Immunohistochemical localization and differentiation of phosphocholine-containing antigens of the porcine, parasitic nematode, *Ascaris suum*

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SUMMARY

The glycolipids of *Ascaris suum* represent either neutral, zwitterionic or acidic structures. The acidic fraction comprises a sulphatide and an unusual phosphoinositolglycosphingolipid (Lochnit *et al*. 1998*b*). The sulphatide was previously localized to the hypodermis, contractile zone of somatic muscle cells and the external musculature of the female uterus, whereas the presence of the phosphoinositolglycosphingolipid species was restricted to the intestine. The neutral and zwitterionic components belong to the arthro-carbohydrate series, which are substituted in their zwitterionic structures by phosphocholine (PC) and in one glycolipid by an additional phosphoethanolamine residue. In previous immunohistochemical localization studies, however, the chemical nature of the PC-substituted biomolecules has not been investigated in detail. Here, we report on the immunohistochemical localization and differentiation of phosphocholine-containing structures into lipid- and protein-bound species in adult *A. suum*. The patterns of immunostaining, obtained with a PC-specific monoclonal antibody and anti-zwitterionic glycolipid hyperimmune serum in the female worm, indicated a parallel organ distribution for glycolipid- and protein-bound PC-epitopes. Immunoreactivity was localized to specific tissues of the body wall, intestine and reproductive tract. This is the first report of surface-located PC-epitopes for ascarids. The patterns of immunolabelling obtained with antibodies directed against the unsubstituted arthro-carbohydrate series backbone suggested that the glycolipid-bound epitope was restricted to the hypodermis, whilst the protein-bound antigenic determinant resembled that for PC.

Key words: *Ascaris suum*, glycosphingolipids, phosphocholine, immunohistochemistry.

INTRODUCTION

A major post-translational modification of parasitic helminth antigens is apparently phosphocholine (PC). This zwitterionic, antigenic determinant has been mainly detected in nematodes (Gualzata, Weiss & Heusser, 1986; Gutman & Mitchell, 1977; Lal *et al*. 1987; Pery *et al*. 1974). In fact, the frequency of serological cross-reactivity between cestodes, trematodes and, in particular, nematodes (Maizels & Selkirk, 1988) may be accounted for by the broad distribution of PC-bearing molecules. The (macro)-molecular identity of the PC-bound moiety is in most cases unknown, but at least in the excretory–secretory product (ES–62) of the adult filarial nematode, *Acanthocheilonema viteae*, it has been found attached to the protein backbone via a *N*-linked glycan (Houston & Harnett, 1996). Our studies have been concentrated on the glycolipids of the porcine nematode parasite, *Ascaris suum*. The isolated neutral glycosphingolipids from *A. suum* were fractionated by silica gel chromatography to yield neutral and zwitterionic fractions, the latter of which contained 2 major glycosphingolipids, designated components A and C. Preliminary chemical and immunochemical characterization with hydrofluoric acid and a PC-specific monoclonal antibody indicated that both components contained phosphodiester substitutions: phosphocholine for component A, and phosphocholine and phosphoethanolamine (PE) for component C (Dennis *et al*. 1995; Lochnit *et al*. 1998*a*). Structural elucidation of these 2 zwitterionic glycosphingolipids has shown the common pentasaccharide core to belong to the arthro-carbohydrate series (as it was originally identified from glycosphingolipids of the blowflies, *Calliphora vicina* and *Lucilia caesar*) (Lochnit *et al*. 1998*a*). The amphoteric substituent PC is linked to C-6 of the third monosaccharide in the oligosaccharide chain, *N*-acetylglucosamine (GlcNAc), of component A and, uniquely, the amphoteric substituents PE and PC are simultaneously linked to C-6 of the second and third monosaccharides in the oligosaccharide chain, mannose (Man) and GlcNAc, respectively, in component C. Component C, therefore, represents the first glycosphingolipid detected to carry 2 zwitterionic substituents (Lochnit *et al*. 1998*a*). The

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carbohydrate and ceramide moieties of these 2 zwitterionic glycosphingolipids correspond exactly to the recently elucidated arthropentaosylceramide of *A. suum* (Lochnit *et al.* 1997).

Zwitterionic glycosphingolipids are not confined to parasitic nematodes, but have been structurally characterized from various members of invertebrate phyla, including, identification of the monosaccharide-amphoteric moiety: in the Sarcomastigophora (Flagellata) as Man-PE (Winter *et al.* 1994); in the Annelida as galactose (Gal)-PC (Noda *et al.* 1992, 1993*a*, *b*; Sugita *et al.* 1992, 1995); in the Arthropoda (Crustacea) as glucose (Glc)-phosphonoethanolamine (Itonori *et al.* 1991*a*); in the Arthropoda (Insecta) as GlcNAc-PE (Helling *et al.* 1991; Itonori *et al.* 1991*b*; Weske *et al.* 1990); in the Mollusca (freshwater Bivalvia) as Man-PE (Itasaka & Hori, 1979); in the Mollusca (marine Gastropoda) as Gal-phosphonoethanolamine (Araki *et al.* 1987*a*, *b*); and, in the Nematoda (Ascaridida or Rhabditida) as GlcNAc-PC and Man-PE or GlcNAc-PC (Gerdt *et al.* 1999; Lochnit *et al.* 1998*a*) respectively.

Here, we report on the immunohistochemical localization and identification of different biomolecules carrying the PC-epitope in *A. suum*, as a pre-requisite for studying the biosynthesis and putative functions of these antigenic determinants.

MATERIALS AND METHODS

Materials

Undamaged, washed adult male and female worms were collected at the local abattoir. As glycolipid-carrier in the generation of specific, polyclonal antibodies, acid-treated *Salmonella minnesota* R595 was prepared and kindly supplied by Dr C. Galanos (Max Planck Institute for Immunobiology, Freiburg, Germany).

Isolation of glycolipids

Worms (800 g wet weight) were pulverized at −20 °C in a pre-cooled Waring blendor and lyophilized. Glycolipids were isolated and purified as described previously (Lochnit *et al.* 1997). In short, glycolipids were extracted with chloroform/methanol/water (10:10:1 v/v/v), chloroform/methanol/0·8 M aqueous sodium acetate (30:60:8 v/v/v) and 2-propanol/*n*-hexane/water (55:20:25 v/v/v), and the pooled extracts were evaporated to dryness. To remove most of the contaminating triacylglycerols, the residue was treated with acetone at 4 °C for 2 h. Neutral (including zwitterionic) and acidic glycosphingolipids were separated by DEAE–Sephadex A-25 column chromatography (Pharmacia, Freiburg, Germany). The column was equilibrated with and the sample taken up in chloroform/methanol/water (30:60:8 v/v/v). Neutral and zwitterionic glycosphingolipids were obtained in the flow-through and the acidic glycosphingolipids were eluted with chloroform/methanol/0·8 M aqueous sodium acetate (30:60:8 v/v/v). The neutral and zwitterionic glycolipid fraction was further separated on a silica gel_{60} column (Merck, Darmstadt, Germany).

High-performance liquid chromatographic (HPLC) isolation of individual zwitterionic glycosphingolipids

The zwitterionic glycosphingolipids were fractionated on a solvent A-equilibrated, i.e. chloroform/methanol/water (40:60:10 v/v/v) silica-gel column (Iatrobeads 10 μm; 500 × 4·6 mm; Macherey & Nagel, Düren, Germany) at 40 °C with a flow rate of 0·5 ml/min and a linear gradient of 0–100 % solvent B, i.e. chloroform/methanol/water (40:70:15 v/v/v) for 100 min, followed by isocratic elution for a further 20 min. Fractions of 1 ml were collected and aliquots of each fraction were analysed for their glycolipid content by high-performance thin-layer chromatography (HPTLC).

HPTLC

For HPTLC separation, HPTLC-silica gel_{60} plates from Merck were used. Glycolipids were dissolved at approximately 2 μg/μl in chloroform/methanol/water (10:10:1 v/v/v). For the reproducibility of runs, HPTLC was performed according to Nores *et al.* (Nores, Mizutamari & Kremer, 1994) using an automatic HPTLC developing chamber (Baron Laborgeräte, Reichenau, Germany). Chloroform/methanol/water (10:10:3 v/v/v) in the case of zwitterionic glycolipids and chloroform/methanol/water (65:25:4 v/v/v) in the case of neutral glycolipids were used as running solvents. Glycosphingolipids were visualized by spraying the plates with orcinol/sulphuric acid. For immunostaining, the developed HPTLC-plates were fixed with polyisobutylmethacrylate (Röhm & Haas, Darmstadt, Germany), blocked with 2 % (w/v) bovine serum albumin-containing 0·1 M phosphate-buffered saline (PBS) and incubated with the PC-specific, monoclonal antibody TEPC-15 (Sigma, Deisenhofen, Germany) or rabbit polyclonal antisera overnight at 4 °C, as described elsewhere (Baumeister *et al.* 1994). Peroxidase-coupled anti-mouse Ig and anti-rabbit IgG (Dako Diagnostics, Hamburg, Germany) were used as the secondary antibodies.

Generation of polyclonal antisera

Glycolipids (2 mg) were suspended in 2 ml of H_2O by sonication and added to a 4 ml suspension of 500 μg/ml acid-treated *S. minnesota* R595 (Galanos, Lüderitz & Westphal, 1971) to yield a bacterial

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carrier-glycolipid mixture at a ratio of 1:1. After lyophilization, the residue was resuspended in 1 ml of isotonic saline, sonicated and 250 μl aliquots were stored at −20 °C, until required. For immunization, the aliquots were diluted to 400 μl and mixed with 400 μl of Freund's incomplete adjuvant by vigorous vortexing for 15 min to produce a stable emulsion. White female rabbits were immunized subcutaneously and boostered twice after 4 and 7 weeks, i.e. 500 μg glycolipid/rabbit/injection. Three weeks after the last booster injection, blood was taken from the ear vein, allowed to coagulate, centrifuged and the resultant serum was stored at −20 °C in 50 μl aliquots.

Isolation of antigen-specific antibodies by affinity chromatography

Affinity chromatography was performed according to Boulanger (Boulanger *et al*. 1994) with minor modifications. Glycolipid antigens (500 μg) were dissolved in 5 ml of chloroform and mixed with 1 g Celite 545 (Merck). Chloroform was removed by rotary evaporation at 37 °C. The glycolipid-matrix was hydrated overnight at room temperature in 5 ml of 0·01 M PBS, pH 7·4, and washed 4 times with PBS. Affinity chromatography was performed by incubating 1 ml of rabbit hyperimmune serum with 1 ml of matrix for 1 h at 4 °C. Unbound antibodies were removed with 40 ml of PBS. Specifically bound antibodies were eluted with 30 ml of 1 M potassium iodide. The eluted antibodies were immediately desalted to remove the chaotropic reagent and concentrated to a final volume of 1 ml by pressure dialysis against PBS with a YM10 filter (cutoff 10 kDa; Amicon, Witten, Germany).

Immunohistochemistry

Segments (1–5 cm) obtained from various regions of frozen adult worms were embedded in Tissue-Tek OCT Compound (Miles, Elkhart, Indiana, USA) and transverse cryosections (5 μm) were obtained with a Jung Frigocut 2800E cryotome (Leica) at −40 °C. The cryosections were mounted on standard glass slides coated with a poly-L-lysine solution (0·01 %, Sigma), treated with acetone for 12 min at −40 °C and air-dried. The sections were rehydrated for 5 min with cold (4 °C) 0·1 M PBS, pH 7·4, blocked with 2 % (w/v) bovine serum albumin-containing 0·1 M PBS for 30 min and incubated for 30 min at 37 °C with the monoclonal PC-specific antibody TEPC-15 or either a polyclonal PC-specific antiserum or an arthro-series-specific antiserum diluted at 1:20, 1:50 and 1:100 with 0·1 M PBS, respectively. After washing 3 times with cold 0·1 M PBS and re-blocking with 2 % (w/v) bovine serum albumin-containing 0·1 M PBS for 30 min, the sections were incubated for 30 min at 37 °C with a fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG or anti-mouse Ig (Dako) diluted with 0·1 M PBS at 1:20, respectively, and containing 0·01 % Evans blue as counterstain. After washing the sections 3 times with PBS and mounting in glycerol buffer (pH 8–9, containing 73 mg $NaHCO_3$, 16 mg Na_2CO_3, dissolved in 10 ml of H_2O, and made up to 100 ml with glycerol). The preparations were examined with a Diaplan fluorescence microscope (Leitz, Wezlar, Germany) and photographed. To investigate the presence of lipid-bound epitopes, the fixed sections were treated for 15 min with chloroform/methanol (1:1 v/v), followed by a 0·1 M PBS wash, before incubation with the respective antibodies. Protein-bound epitopes were degraded by incubating the fixed sections in a proteinase K solution (1·4 mU/ml 0·01 M PBS) for 25 min at room temperature with gentle shaking, followed by a 0·01 M PBS wash, prior to incubation with the respective antisera. The following sources were consulted for the interpretation of *A. suum* cryosections: general anatomy (Liebau *et al*. 1997); female reproductive tract (Ishii & Yanagisawa, 1954); and, egg embryonic membranes (Foor, 1967). For a survey of the anatomy of the adult female worm an annotated diagram is included (Fig. 7).

To determine the tissue distributions of (glyco)protein-bound epitopes at higher resolution, processed, paraffin-embedded sections were exploited after decreasing and increasing graded dilutions of aqueous ethanol for dehydration and rehydration, respectively, had leached out the glycolipid content. These sections were either subjected to the same TEPC-15 primary and FITC-conjugated, secondary antibody dilutions and protocol as above or submitted to the same incubation conditions but with anti-arthro series primary (1:100 dilution) and horseradish peroxidase-conjugated, goat anti-rabbit IgG secondary antibody (Dako; 1:20 dilution). Following a 5–10 min visualization step with the peroxidase substrate 3-amino-9-ethylcarbazole (AEC; AEC-Kit, Sigma), to yield a red-positive chromogen, the sections were counterstained for 1–5 min with Mayer's haematoxylin (Sigma).

To facilitate the anatomical interpretation, especially of cryosections, a set of processed, paraffin-embedded sections were histologically treated with Goldner's trichrome stain (Böck, 1989).

RESULTS

Generation of polyclonal antibodies against zwitterionic glycosphingolipids

Four rabbits were individually immunized with zwitterionic glycolipid fractions A–C (Fig. 1), respectively, obtained by HPLC separation of the zwitterionic fraction of *A. suum*. Fractions A and C consisted of the pentaosyl glycosphingolipid (Galα3GalNAcβ4GlcNAcβ3Manβ4Glc-Cer) carrying a PC-substituent at C-6 of GlcNAc in

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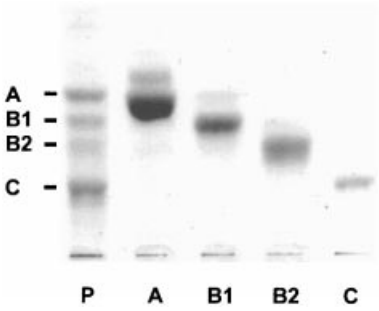

Fig. 1. Zwitterionic glycolipid antigen fractions used for immunization. The isolated glycolipid fractions A, B1, B2 and C were separated on silica gel_{60}-HPTLC plates with chloroform/methanol/water (10:10:3, v/v/v) as running solvent. Glycosphingolipids were visualized chemically by spraying with an orcinol/sulphuric acid reagent. P, zwitterionic glycolipid pool from adult worms.

component A, whilst containing an additional PE-substituent at C-6 of Man in component C (Lochnit *et al*. 1998*a*). The fractions B1 and B2 comprised PC-substituted glycosphingolipids with extended carbohydrate chains (unpublished results). The 4 polyclonal, hyperimmune sera obtained exhibited complete serological cross-reactivity with all the above zwitterionic fractions on HPTLC plates and the resultant staining patterns were identical to that of the monoclonal PC-specific antibody TEPC-15 (Fig. 2). The cross-reactivity could not be eliminated by affinity purification of the polyclonal antisera on Celite 545 with the different glycolipid antigens as ligands, which indicated the immunological dominance of the PC-epitope and predominance of resultant anti-PC antibodies (data not shown). As exemplified for anti-component A hyperimmune serum, inhibition assays of the 4 polyclonal antisera-immunoreactivities using phosphocholine (PC), phosphodimethylaminoethanol (PDMAE) and phosphoethanolamine (PE) revealed competition only in the case of PC (Fig. 3). Immunochemical staining of the zwitterionic glycolipids of *A. suum* resulted in similar patterns, as above, when infection sera of either human (*Ascaris lumbricoides*), pig (*A. suum*), horse (*Parascaris equorum*) or raccoon (*Baylisascaris procyonis*) were used (Fig. 4).

Generation of arthro-series-specific antibodies

Immunization of a rabbit with a preparation containing unsubstituted *A. suum* pentaosyl glycosphingolipids resulted in a polyclonal antiserum recognizing the neutral glycolipids ceramide trihexoside (CTH), ceramide tetrahexoside (CTetH) and ceramide pentahexoside (CPH) of *A. suum* (Lochnit *et al*. 1997), as well as the zwitterionic glycosphingolipids both before and after hydrofluoric acid treatment. The latter treatment would have removed the zwitterionic substituents PC and PE (data not shown). Subsequent immunoaffinity fractionation of the polyclonal antiserum using hydrofluoric acid-treated zwitterionic glycosphingolipids as ligands resulted in an antibody fraction specific for arthro-series glycosphingolipids of *A. suum* carrying the carbohydrate sequence GlcNAcβ3Manβ4Glc (Fig. 5).

Immunohistochemical localization of the PC-epitope(s)

In order to improve the anatomical interpretation of fluorescein isothiocyanate (FITC)-immunostained cryosections, relevant paraffin-embedded tissue sections were histologically prepared using Goldner's trichrome stain (Fig. 6A, E, G), prior to light microscopy. The yellow fluorescence observed on positive, immunofluorescent staining and normally considered negative, resulted from a combination of the red autofluorescence induced by Evans blue counterstain and green fluorescence of FITC (Fig. 6B–D, F, H, I).

The staining patterns exhibited by the monoclonal antibody TEPC-15 and the polyclonal, monospecific A, B1, B2 and C hyperimmune sera defined a comparable, restricted distribution of label within the tissues and organs of the adult female worm. Because of the intensity of the TEPC-15 immunological signal, this antibody was given preference in the presentation of the tissue-distribution of the PC haptenic determinant in this study. For cryosections, the body wall of cuticle, hypodermis and somatic muscle cells was characterized by labelling of the following structures: all 3 zones and the epicuticular layer of the cuticle; the syncytial hypodermis; and the basal lamina surrounding the somatic muscle cells (Fig. 6B, D). The intestine displayed a strong fluorescent signal for the basal lamina and a weak signal for the poorly preserved intestinal epithelium and microvilli of the brush border (Fig. 6F). The female reproductive tract exhibited the following immunostaining pattern: ovarial epithelial cell longitudinal striations and basal lamina of the ovary (data not shown); the external musculature and basal lamina of the uterus, which could not be clearly differentiated by this technique (Fig. 6H); the uterine epithelium, which demonstrated one of the few differences between monoclonal and polyclonal, monospecific antibodies, in that, it was labelled only by the latter (Fig. 6H, I); and the embryos and embryonic layers of eggs, whereby a discrimination of the latter was not possible by this method (Fig. 6H). To investigate the glycolipid nature of the PC antigenic determinant in the tissues and organs of the female worm, a pre-treatment of cryosections with organic solvents was undertaken. This resulted in the incomplete reduction of specific immunofluorescence in all organs tested, as exemplified by the cuticle (Fig. 6C). A resultant proteinase K-pre-

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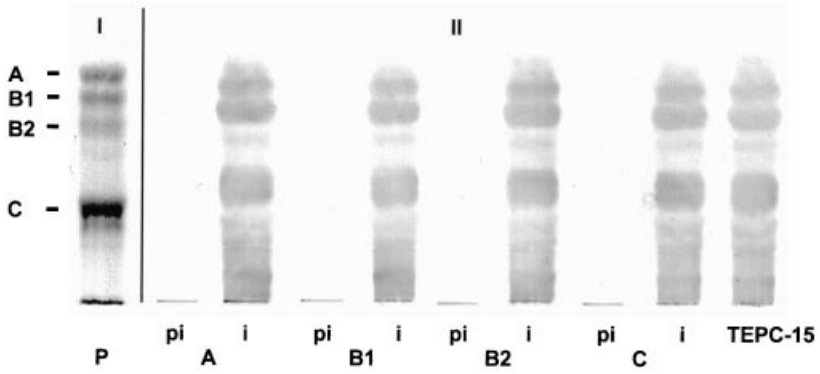

Fig. 2. Chemical and immunochemical characterization of zwitterionic glycolipids from *Ascaris suum*. Isolated glycolipid fractions were separated on silica gel_{60}-HPTLC plates with chloroform/methanol/water (10:10:3, v/v/v) as running solvent. Glycosphingolipids were detected chemically (I) or immunologically (II) with either pre-immune (pi) or hyperimmune (i) sera from rabbits immunized with component A, fraction B1, fraction B2 and component C, respectively, and the monoclonal PC-specific antibody TEPC-15. P, zwitterionic glycolipid pool from adult worms.

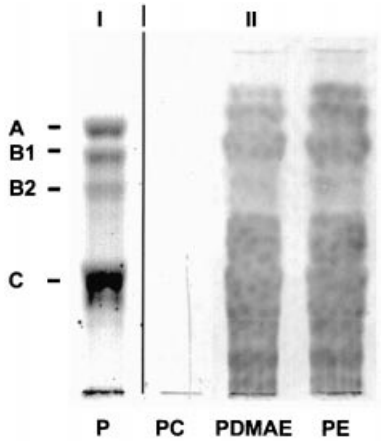

Fig. 3. Immunochemical characterization of the epitope(s) recognized by the hyperimmune serum generated against *Ascaris suum* zwitterionic glycolipids. Glycolipids were separated on silica gel_{60}-HPTLC plates with chloroform/methanol/water (10:10:3, v/v/v) as running solvent. Glycosphingolipids were detected chemically (I) or immunologically (II) as exemplified with the hyperimmune serum raised against component A. The immune serum was pre-incubated with either 50 mM of phospocholine (PC), phosphodimethylamino-ethanol (PDMAE) or phosphoethanolamine (PE) for 1 h at room temperature. P: zwitterionic glycolipid pool from adult worms.

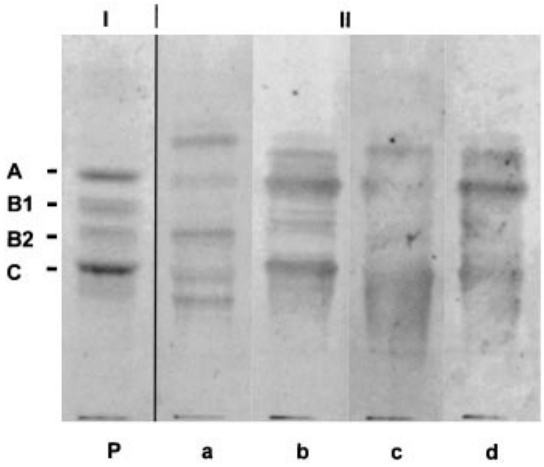

Fig. 4. Immunochemical detection of anti-PC antibodies in sera of (a) human infected with *Ascaris lumbricoides*, (b) pig infected with *A. suum*, (c) horse infected with *Parascaris equorum* or (d) raccoon infected with *Baylisascaris procyonis*. Zwitterionic glycolipids of *A. suum* were separated on silica gel_{60}-HPTLC plates with chloroform/methanol/water (10:10:3, v/v/v) as running solvent and detected chemically (I) or immunologically (II) using the respective infection sera. P, zwitterionic glycolipid pool from adult worms.

treatment, besides a loss in tissue integrity, resulted in the complete elimination of specific immuno-fluorescence in all organs tested. Taken together, these results indicated that all organs expressed glycolipid- and protein-bound PC in different proportions.

To examine the distribution and protein, i.e. non-lipid, nature of the PC-epitope in the tissues and organs of the female adult worm at a higher resolution, TEPC-15 incubation and FITC-immunofluorescence was performed on processed, paraffin-embedded sections. The results confirmed the premise, that protein-bound PC was similarly distributed as to glycolipid-bound PC in the adult female *A. suum*. The body wall was characterized by immunostaining the ensuing structures: the epi-cuticle, the median and basal zones of the cuticle; the fibrillar hypodermis; and the basal lamina and sarcoplasm of the somatic muscle cells (Fig. 6J). In addition, the thickened basal lamina/basement membrane (regularly found underlying the cuticle in Goldner trichrome-stained sections) (Fig. 6A) was also PC positive (Fig. 6K). The intestine manifested specific immunolabelling on the surface of the basal lamina, the intestinal epithelium, apart from the nuclei and terminal web, and the microvilli of the brush border (Fig. 6L). The reproductive tract revealed the following sites of immunoreactivity: the undifferentiated external musculature and basal lamina of the uterus; uterine epithelial cells,

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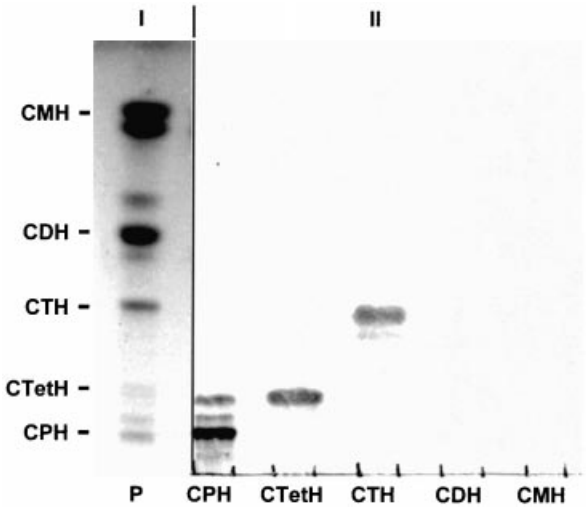

Fig. 5. Immunochemical characterization of arthro-series glycolipids from *Ascaris suum*. Individual neutral glycolipid fractions were separated on silica gel_{60}-HPTLC plates with chloroform/methanol/water (65:25:4, v/v/v) as running solvent. Glycosphingolipids were visualized chemically (I) or immunologically with arthro-series-specific antibodies (II). CMH: ceramide monohexoside, Glcβ1Cer; CDH: ceramide dihexoside, Manβ4Glcβ1Cer; CTH: ceramide trihexoside, GlcNAcβ3Manβ4Glcβ1Cer; CTetH: ceramide tetrahexoside, GalNAcβ4GlcNAcβ3Manβ4Glcβ1Cer; CPH: ceramide pentahexoside, Galα3GalNAcβ4GlcNAcβ3Manβ4Glcβ-1Cer; P, neutral glycolipid pool from adult worms.

excluding the nuclei; apparent secretion of PC-positive material present on the epithelial cell-plasma membrane, in the uterine cavity and covering the contained eggs; and, the embryos and the apparent inner lipid layer, but not intermediate chitinous layer, of the eggs themselves (Fig. 6L, M).

Immunohistochemical localization of the arthro-series epitope(s)

A reasonable inference to the finding of a restricted distribution of the PC-epitope in the tissues and organs of the studied female worm would be an expected, parallel location of the backbone arthro-series glycoconjugate, as PC- and PE-modified zwitterionic glycolipids of *A. suum* are based on the arthro-carbohydrate series of neutral glycosphingolipids. To assess this supposition, the binding of the affinity-purified, polyclonal anti-arthro-series antibodies to processed, paraffin-embedded sections was visualized with the corresponding horseradish peroxidase-conjugated secondary antibody and the substrate AEC. The pattern of immunolabelling closely paralleled that for both the glycolipid- and protein-bound PC-epitope in the cuticle, hypodermis, somatic muscle cells, intestine and female reproductive tract; as exemplified by the body wall (Fig. 6N). The only additional immunostaining by anti-arthro-series antibodies was of a fine, membranous network in the oocytes of the ovary (data not shown).

Under the conditions used, immunohistochemical staining of anti-arthro-series antibodies-treated and FITC-immunofluorescence-visualized cryosections revealed the presence of arthro-series glycoconjugates solely in the thickened basal lamina/basement membrane underlying the cuticle and hypodermis. Chloroform/methanol pre-treatment abolished the antibody binding completely (data not shown), to suggest the restriction of antigens of the glycolipid-bound arthro-series to these tissue compartments.

DISCUSSION

Glycosphingolipids of nematodes studied so far may be structurally divided into 3 classes: neutral, zwitterionic and acidic glycolipids. Whilst neutral and zwitterionic compounds belonged to the so-called arthro-series of protostomial glycosphingolipids, acidic species were found to consist of a phosphoinositolglycosphingolipid and sulphatide. Zwitterionic glycolipids have been further characterized by the presence of a phosphodiester-bound PC-substituent, which has been assigned to C-6 of GlcNAc (Lochnit *et al.* 1998*a*). Moreover, the ceramide pentahexoside has been found to carry, in part, PE linked to C-6 of the Man residue in addition to PC. The occurrence of similar neutral and/or zwitterionic arthro-series glycosphingolipids has been verified, mainly by serological means, in different orders of the Nematoda including, *Nippostrongylus brasiliensis* (Dennis *et al.* 1995), *Litomosoides sigmodontis* (Baumeister *et al.* 1994), *Onchocerca volvulus* and *Setaria digitata* (Wuhrer *et al.* 2000), as well as the free-living nematode, *Caenorhabditis elegans* (Gerdt *et al.* 1997, 1999). This would indicate that arthro-series glycosphingolipids carrying, in part, PC-substituents represent highly conserved glycolipid markers within the nematode phylum. It has to be pointed out, however, that zwitterionic glycosphingolipids are not a unique feature of nematodes, but have also been characterized in the phyla Annelida, Arthropoda (Crustacea and Insecta) and Mollusca (freshwater Bivalvia and marine Gastropoda).

In *A. suum*, PC-substituted glycolipids and protein-bound PC-epitopes were found in the cuticle, the hypodermis, the basal lamina investing the contractile zone of the somatic muscle cells, the external musculature/basal lamina and epithelium of the uterus and enclosed embryos, the epithelium of the ovaries and the gut. Therefore, it is the first report for PC-epitopes located at the surface of this nematode. In *A. suum* lung-stage larvae, PC-epitopes appeared to be confined to internal membranous structures and the lining of the intestinal tract

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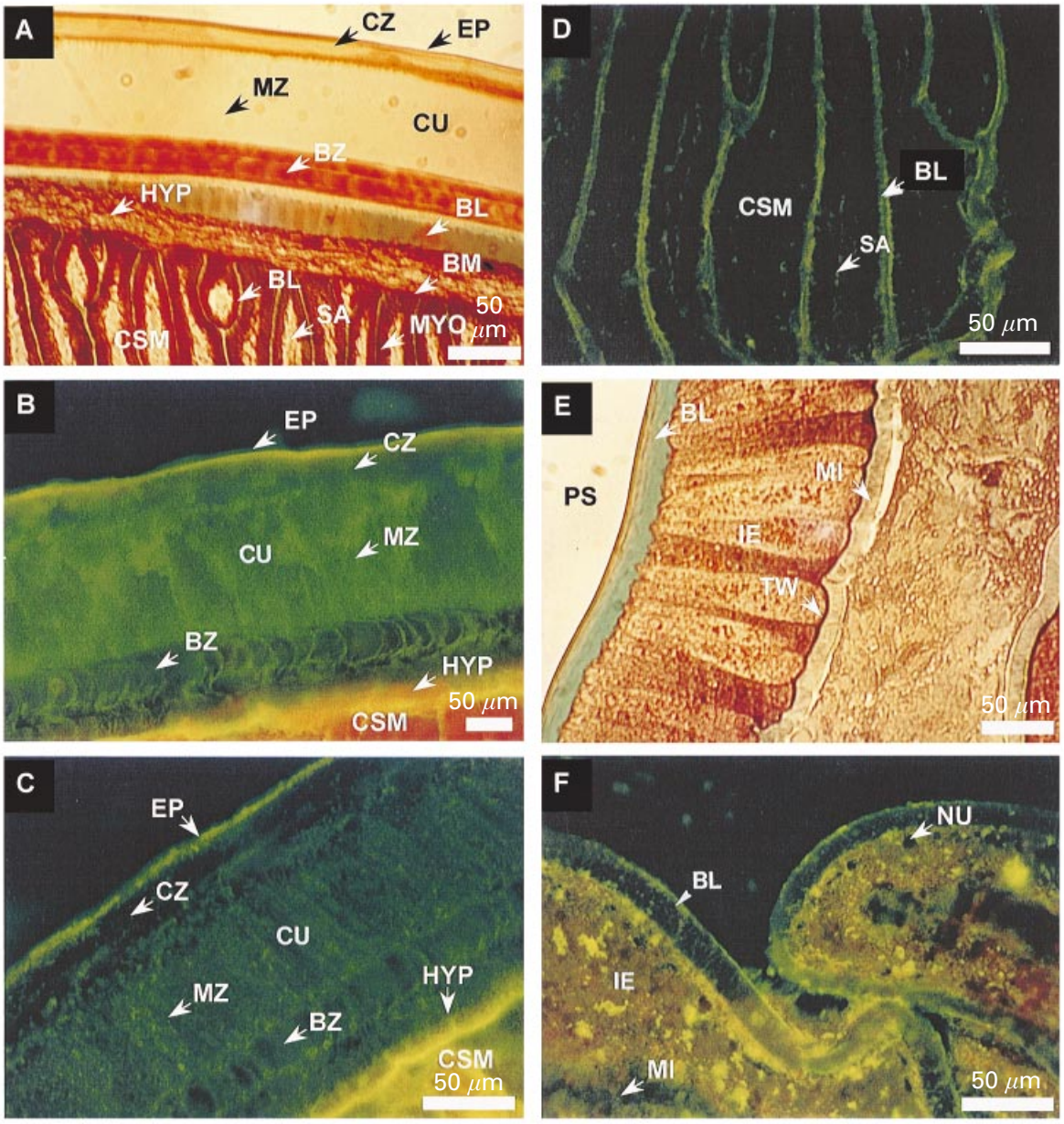

Fig. 6. Histological and immunohistological staining of *Ascaris suum* sections (5 μm) of adult female worms were obtained and treated in various ways: as paraffin-embedded, Goldner trichrome-stained sections (A, E, G); as Tissue-Tek OCT compound-embedded, monoclonal antibody TEPC-15 (B–D, F, H)- or polyclonal anti-B1 antiserum (I)-incubated and FITC immunofluorescence-stained cryosections; as paraffin-embedded, TEPC-15 (J–M)-incubated and FITC immunofluorescence-stained sections; and as paraffin-embedded, anti-arthro-series antiserum (N)-incubated and chromogen AEC-stained section. The TEPC-15-incubated and FITC immunofluorescence-stained cryosection (C) was pre-treated with organic solvent. BL, basal lamina; BM, basement membrane, BZ, basal zone; CL, chitinous layer; CSM, contractile portion of somatic muscle cell(s); CU, cuticle; CZ, cortical zone; E, embryo(s); EMU, external musculature of uterus; EP, epicuticle; HYP, hypodermis; I, intestine; IE, intestinal epithelium; LL, lipid layer; MI, microvilli of brush border; MYO, myofibrils of obliquely striated, coelomyarian muscle cells; MZ, median zone; NU, nuclei; O, ovary; OE, ovarial epithelium; OO, oocyte(s); PS, pseudocoelomic space; SA, sarcoplasm; TW, terminal web; U, uterus; UE, uterine epithelium; UL, uterine layer.

(Gutman & Mitchell, 1977). Anti-PC antibodies raised against *Onchocerca gibsoni* eggs revealed the presence of PC-epitopes in *Brugia malayi* egg-bearing regions and the intestines of adult worms (Wenger, Forsyth & Kazura, 1988). In the case of *N. brasiliensis*, PC-antigens of the L3 infective larvae, adult worms and eggs were restricted to internal structures of the parasite, especially the gonads and intestinal tract (Pery *et al*. 1979). In the filarial parasite *Dipetalonema* (= *Acanthocheilonema*) *viteae*, PC-epitopes were restricted to internal structures, such as the egg and uterine and

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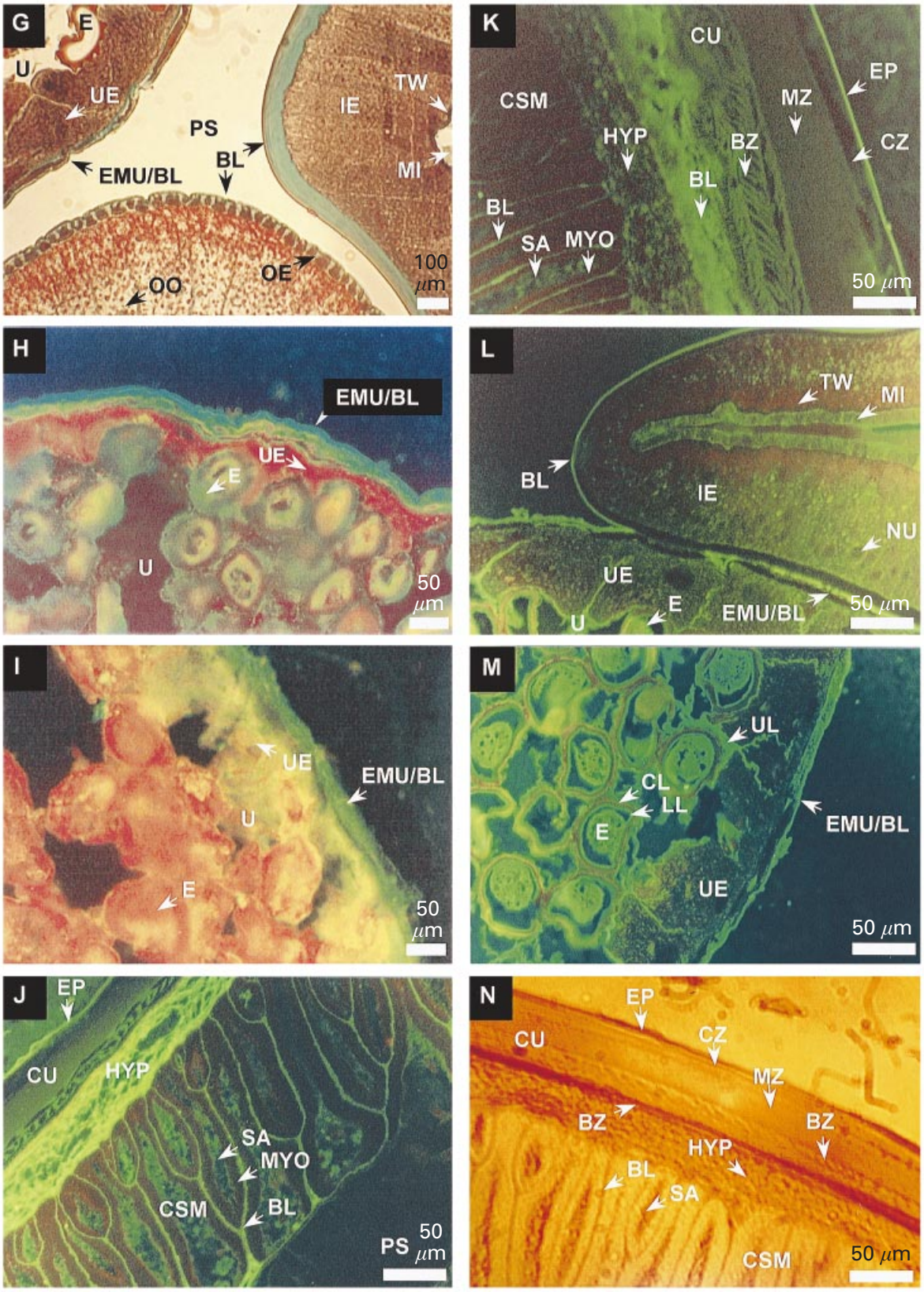

Fig. 6. For legend see page 365.

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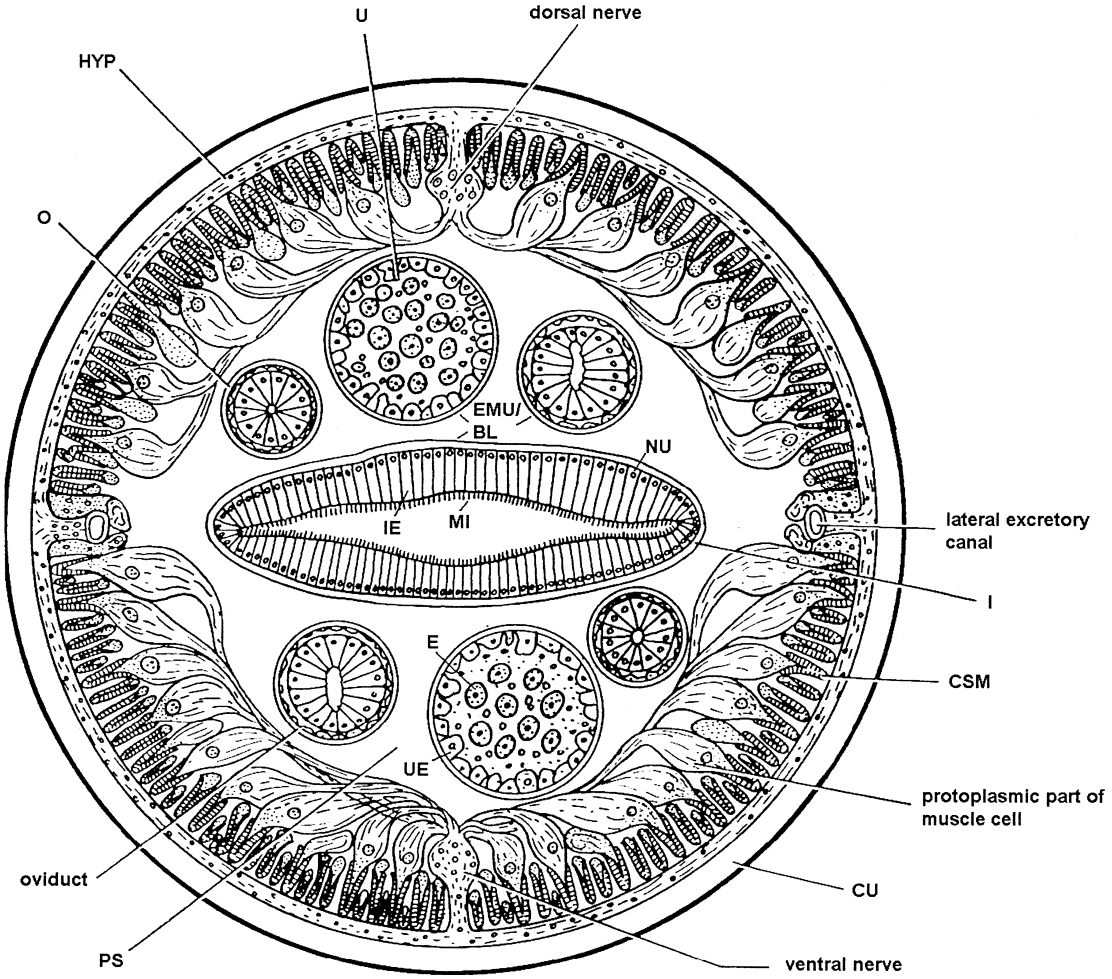

Fig. 7. Annotated diagram of the anatomy of the adult female *Ascaris suum* worm. For abbreviations see Fig. 6.

intestinal membranes, but not on the cuticle, whereas PC-devoid antigens were predominantly expressed on the cuticle (Gualzata *et al*. 1986). Stage-specific expression of the PC-epitope has also been observed in *Trichinella spiralis*, with an abundance on internal structures of the muscle larva and the adult worm, but absence from the foetal larva (Sanmartin *et al*. 1991; Takahashi, Homan & Lim, 1993). PC-antigens in the cuticle of muscle larvae were restricted to the inner layer, whereas this epitope was absent from foetal larvae and adult worms. As a first approximation, the membrane-associated tissue and organ distribution of the PC-epitope in L1 muscle larvae of *T. spiralis* (Hernandez *et al*. 1995) would appear to resemble the pattern in the adult female nematode, *A. suum*, described in this publication.

Phosphocholine (PC-) substituted macromolecules have been regularly identified in extracts of numerous species of parasitic nematode by immunological means (Crandall & Crandall, 1971; Pery *et al*. 1974, 1979; Brown & Crandall, 1976; Mitchell *et al*. 1976; Gutman & Mitchell, 1977). So far, however, little is known as to the biological activity of glycolipids, in general, and parasitic helminth-derived glycolipids, in particular, as regards their putative modulation of the host's immune system via the cytokine network (O'Garra, 1998). Whilst neutral glycosphingolipids of the cestode, *Echinococcus multilocularis*, inhibited the production of interleukin-2 (Persat *et al*. 1996), the zwitterionic glycosphingolipids of *A. suum* appeared to stimulate rather than suppress human peripheral blood mononuclear cells' production of the cytokines tumour necrosis factor-α and interleukin-6 in a concentration range similar to that of lipopolysaccharide, whereas these molecules were at least a factor 100-fold less potent than lipopolysaccharide in the stimulation of interleukin-1 (Lochnit *et al*. 1998*a*). The frequent observation of host T-cell hyporesponsiveness to filarial nematode infection (Allen, Lawrence & Maizels, 1996) may involve PC, because of its ability to block both T- and B-cell antigen-specific proliferation (Harnett & Harnett, 1993; Lal *et al*. 1990).

At present, the biological significance for the predominant localization of PC-nonsubstituted arthroseries glycolipids in the hypodermis remains unclear, but may be hypothesized to be due to the function of the biosynthetically active hypodermal tissue in maintaining an intact cuticular shield for the para-

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site. In addition, the nature of the protein-bound PC-substituents in *A. suum* has to be elucidated. It may be speculated as to whether the PC-substituent is linked via carbohydrates, either *N*- or *O*-linked, or directly to distinct polypeptide backbone-amino acids. This also raises the question as to the biosynthesis of these modifications.

In contrast to protein determinants, glycolipids undergo antigenic shifts to a much lesser extent. Since all the parasitic helminths investigated so far possess at least one highly antigenic glycolipid fraction, parasite glycolipids and their host-derived antibodies represent powerful tools for serodiagnosis, epidemiological studies, and control programmes as to the efficacy of anthelminthic treatment (Baumeister *et al*. 1994; Dennis *et al*. 1996).

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