then with gold-conjugated WGA. Molecular weight markers (kDa) are indicated.

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. Labelling was visualized with 4-chloro-1-naphthol for the streptavidin-HRP. A more positive ECL kit (Amersham Life Science, Bucks., UK) was used as a substrate for the anti-biotin-HRP, to visualize the label on X-ray film.

SC lability

Dynamics of HRBC adherence to nematode SC. Preparation of HRBC and assay performance were according to Spiegel *et al*. (1995). 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 in PBS/2. The HRBC-binding assay was performed with each harvest on day 6; J2 used in these assays were pre-incubated for 1 h in PBS/2 containing Ca^{2+} ions.

SC recovery after detergent stripping. 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.

To demonstrate SC-protein recovery on gels, SDS-extracted J2 were washed thoroughly and divided into 2 groups for a 2-day incubation at 4 °C or 25 °C, and a further extraction with SDS. The extracts were then loaded onto SDS–PAGE gels.

RESULTS

Separation of SC extracts by SDS–PAGE

A typical SDS–PAGE pattern of the SC proteins extracted with SDS is presented in Fig. 1, lane 1. The extraction yielded bands at 6, 9, 14, 22, 26, 31, 46, 49, 58, 66, 80, 205 and 250 kDa.

Labelling of glycoproteins on blots

On Western blots, Con A (data not shown) and WGA (Fig. 2) labelled the 6, 14, 31, 58 and 66 kDa

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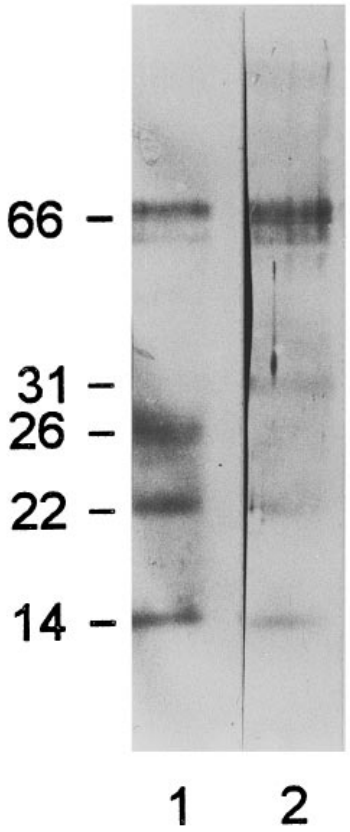


Fig. 3. Western blots of SDS-extracted surface coat proteins of *Meloidogyne javanica* 2nd-stage juveniles. The blots were labelled with gold-conjugated neoglycoproteins. Lane 1, glucosylated-bovine serum albumin; Lane 2, the neoglycoprotein was pre-incubated with concanavalin A before labelling the blot. Molecular weight markers (kDa) are indicated.

bands; WGA labelled also the 22 and 26 kDa bands (Fig. 2). Pre-treatment of the WGA blot with gluc*N*Ac resulted in almost complete inhibition of the labelling (Fig. 2), while with Con A, labelling was only partially inhibited by α-methyl mannoside (data not shown).

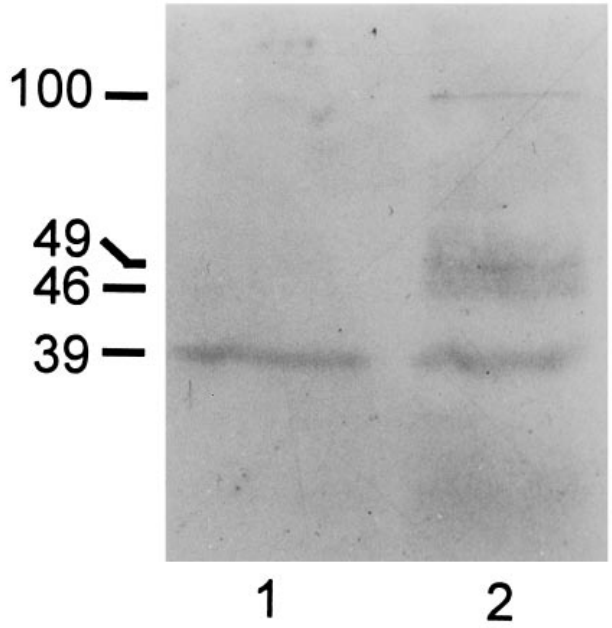


Fig. 5. Western blots of biotinylated and non-biotinylated *Meloidogyne javanica* 2nd-stage juvenile extract. Lane 1, biotinylation with sulfo-*N*-hydroxysuccinimide (NHS)-biotin; Lane 2, biotinylation with ϵ-aminocaproic acid NHS-biotin. Molecular weight markers (kDa) are indicated.

Labelling of CBPs on blots

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 (e.g. glucosylated-BSA in Fig. 3). Pre-incubation with the appropriate carbohydrates did not inhibit or reduce neoglycoprotein binding to the membrane; however, compatible lectins pre-incubated with the neoglycoproteins did reduce the labelling intensity of the 14, 22 and 26 kDa bands with fucosylated-, mannosylated- and glucosylated-BSA (Fig. 3). When Con A was

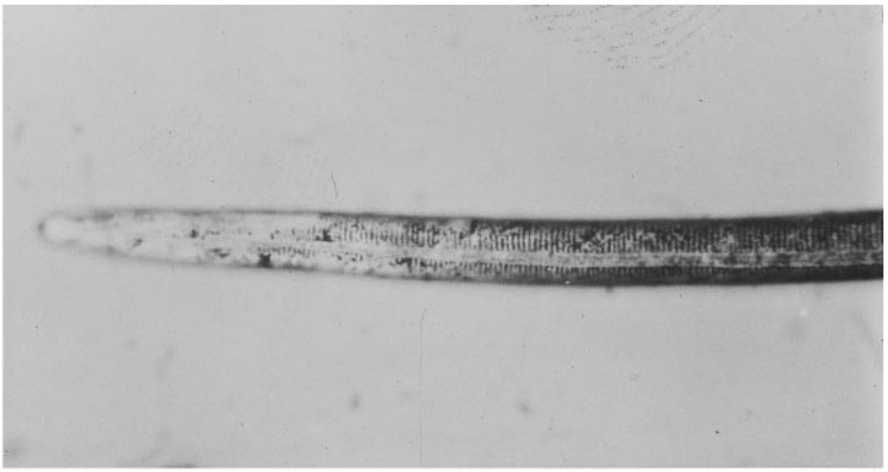


Fig. 4. Anterior part of a 2nd-stage juvenile of *Meloidogyne javanica* labelled with sulfo-*N*-hydroxysuccinimide-biotin, visualized with gold-conjugated anti-biotin combined with silver enhancement.

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Table 1. Binding intensity of human red blood cells (HRBC) to the outer cuticle of *Meloidogyne javanica* 2nd-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 and 10 individuals were monitored/well. Experiments were repeated at least 3 times with consistent results. Means followed by the same letter are not different, according to Tukey's HSD ($P = 0{\cdot}05$).)

Nematode age (h)	Binding intensity (No. of HRBC/nematode ± S.E.)
0–24	6·3 ± 1·6 d
24–48	42·7 ± 5·5 a
48–96	37·2 ± 5·9 ab
96–120	28·3 ± 4·8 bc
120–144	19·8 ± 3·5 c
144–168	1·4 ± 1·4 e

used for inhibition, a new 31 kDa band appeared, probably resulting from binding of Con A to the SC protein on the blot (see Fig. 2).

Biotin labelling

The transverse annulations over the surface of 2nd-stage juveniles were labelled when the nematodes were exposed to the biotin reagents (e.g. NHS-biotin in Fig. 4). 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 specific labelling at 46 and 49 kDa only with the ϵ-aminocaproic acid NHS-biotin (Fig. 5).

SC lability

Dynamics of HRBC adherence. HRBC did not adhere to freshly hatched 0 to 24-h-old. Maximum adherence of J2 kept at 25 °C was observed with 24 to 48-h-old J2 and this adherence then decreased gradually (Table 1).

SC recovery after detergent stripping. Less HRBC adhered to the surfaces of detergent-treated J2 than to those of non-treated J2 (Spiegel *et al*. 1995). 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 re-occurred.

The electrophoretic profile of the SDS-stripped SC of nematodes kept at 25 °C for 2 days was much as that of the original SDS extraction's protein pattern, whereas the profile of the SDS-stripped SC of nematodes kept at 4 °C for 2 days presented only 2 bands at 58 and 66 kDa (Fig. 1).

DISCUSSION

In this work we characterized surface proteins on pre-parasitic J2 of the root-knot nematode *M. javanica*. Politz & 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. Several methods have been used to characterize and map SC proteins (e.g. Proudfoot *et al*. 1991; Blaxter, 1993). After comparison with other detergents such as cetyltrimethylammonium bromide (CTAB), or *N*-octylglucoside (data not shown), we decided to choose the SDS extraction method for our analyses because it resulted in more protein fragments on an SDS–PAGE while having no effect on J2 viability.

Biotin derivatives have been used to detect surface-associated proteins (Spiegel & McClure, 1995). 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 Western blots. However, substituting an anti-biotin conjugate for the streptavidin reagent enabled the detection of specific labelling at 46 and 49 kDa. These bands appeared also in the SDS SC extraction profile.

Previously it has been shown that surfaces of intact J2 reacted with lectins and neoglycoproteins (Sharon & Spiegel, 1996). These reagents also react on Western blots with the SDS-extracted SC proteins , indicating that these labelled proteins are part of the nematode's surface complex.

On Western blot, the 6, 9, 14, 22, 26, 31, 58 and 66 kDa bands could be related to glycoproteins because these bands reacted with lectins. 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.

Evidence supporting the presence of CBPs on *M. javanica*'s surface has already been presented by Spiegel *et al*. (1995), using HRBC; further support was later provided whereby J2 were exposed to neoglycoproteins and revealed a binding pattern similar to that recorded for HRBC (Sharon & Spiegel, 1996). In the current report, these neoglycoproteins also reacted with the SC protein extract on a Western blot.

SC lability has been well documented in several parasitic and free-living nematodes and it is generally accepted that one of the biological functions of this

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feature is to avoid detection by the host's immune system (Blaxter *et al*. 1992; Page *et al*. 1992; Politz & Philipp, 1992).

This report explores a few aspects of SC lability in J2 of a plant-parasitic species, *M. javanica*. The dynamics of HRBC adherence to J2 surfaces indicates changes occurring in the SC during the juvenile life. HRBC barely adhered to freshly hatched 0 to 24-h-old J2, indicating that the CBPs are probably absent at this early life-stage being produced only later on. Maizels *et al*. (1987) reported that antibodies did not bind to freshly hatched *Toxocara canis* larvae. Change in the SC during the life of *M. incognita* race 3 J2 was demonstrated by Lin & McClure (1996); J2 labelled with radio-iodine and biotin released labelled molecules into the water after 20 h incubation, indicating that the surface proteins may be loosely attached to the nematode. Consequently, nematode age and SC lability should always be considered when SC labelling or extraction are performed.

The ability of the SC to regenerate after external removal was previously demonstrated for *Anguina agrostis* by Bird & Zuckerman (1989). These authors have shown that the SC can regain proteins lost by exposure to proteolytic enzymes when the phytonematodes are removed from these enzymes and given time to recover. This renewing ability, which reflects another aspect of the SC's transitory nature, was also demonstrated by us. Detergent-treated *M. javanica* J2 were capable of re-adhering to 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 reproduce 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. SC recovery after detergent stripping has been demonstrated with antibodies for *Nippostrongylus brasiliensis* by Preston-Meek & Pritchard (1991).

The lability of the SC 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.

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This manuscript is dedicated to the memory of the late Professor Itzhak Kahane, our dear colleague and friend.

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