The surface coat of infective larvae of *Trichinella spiralis*

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**SUMMARY**

The surface coat of the infective larvae of the parasitic nematode *Trichinella spiralis* was characterized with respect to its biophysical properties, morphology and composition. Labelling of larvae with the fluorescent surface probe PKH26 was lost after activation (by incubation in mammalian medium containing trypsin and bile), or following pronase treatment. Electron microscopical examination revealed that pronase treatment resulted in the loss of an amorphous surface layer only, further demonstrating the specificity of PKH26 for the larval surface coat. Surface coat shedding was inhibited by sodium azide and carbonyl cyanide, or by incubation of larvae at 4°C, suggesting the shedding process required metabolic energy. Pre-labelled, unactivated larvae demonstrated continuous slow surface coat shedding and could be re-labelled with PKH26, indicating that the shed coat is replaced in these parasites. However, pre-labelled larvae which were activated failed to re-label with the probe, suggesting that activation provides an irreversible trigger for surface changes. PKH26, therefore, is a useful marker for larval activation. Examination of the shed coat material by scanning electron microscopy revealed 2 types of morphologies; one comprising thin multilaminate sheets and the other of amorphous material with ridges producing a fingerprint-like motif. Western- and lectin-blotting of the shed coat material demonstrated 2 prominent entities; a 90 kDa glycoprotein, which bound *Datura stramonium* agglutinin and was resistant to N- and O-glycanase treatment and a 47–60 kDa set of protein(s). Analysis of the surface lipids by electrospray mass spectrometry revealed the presence of lysophosphatidic acid (lysoPA, C14:2) and an unidentifiable component of 339 Da. These two lipids constituted 36.9% and 36% by mass of surface coat lipids respectively. The presence of lysoPA was confirmed by thin layer chromatography, which also detected phosphatidic acid (PA). The polar lipids detected in solvent rinses of intact parasites by electrospray mass spectrometry were PI (C48:4), PE (C40:4 and C38:4), PS (C40:4), lysoPC (C20:2 and C18:2) and lysoPA (C14:2). These observations are discussed with respect to the role of the surface coat and its shedding in the *T. spiralis* host–parasite relationship.

Key words: *Trichinella spiralis*, surface coat, *Datura stramonium* agglutinin, lysophosphatidic acid, electrospray mass spectrometry, PKH26, 5N-octadecanoylaminofluorescein.

**INTRODUCTION**

The nematode cuticle is overlain by an amorphous surface coat (Nerbonne *et al.*, 1984; Wright, 1987; Maizels, Blaxter & Selkirk, 1993; Probert, Zhang & Bundle, 1996). This structure, although often loosely attached to the surface, has been identified in all types of nematodes, including free-living and plant- and animal-parasitic forms, indicating its importance in this group of organisms (Blaxter *et al.*, 1992). It can be visualized by a variety of fluorescent conjugated proteins, antibodies, stains, dyes and probes, such as cationized ferritin, ruthenium red and PKH26 (Blaxter *et al.*, 1992; Maizels *et al.*, 1993; Modha, Kennedy & Kusel, 1995).

An interesting feature of the nematode surface coat is that it is shed from the parasite surface upon exposure of the parasite to host signals (Denham & Martinez, 1970; Grove *et al.*, 1987; Stewart *et al.*, 1987; Maizels *et al.*, 1993; Modha *et al.*, 1995). The surface coat of the infective larvae of *Trichinella spiralis* seems to be shed continuously (Modha *et al.*, 1995), but appears to be substantially lost upon larval activation and host infection (Despommier, 1983; Stewart *et al.*, 1987; Modha *et al.*, 1994a). The loss of the surface coat in *T. spiralis* larvae coincides with a change in parasite motility pattern, from coiling–uncoiling to sinusoidal (Modha *et al.*, 1994a; Stewart *et al.*, 1987), increased glucose uptake (Stewart *et al.*, 1987) and with increased insertion of the fluorescent lipid probe AF18 (Kennedy *et al.*...
The surface changes, as detected using the above fluorescent probes, constitute the earliest, and most easily detected, indication of activation.

We have previously reported identification of a second messenger pathway, involving the sequential release of inositol triphosphate, calcium ions and cyclic AMP, thought to be pertinent to the alteration of surface lipophilicity that follows surface coat shedding in activated T. spiralis larvae (Modha et al. 1995). This communication concerns characterization of the surface coat, describes the nature and composition of the coat material, and suggests novel roles for surface coat shedding in the host–parasite relationship.

**Materials and methods**

**Chemicals and reagents**

All chemicals, reagents, PKH26 labelling kit and TLC plates were purchased from Sigma (Poole, Dorset) unless otherwise stated; 5N-octadecanoylaminofluorescein (AF18) was purchased from Molecular Probes Inc. (Eugene, Oregon, USA). A stock of 2 mg/ml was prepared in ethanol and diluted in PBS for use at a final concentration of 10 μg/ml. Butyl hydrophobic interaction (HIC) beads were purchased from Bio-Rad (Hemel Hempstead, Hertfordshire). *Datura stramonium* agglutinin (DSA) was purchased from Boehringer Mannheim (Germany). ‘Color’ markers were purchased from Sigma and ‘Rainbow’ markers from Amersham.

**Preparation, activation and labelling of larvae**

Infective muscle-stage larvae of *T. spiralis* were isolated from BALB/c mice by homogenization and digestion in 0.5% HCl and 5 mg/ml pepsin as described previously (Kelly et al. 1991). Larvae were activated by incubation in RPMI 1640 at pH 7.5 containing 0.25% trypsin and 5% bile for 30–45 min and 37 °C (Modha et al. 1995). They were labelled with the fluorescent lipid probe PKH26 as described in the manufacturer’s instructions (Horan et al. 1990), with modifications outlined previously (Modha et al. 1995). Larvae were labelled with 10 μg/ml AF18 (in PBS) for 10 min at 37 °C and washed 3 times before examination. Trypan blue (0.25%) was used as a quenching agent to check on surface proximity of fluorescent labelling. Quantification of fluorescence (peak excitation and emission wavelengths as follows; PKH26 λex = 551 nm λem = 567 nm, AF18, λex = 495 nm λem = 521 nm) was performed as described previously (Modha et al. 1995).

**Preparation of surface coat material**

Surface coat material was collected from 20000–50000 freshly-isolated *T. spiralis* larvae by incubation in distilled water (the volume varied from 200 to 500 μl, see figure legends for details) at ambient temperature (18–20 °C) for 8 h. Larvae were sedimented by gentle centrifugation and the parasite-free supernatant was aspirated and kept. Its protein content (approximately 0.6 mg/ml from 20000 larvae shedding in 200 μl of distilled water) was determined using the dye-based protein estimation assay (Bradford, 1976) from Bio-Rad, but often material was concentrated by lyophilization and resuspension in a smaller volume.

**Preparation of rabbit antiserum**

To raise a rabbit antiserum 150 μg surface coat material (0.25 ml at 0.6 mg/ml) was diluted with an equal volume of PBS, made up to 1 ml with Freund’s Complete Adjuvant and emulsified by vigorous shaking. An experimental New Zealand White rabbit (Harlan, Oxon) was immunized with 0.1 ml of emulsion at 10 different sites subcutaneously with this preparation. One month later a booster immunization was administered with a similar preparation, using Freund’s Incomplete Adjuvant. The rabbit was exsanguinated 1 month after the booster, the antiserum being isolated by centrifugation and stored at −20 °C.

**Immunoblotting and lectin blotting**

Surface coat samples for immunoblotting and lectin blotting were run on 10% mini-gels (Bio-Rad) by SDS–PAGE (Kennedy et al. 1987d) and transferred onto nitrocellulose (Towbin, Staehelin & Gordon, 1979) using ‘Immune-Lite’ blotting membrane (Bio-Rad) for immunoblotting and ‘Trans-blot transfer medium’ (Bio-Rad) for lectin blotting, as described previously (Modha et al. 1994b). For immunoblotting, after electrophoretic transfer of proteins, the membrane was blocked with 5% Marvel in blotting buffer (PBS containing 0.03% Tween 20), probed with 1/400 dilution (in blotting buffer) of rabbit anti-surface coat antiserum and developed as described previously (Modha et al. 1994b). For lectin studies, the lectin differentiation kit (Boehringer Mannheim, Germany) was used according to the manufacturer’s instructions. After electrophoretic transfer of (glyco)proteins, the blotted membrane was blocked overnight at 4 °C, washed extensively (4 × 10 min) and probed with 500 ng/ml DSA in buffer 1 (see kit for details).

For competition blots, approximately 3 μg surface coat material was spotted directly onto the membrane, which was blocked for 30 min, washed extensively with buffer, probed with 500 ng/ml DSA and developed in the usual manner (according to kit instructions). In sugar competition studies, DSA was pre-incubated (37 °C, 30 min) with 4 mM N-acetyllactosamine.
Fig. 1. Effect of activation and pronase treatment on PKH26 labelling of *Trichinella spiralis* larvae. (A) Unactivated (A) and activated (B) *T. spiralis* larvae were labelled with PKH26 and surface fluorescence quantified. (B) Unactivated *T. spiralis* larvae were incubated with PBS (A), or 1 mg/ml pronase (B), for 10 min at 18–20 °C. Larvae were then washed extensively, labelled with PKH26 and surface fluorescence quantified.

**Indirect immunofluorescence and immuno-gold electron microscopy (EM)**

Indirect immunofluorescence was performed as described previously (Modha et al. 1994b), using the rabbit anti-surface coat serum at 1/50 dilution. Immuno-EM was performed on intact larvae or 50 nm thick sections embedded in ‘Emix’ resin (Robertson, Topham & Smith, 1987). The rabbit anti-surface coat serum was used as the primary antibody and a sheep anti-rabbit IgG conjugated to 10 nm gold particles (Sigma) as a second antibody for visualization.

**Folch extraction**

Solvent extractions for analysis of total parasite surface lipid by electrospray mass spectrometry and thin layer chromatography were performed by the Folch method (Folch, Lees & Sloane Stanley, 1957). Briefly, freshly-isolated larvae (number of parasites varied according to experiment, see figure legends for details) were suspended in 200 µl of saline in a clean glass tube, to which 50 µl of chloroform: methanol (2:1) mixture were added. Larvae were gently shaken for 10 sec and centrifuged briefly (5 sec). The sample separated into 2 phases, with the larvae at the interface. Following extraction the solvent was evaporated under nitrogen gas and the extracted lipids resuspended in the required volume of solvent for analysis.

**Electrospray mass spectrometry (ES–MS)**

Approximately 100 µg of freeze-dried surface coat material was resuspended in 50 µl of 20% methanol. A sample containing 10 µl was injected for analysis by ES–MS (Han & Gross, 1994) with modifications as previously described (Sweetman et al. 1996). Briefly, ES–MS was performed on a VG Quatro BQ Instrument (VG Organic, Manchester) using negative ion mode. Samples were infused into the source at a flow rate of 10 µl/min using a syringe pump (Harvard Apparatus Ltd, Edenbridge). MS spectra were acquired as previously described (Sweetman et al. 1996).

**RESULTS**

**Labelling the larval surface**

*T. spiralis* infective larvae elaborate a surface coat, which has previously been shown to label with the fluorescent lipid probe PKH26 (Modha et al. 1995). In this study fluorescence quantification shows substantial loss of PKH26 labelling following activation of larvae by incubation in medium containing trypsin and bile. PKH26 labelling could also be reduced by treating larvae with pronase (Fig. 1). Electron microscopical examination of pronase-treated parasites indicated that the larval surface coat was removed (Fig. 2). Other structures in the cuticle, or its general arrangement, did not seem to be affected by pronase treatment, but treated larvae were blackened by osmium tetroxide during the counter-staining process, suggestive of increased permeability of larvae. Pronase removal of the surface coat, and the subsequent loss of PKH26 labelling, did not increase insertion of AF18 into the underlying epicuticle (data not shown) as is the case following trypsin treatment (Proudfoot et al. 1993; Modha et al. 1995). This suggests that whilst tryptic digestion of the surface coat occurs when the parasite becomes activated (Despommier, 1983; Stewart et al. 1987), surface changes other than mere exposure of the epicuticle must contribute to the concurrent increase in surface lipophilicity detected using AF18.
Fig. 2. Effect of pronase treatment on larval ultrastructure. Infective larvae were incubated in buffer (A), or with 1 mg/ml pronase (B), for 10 min at 18–20 °C, washed extensively in cold PBS and fixed in 2% paraformaldehyde/3% glutaraldehyde for 2 h for examination by electron microscopy. Arrow highlights removal of surface layers.

(Kennedy et al. 1987a; Modha et al. 1995; Proudfoot et al. 1993).

Surface coat shedding

The shedding of the surface coat of *T. spiralis* larvae is a phenomenon that has been previously described by other workers (Smith et al. 1981; Bird & Bird, 1991), and PKH26 is a more convenient label than immunofluorescence (Modha et al. 1995). This has permitted studies on the dynamics of the shedding process, which demonstrated that surface coat shed-
Fig. 3. Labelling and relabelling *Trichinella spiralis* larvae with PKH26. Unactivated larvae were labelled with PKH26 (A) and allowed to shed the surface coat at room temperature (B). They were then either relabelled with PKH26 (C), or activated with trypsin and bile (D) and then re-labelled with the probe (E). This is summarized as follows: (A) PKH26 labelled larvae; (B) group A after surface coat shedding at ambient temperature; (C) group B relabelled with PKH26; (D) group A after activation with trypsin and bile; (E) group D relabelled with PKH26.

Fig. 4. Loss of surface PKH26 in larvae recovered from the small intestine. PKH26 labelled larvae (A) were orally administered to mice and the intraepithelial larvae recovered from the isolated small intestine 30 min after oral infection. For larval recovery, the mouse small intestine was isolated and flushed with PBS, to remove unwanted parasites from the lumen. The gut was then slit open longitudinally, rinsed in buffer and incubated in fresh buffer. Larvae emerging from the intestinal epithelium were collected and examined by fluorescent microscopy for the presence of the label (B). Control larvae (i.e. group A) were kept at 8–10 °C after PKH26 labelling to minimize surface coat shedding for comparative purposes.

Nature and morphology of the shed material

Scanning electron microscopical examination of material shed from the larval surface and negatively stained on EM grids revealed thin sheet-like material with ridges of regular periodicity which formed fingerprint-like patterns (arrowed in Fig. 5A). Other flecks of shed material appeared like stacked sheets, each stack comprising up to 8 layers (arrowed in Fig. 5B). It was not possible to determine the thicknesses of the sheets, but they seemed to be of varying electron densities. It was also difficult to examine copious amounts of shed material by electron microscopy because it tended to dissolve into the buffer after release from its insoluble sheet-like state on the larval surface. This problem was partially that the shedding process requires metabolic energy. Carbachol (20 mg/ml), however, did not affect the dynamics of the shedding process (not shown).

Experiments exploiting the ability of PKH26 to label the surface coat were carried out to determine whether it is replaced after shedding. Thus, larvae (unactivated) were labelled with PKH26 (Fig. 3A), then allowed or induced to shed, either by leaving them in buffer at ambient temperature (18–22 °C), or by activating them in medium containing 0.25% trypsin and 5% bile at 37 °C, respectively. Larvae were then re-labelled with the probe and their surface PKH26 fluorescence quantified. These experiments revealed that although pre-labelled unactivated larvae shed the surface coat, and consequently decreased in PKH26 fluorescence (Fig. 3B), they could be re-labelled, even to original levels (Fig. 3C), suggesting that shed material was replaced in these organisms. When pre-labelled larvae were activated, however, they lost the label almost entirely (Fig. 3D) and could not be re-labelled (Fig. 3E).

This loss of surface bound PKH26 was also observed *in vivo*, when mice were orally infected with PKH26-labelled larvae. Parasites recovered from the intestine 30 min later were no longer fluorescently labelled, and could not be re-labelled with the probe (Fig. 4). In contrast, PKH26-labelled butyl hydrophobic interaction chromatography beads remained fluorescent 60 min after oral dosing (data not shown). Whilst by no means conclusive, this implies that PKH26 is not digested from the larval surface during passage through the host digestive tract.

Interestingly, larvae treated with photoactivated caged cyclic AMP, which increases the rate of surface coat shedding, surface lipophilicity (Modha et al. 1995) and surface fluidity (Modha et al. 1997), could not be re-labelled well with PKH26 (data not shown), indicating that exposure to this second messenger simulates the effects of exposure to natural host stimuli.
Fig. 5. Electron micrographs of material shed from the larval surface. Freshly isolated larvae were placed on electron microscope grids and permitted to shed the surface coat. Surface coat shed material and larvae on the grids were then fixed in 2% paraformaldehyde/3% glutaraldehyde and negatively stained with 2% phosphotungstic acid for 10 min for clearer observation. (A) Electron micrograph of a fragment shed from the larval surface. (B) Electron micrograph of a sheet of surface coat.

alleviated by placing shedding larvae directly on EM grids and fixing quickly.

Immunofluorescence analysis of intact larvae using a rabbit antiserum against the surface coat confirmed surface labelling (Fig. 6A), but within 2–5 min, surface-bound antibodies began to shed in aggregated sheets with the imprint of the cuticle (Fig. 6B). The manner of this shedding, and its
Fig. 6. Immunofluorescence of *Trichinella spiralis* larvae probed with anti-surface coat serum. Larvae were probed with a rabbit anti-surface coat serum and then incubated with a FITC-conjugated anti-rabbit antibody. Labelled larvae were left on the slide for a few min to observe surface coat shedding and the shed material. (A) Labelled larvae (magnification: ×500). (B) Material shed from the surface of a labelled larva. The intensity of the labelled larva (top right of picture) appears much brighter than larvae shown in (A) because the exposure time for the picture was increased to enable labelled shed material to be photographed clearly.

time-scale, was similar to the shedding of PKH26-labelled material from the larval surface (Modha *et al.* 1995), suggesting that shedding of the immune conjugate occurs as a result of surface coat shedding and not merely shedding of surface-bound fluorescent immune conjugates. Immunoelectron microscopy using rabbit anti-surface coat antiserum followed by gold-conjugated anti-rabbit antibody demonstrated gold particles on the larval surface and on material lying distal to the surface, which
presumably was material released from the parasite surface (Fig. 6A). The same methodology on cryostat sections of larvae demonstrated binding of gold particles not only to the larval surface, but also throughout the cuticle and hypodermis (Fig. 6B).

**Composition of the shed material**

Western blotting analysis of surface coat material revealed major bands at 90 kDa and 47–60 kDa positions (Fig. 7, lane 1). A molecule of approximately 90 kDa was also recognized by *Datura stramonium* agglutinin (DSA, Fig. 7, lane 2) and lectin binding was reduced by 90% when it was preincubated with 4 mM N-acetyllactosamine. However, there was no evidence of DSA binding to the surface of intact living larvae when parasites preincubated with digoxigenin (DIG)-conjugated DSA were probed with FITC-conjugated anti-DIG antibodies (data not shown). The target for DSA is therefore either not on the surface, or represents a hidden determinant when in an intact surface, as observed for certain monoclonal antibody epitopes in *Toxocara* spp. (Kennedy *et al.* 1987; Maizels *et al.* 1993). Treatment of surface coat material with N- and O-glycosidases (Maizels *et al.* 1993) did not detectably affect the 90 kDa antigen in Western blotting whether lectin- or antibody probes were used (data not shown). The smear comprising antigens at 47–60 kDa did sometimes resolve into 3 or 4 distinct bands, but none of these were recognized by a panel of lectins which included concanavalin A, peanut agglutinin, wheatgerm agglutinin, *Galanthus nivalis* agglutinin or DSA.

In order to identify lipid components in the larval surface electrospray mass spectrometry (ES–MS) was performed on solvent rinses of intact larvae. These revealed phospholipids ranging in size from 200 to 900 Da, and an acetylated hexosamine (Fig. 8A). The dominant phospholipids in the sample were phosphatidyl inositol (PI, C48:2) and lysophosphatidyl choline (lysoPC, C20:2 and C18:2), phosphatidyl ethanolamine (PE, C40:4 and C38:4) and phosphatidyl serine (PS, C40:4) with a small amount of lysophosphatidic acid (lysoPA, C14:2) (see Table 1). The presence of these lipids in intact larvae was confirmed by thin layer chromatography (Fig. 9, lane 3).

ES–MS analysis of surface coat material revealed the presence of relatively low molecular mass (<400 Da) components only, with the major lipid components eluting within a narrow size range, from 340 to 380 Da (Fig. 8B). There were 2 predominant lipid peaks; one (36%) which belonged to an unidentified molecule of 339 Da, and the other (36-9%) was lysophosphatidic acid (lysoPA, C14:2). The 339 Da component was exclusive to the shed material and was not detectable in Folch extractions of intact larvae. The detection of lysoPA as one of the major components of the larval surface coat was confirmed by thin layer chromatography (TLC), which also indicated the presence of phosphatidic acid (PA) in the surface coat (Fig. 9).

A component of mass 215 Da, which was less abundant in intact larvae (Fig. 8A), was detectable in the surface coat as well (Fig. 8B). The mass of this material approximates that of an acetylated hexosamine and it is presumed to be N-acetyl glucosamine. This would concur with the lectin binding data above (Fig. 7) and with previous reports (Wisnewski *et al.* 1993), but further analysis is necessary for confirmation.

**DISCUSSION**

**Labelling of the surface coat**

We now possess 2 fluorescent lipid probes with reciprocal labelling pattern that are able to mark the surface of *T. spiralis* larvae; PKH26 which labels the surface coat and AF18 which identifies the epicuticle. Using antibody to mark the surface coat, it has been demonstrated for other nematodes that AF18 labels the epicuticle and not the surface coat (Kennedy & Proudfoot, 1993). Unactivated *T. spiralis* larvae demonstrate high PKH26 fluorescence and low AF18 insertion (Modha, Kennedy & Kusel, 1995), whereas activated larvae demonstrate increased AF18 insertion but decreased PKH26 binding. Consequently, these 2 probes can be used to monitor activation of *T. spiralis* larvae during the infection event.

In addition to being useful as a marker for the surface coat, PKH26 has also been useful in enabling the phenomenon of surface coat shedding to be
T. spiralis surface coat

Fig. 8. Electrospray mass spectrograms of larval lipids. (A) 10 µl of the solvent phase from a Folch extraction of intact larvae. (B) 10 µl of 2 µg/µl (in 20% methanol) surface coat material. Solvent extraction procedures and analysis of lipids by electrospray mass spectrometry were as described previously by Sweetman et al. (1996) with the modifications outlined in the Materials and Methods section. The percentage abundance of lipids described in Table 1 was determined by calculating the surface area under each corresponding peak from the profiles shown in this figure. Profile B is a composite of 2 traces derived from the same run.

visualized quickly i.e. within a few minutes, thus eliminating the need for fluorescent immune conjugates to visualize this process (Smith et al. 1981; Bird & Bird, 1991). Therefore, studies on the kinetics of shedding and the amount of material shed (quantitative fluorescence) can now be performed using this probe. Here we have shown that the shedding process demonstrated by T. spiralis larvae is dynamic and requires metabolic energy, as has also been observed in nematode larvae using fluorescent immune conjugates (Smith et al. 1981; Bird & Bird, 1991; Maizels et al. 1993).

The PKH26-labelled surface coat has previously been reported to be shed in aggregated sheets, with the imprint of the cuticle (annulce) remaining on them (Modha et al. 1995). However, the aggregated sheet-like organization disperses during observation under the microscope, suggesting although the coat material is maintained in an insoluble form on the parasite surface, but shedding causes it to become soluble. Our previous findings indicate that intracellular calcium ions may be important in the maintenance of the aggregated sheet-like structure on the parasite surface, since their removal results in an altered morphology of the shed material (Modha et al. 1995).

Structure of the surface coat

The surface of T. spiralis larvae has also been studied by transmission EM (TEM) (Despommier, 1983; Stewart et al. 1987; Wright & Hong, 1988; Modha...
Table 1. Lipids identified in larvae and surface coat material by electrospray mass spectrometry

<table>
<thead>
<tr>
<th>Size (Da)</th>
<th>Abundance* (%</th>
<th>Lipid type</th>
<th>Chain length: double bonds†</th>
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<tbody>
<tr>
<td>Whole larvae</td>
<td></td>
<td></td>
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<tr>
<td>2151</td>
<td>1.3</td>
<td>—</td>
<td>N-acetyl hexosamine</td>
</tr>
<tr>
<td>3771</td>
<td>2.6</td>
<td>Lyso PA</td>
<td>C14:2</td>
</tr>
<tr>
<td>5196</td>
<td>10.8</td>
<td>Lyso PC</td>
<td>C18:2</td>
</tr>
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<td>5475</td>
<td>12.4</td>
<td>Lyso PC</td>
<td>C20:2</td>
</tr>
<tr>
<td>7669</td>
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<td>12.8</td>
<td>PE</td>
<td>C40:4</td>
</tr>
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<td>8.4</td>
<td>PS</td>
<td>C40:4</td>
</tr>
<tr>
<td>8856</td>
<td>16.0</td>
<td>PI</td>
<td>C48:4</td>
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<tr>
<td>Surface coat</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>2151</td>
<td>9.3</td>
<td>—</td>
<td>N-acetyl hexosamine</td>
</tr>
<tr>
<td>3394</td>
<td>36.0</td>
<td>?</td>
<td></td>
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<tr>
<td>3773</td>
<td>36.9</td>
<td>Lyso PA</td>
<td>C14:2</td>
</tr>
</tbody>
</table>

* The abundance of each lipid component was determined by calculating the surface area under the corresponding peak in the spectrograms shown in Fig. 8.
† The acyl chain length and the number of double bonds for each lipid species was determined by consulting reference tables which can pinpoint the identity of each common lipid species from the m/z value obtained from ES–MS spectra. These tables were produced after running numerous lipid standards as part of a major program involving ES–MS standardization (G. Sweetman).

et al. 1994b) and freeze fracture (Lee, Wright & Shivers, 1984, 1986; Wright & Hong, 1988, 1989; Gounaris, Smith & Selkirk, 1996). It is suggested to comprise an outermost layer of globular proteins enveloped in a matrix and an inner layer of filaments consisting of phospholipid tubular micelles (Lee et al. 1984; Wright & Hong, 1988). TEM studies identify 4 layers on the larval surface (Lee et al. 1984; Despommier, 1983). Layer 4 is the outermost layer and seems to be removed, with layer 3, by trypsin and/or bile treatment (Despommier, 1983; Stewart et al. 1987). We interpret layer 2 (the most electron-dense layer) as the epicuticle, into which our lipid probe AF18 inserts (Proudfoot et al. 1993; Modha et al. 1995), and layer 1 as the upper extremity of the cuticle. Based on this understanding and nomenclature, our investigations indicate the surface coat is equivalent to layer 4, and possibly, some part of layer 3. Therefore, it is likely to contain the 47–60 kDa protein and 90 kDa glycoprotein in a globular matrix (Wright & Hong, 1988). Layer 3 included as possibly being a component of the surface coat because the shed material has the same fingerprint-like morphology as layer 3 described by freeze-fracture (see Figures 6 and 9 in Lee et al. 1984 and Figures 3 and 9 in Wright & Hong, 1988). This pattern, reminiscent of rows of phospholipid tubular micelles is similar to that seen in the shed material by scanning EM. Interestingly, when flecks released from the parasite surface were analysed closely by scanning EM, they appeared to comprise up to 8 distinct sheets (arrowed in Fig. 5B), indicating that layers 3 and 4 may constitute an octalaminate structure. This perhaps extends earlier views of the accessory layer being trilaminate (Wright & Hong, 1988; Lee et al. 1984), and highlights the structural complexity of this region of the larval surface.

Shedding of the surface coat before, and after, activation

It is noteworthy, however, that shedding of the surface coat by unactivated larvae is distinct from its loss or removal during activation. In unactivated larvae, the loss of surface-bound PKH26 (via surface coat shedding) can be replaced by re-labelling larvae with the probe. Thus, coat material which is lost through shedding is replaced onto the surface. However, our investigations demonstrate that pre-labelled larvae which have undergone activation cannot be re-labelled with PKH26. Hence, it seems that additional surface layers are lost or altered following larval activation such that the layer which is identified by PKH26 is no longer available to the probe after activation. This suggests that activation provides important signals or triggers which have irreversible consequences in the development of the parasite.

Role of shedding of surface coat in infection biology

Whether the surface coat is removed or merely reduced following activation does, however, require consideration. Some reports suggest that the surface coat is removed (Stewart et al. 1987), whilst others detect the surface coat (termed accessory layer) on intestinal- and intraepithelial larvae and is only removed at the first moult (Wright & Hong, 1989; Ellis et al. 1997). They also argue that the loss of the
surface coat prior to the moult observed by others (Stewart et al. 1987; Modha et al. 1994b) may be a technical artefact, due to alcohol dehydration during the processing of the samples for EM (Wright & Hong, 1989). However, alcohol dehydration procedures per se cannot be the reason for the absence of the surface coat in activated larvae because it is present on the surface of control (i.e. unactivated) larvae (see Modha et al. 1994a, b), which have also undergone the same processing procedures during preparation for EM. Clearly, this issue requires further attention so that a reliable evaluation of the role of the surface coat in the T. spiralis host–parasite relationship can be made. Nevertheless, a number of roles have been proposed. Some suggest that it permits evasion of host immune effector mechanisms (Blaxter et al. 1992) by the sloughing off of bound host molecules endangering the parasite. Others speculate it provides protection for larval surface receptors from damage by acidified pepsin in the stomach (Stewart et al. 1987). According to this view, its shedding in the small intestine exposes these receptors for receipt of new signals from host environmental cues for further development and maturation of the parasite. Shedding of the surface coat may also prime intestinal immune mechanisms for adult worm expulsion and protective immunity (Grencis, Hultner & Else, 1991; Jalink, Hordijk & Moolenaar, 1994). Certainly cells adjacent to invading larvae, and those which have been invaded, have been reported to take up larval products (Capo, Silberstein & Despommier, 1986; ManWarren et al. 1997). Moreover, recent reports show that epithelial cells invaded by T. spiralis larvae demonstrate increased transcription of mRNA for IL-1β, IL-8 and neutrophil activating peptide 78 (Li et al. 1998).

It is also interesting to note that surface components isolated by cetyltrimethyl-ammonium bromide (CTAB) treatment, which includes the surface coat (Modha et al. 1994b), stimulate protective immune responses in mice (Grencis et al. 1986). These observations considered together would suggest an immunological role for the surface coat. This possibility has been investigated and recently we have been able to demonstrate that immunoglobulin receptor-mediated internalization of the surface coat by murine macrophages results in cell activation and interleukin 12 production (manuscript submitted). This observation is significant because although T. spiralis infection is generally characterized by a Th2 response (Grencis et al. 1991; Pond, Wassom & Hayes, 1992; Urban et al. 1992), the early response to larval invasion of the epithelium appears to be mediated by Th1 mechanisms (Jalink et al. 1994; Edwards et al. 1990; Ishikawa et al. 1998; Li et al. 1998), which can be facilitated by IL-12 (reviewed by (Trinchieri, 1997, 1998)). Therefore, it may be that the initial Th1 response to T. spiralis infection (Jalink et al. 1994; Edwards et al. 1990) is stimulated by components of the surface coat of infective larvae, which indicates that further investigation of the immunological role of the 47–60 kDa and 90 kDa antigens of the surface coat in the host–parasite interaction is warranted.

The identification of lysophosphatidic acid (lyso-PA) as one of the major lipid components of the

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**Fig. 9.** Thin layer chromatogram of larval lipids. A total of 20 µl of 2 µg/µl (in 20 % methanol) surface coat (lane 2) and 25 µl of the solvent phase from a Folch extraction of intact larvae (lane 3) were applied to a TLC plate with the reference lipids indicated on the plate as described in the Materials and Methods section. The plate was run using a solvent system for polar lipids (chloroform:methanol:acetic acid:water – 65:5:1:4). The abbreviations for the lipid standards are FFA, free fatty acids; PG, phosphatidyl glycerol; PE, phosphatidyl ethanolamine; PI, phosphatidyl inositol; PA, phosphatidic acid; PS, phosphatidyl serine; LPA, lysophosphatidic acid (lane 1); LPS, lysophosphatidyl serine; PC, phosphatidyl choline; LPC, lysophosphatidyl choline. Lipid standards were resuspended (where necessary) in 100 % methanol, to obtain a stock solution of 5 mg/ml, of which 3 µl were applied per lane. After running the plate was developed with iodine vapour. The arrow marks the origin and the arrowhead identifies the solvent front. Rf values are given for each lipid standard.
surface coat is interesting and suggests other roles for this structure. This lipid has been identified as an important signalling molecule involved in a wide range of phenomena (reviewed by Horan et al. 1990; Moolenaar, 1995 and Laemmli, 1970), one of which is facilitating tumour cell metastasis (Imamura et al. 1993). It would be premature at this stage to suggest that surface-associated lysoPA in T. spiralis larvae plays a role in facilitating invasion of the intestinal epithelium, but this point is noteworthy.

### Chemical and immunological properties

Much work has been carried out to define the chemical and immunological properties of the larval surface, not only for a greater understanding of the biochemistry of the surface, but also for potential immunological targets against infection (Appleton et al. 1991). The surface coat appears to comprise protein, carbohydrate and lipid. The protein component consists of 2 entities; one at 47–60 kDa (which may be a related set of proteins) and a 90 kDa glycoprotein containing N-acetyllactosamine. The former was detectable as a smear in Western blots when the surface coat was collected in water. However, if it is collected in PBS, it is detectable as a cluster of smaller antigens between 35 and 55 kDa. Perhaps the salt in the buffer causes dissociation of the 47–60 kDa antigen complex (or components within it) to smaller fragments. We have not been able to determine how the N-acetyllactosamine is linked to the protein backbone of the 90 kDa antigen. The sugar was not affected by N- and O-glycosidase treatment and, clearly, further studies are required for a detailed characterization of this molecule. The 215:1 Da component detected in the surface coat material by ES–MS correlates well with the molecular mass of an acetylated hexosamine. It may be derived from the breakdown of N-acetyllactosamine, which consists of N-acetyl glucosamine-linked (β1–4) to galactose.

Surprisingly, although DSA recognized components from the larval surface coat in lectin blots, there was no evidence of digoxigenin (DIG)-conjugated DSA having bound to the surface of intact larval parasites. The differences between our studies and those of other workers is that we analysed coat material shed from intact larvae, whereas other studies involved rinsing the larval surface with organic solvents. In our studies therefore, the dangers of inadvertently analysing somatic lipids did not arise, since the coat material is free from intact larvae by virtue of it having being shed into aqueous solvent (water or PBS) in which it is collected. Previous reports highlight some destruction of internal structure by organic solvents (e.g. ethanol, acetone and chloroform:methanol mixtures) used to rinse nematode surfaces (Wright & Hong, 1988; Gounaris et al. 1996). Consequently, lipids which are found in whole parasite extracts, and putatively on the surface (e.g. PE and lysoPE, Wright & Hong, 1988) cannot be guaranteed to be of surface origin. Although in our studies a very small amount of lysoPA was detected in the whole parasite extract by ES–MS, since there were no intact parasites in the sample, and since no extraction procedure using organic solvents was employed to collect the sample, we are confident that the lysoPA detected in the coat material was genuinely from the surface and was not a somatic contaminant. Our ability to detect lysoPA in intact parasite surface by ES–MS, but not by TLC, was presumably due to the sensitivity of the former technique over the latter. Gounaris et al. (1996) found a range of polar (PE, PG, PS, PC, lysoPE, lysoPC) and non-polar lipids (cholesterol, free fatty acids and mono/triglycerides) in the epicuticular accessory layer by TLC, but since there is no indication which lipids were detected in whole parasite extracts, the likelihood of contamination by somatic lipids cannot be evaluated. This is presumed not to have taken place because of prior labelling of the lipsids using putatively surface-restrictive iodination procedures. It is noteworthy that we found none of these polar lipids in coat material shed from the larval surface, but we did find most of them (except for PE and lysoPE), and additionally PI and lysoPA (which have not previously been reported in these larvae), in surface solvent rinses of intact larvae. Thus, some lipids seem to be peculiar to the surface coat (e.g. lysoPA), whilst others (e.g. PE, PG, PS, PC, PI, lysoPE and lysoPC) to deeper layers such as the epicuticle and cuticle.

In conclusion, we have shown that the surface coat of T. spiralis infective larvae can be very readily labelled by the fluorescent probe PKH26, which is likely to be associated with the lipid components of this structure. The shedding of this surface coat has been measured and the lipoprotein macromolecules released can aggregate to produce a structure reminiscent of liposomal membranes (Wisnewski et
al. 1993). The identification of lysophosphatidic acid as one of the major lipid components of the surface coat is of particular interest (Laemmli, 1970; Horan et al. 1990; Moolenaar, 1995). Any interaction of the surface coat lipoproteins with enterocytes or macrophages of the host intestine (Capo et al. 1986; ManWarren et al. 1997) may have profound effects on their metabolism and/or activation of these cells. This modification of host cells by the parasite surface coat may have implications for successful invasion by the parasite.

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