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Thin-layer and liquid column chromatographic analyses of the lipids of adult *Onchocerca gibsoni*

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Abstract. Lipids were extracted from adult *Onchocerca gibsoni* with chloroform/methanol and the total lipid content was characterized. Glycolipids were isolated from other lipid classes by Florisil column chromatography and were then fractionated by DEAE-Sephadex ion-exchange chromatography. HPTLC revealed the presence of 9 neutral glycolipid bands and of 15 acidic glycolipid bands that stained for sialic acid with resorcinol. Lipids that contained no carbohydrates were analyzed by a combination of TLC and amino column chromatography. Triacylglycerols, cholesterol, cholesterol esters, and free fatty acids were found to be major components of the neutral lipid fraction, and diacylglycerols and monoacylglycerols were minor components. Phosphatidylethanolamine and phosphatidylcholine were the predominant phospholipids. Phosphatidylserine, phosphatidylinositol, sphingomyelin, lysophosphatidylcholine, and lysophosphatidylethanolamine were also present in significant amounts, whereas only traces of cardiolipin and phosphatidic acid were detected. Several minor lipids and phospholipids remained unidentified. These results indicate that adult *O. gibsoni* have a nonglycosylated lipid composition resembling that of other parasitic nematodes as well as a substantial repertoire of glycolipids, including many with the characteristics of gangliosides.

Onchocerciasis is one of the major causes of blindness in the world and a severe dermatological disease affecting >40 million people in Africa and Latin America (MacKenzie 1987). The disease is caused by the parasitic nematode *Onchocerca volvulus*, which enters the human host during the ingestion of a blood meal by the black fly vector. Adult parasites live for many years in immunologically competent hosts without inducing significant pathology or helminthicidal immune responses, the pathology of infection arising primarily as the result of host allergic responses to skin microfilariae (MacKenzie 1987). Current means of limiting pathology and transmission primarily rely upon antifilaricides and antivector campaigns. Nevertheless, it is recognized that the development of safe and effective agents to kill adult worms could be instrumental in limiting or eradicating this parasite (Duke 1990).

Although considerable effort has been made to characterize the proteins of adult *Onchocerca* and determine their roles in disease and resistance, onchocercal lipids have received limited attention. Lipids and their breakdown products have been shown to perform diverse biological functions, including those as structural components of membranes, mediators of signal transduction, and modulators of cell function (Berridge 1987; Bloch 1983; Cockcroft 1987; Hannun and Bell 1989; Nishizuka 1986; van Corven et al. 1989). They have been reported to be targets of immune effectors (Costello and Green 1988; Feizi and Childs 1985; Hakomori and Kannagi 1983) and to inhibit or enhance the activity of cells of the immune system (Bellini et al. 1990; Ginsburg et al. 1989; Hannun and Bell 1989; Lindahl et al. 1988; Quinn et al. 1988; Traill and Wick 1984). Certain lipids and lipid derivatives cause significant damage to cell membranes (Corr et al. 1987; Golan et al. 1988; Gregson 1989; Kagan 1989; Ungemach 1989; Weltzien 1979). Parasite lipids could obviously play a significant role in the parasite's capacity to survive and cause disease and could provide targets for chemotherapeutic and immunoprophylactic agents.

The lipid component of multicellular eukaryotic organisms such as *Onchocerca* is composed of a diverse array of molecules belonging to several classes of lipids. Many lipids, especially the glycolipids, are typically present only as minor constituents of the lipid fraction. Therefore, the isolation and characterization of the total lipids require significant amounts of tissue for extraction. Because *O. volvulus* adults are a limited resource, being available only from human surgical material, the initial lipid analysis of adult onchocercal tissues was per-

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formed using a model species. The bovine parasite *O. gibsoni* was chosen because of its striking similarities to *O. volvulus* with regard to protein composition (Cabrera and Parkhouse 1986), ultrastructure – especially of the worm surface and gut (Deas et al. 1974; Franz et al. 1987), and host-parasite relationship (adult worms of both species elicit connective tissue nodules). In addition, methods are available for quantitative isolation of pristine *O. gibsoni* adult tissue from bovine connective tissue nodules (Semprevivo and Maloney 1989). The objective of the present study was to perform a general lipid analysis of *O. gibsoni* adult tissues using liquid column and thin-layer chromatography (TLC).

Materials and methods

Source of worm material

Bovine connective tissue nodules containing *Onchocerca gibsoni* adults were provided courtesy of Dr. D.B. Copeman. The nodules were collected at abattoirs in Australia and shipped frozen on solid CO_2. They were then stored at −100° C until their processing for adult worm tissue.

Isolation of adult worm tissue

The technique used to isolate adult *O. gibsoni* tissue for biochemical analysis has been described in detail elsewhere (Semprevivo and Maloney 1989). Briefly, the frozen nodules were sectioned into 3-mm-thick slabs. These were thawed and the worm sections were immediately harvested, rinsed in excess ice-cold Hanks' balanced salt solution, and pelleted. The pellets were flash-frozen in liquid nitrogen and stored at −100° C until their use for extraction of lipids.

Lipid standards

Cholesterol, cholesteryl palmitate, oleic acid, mono-, di-, and triolein, and the individual phospholipid standards were obtained from Sigma (St. Louis, Mo.). A serum-lipid standard mixture was obtained from Supelco (Bellefonte, Pa.), as were all glycolipid standards except GM1, which was purchased from Sigma, and lactosyl ceramide (ceramide dihexoside), which was generously provided by Dr. T. Toyokuni of the Biomembranes Institute and E. Nudelman of the University of Washington, Seattle.

Lipid extraction

First, 2 g worm tissue was extracted with 80 ml chloroform/methanol/water (4:8:3, by vol.). The extract was then filtered through a sintered glass funnel and the worm tissue residue was reextracted in 40 ml of the above solvent. This extract was also filtered and the residue on the filter was washed with 10 ml chloroform. The filtrates were combined and taken to dryness on a rotary evaporator. The lipid residue was resuspended in 4 ml chloroform/methanol/water (2:1:0.05, by vol.) and applied to a column of Sephadex LH 20 (1.5 × 95 cm) in the same solvent (Baumann et al. 1979). The column was then eluted with this solvent and the fractions containing lipid, as determined by TLC, were pooled, dried on a rotary evaporator, and resuspended in 2 ml chloroform. This was designated the "purified total lipid extract."

Glycolipid analysis

In preliminary thin-layer analyses of the total lipid extract it was found, as is characteristic of most organisms, that the glycolipids were minor constituents of the parasite lipid component. Because other lipids, especially the phospholipids, interfered with the analysis of glycolipids by TLC, it was necessary first to isolate the total glycolipids from the other lipid classes. Glycolipids were acetylated prior to isolation by Florisil column chromatography (Saito and Hakomori 1971). The purified glycolipids were then deacetylated and analyzed by high-performance thin-layer chromatography (HPTLC). The carbohydrate content of the glycolipid fraction was estimated using an anthrone procedure (Yamamoto and Rouser 1970), and samples containing 20 µg sugar (corresponding to 65 µl purified total lipid extract) were spotted onto HPTLC plates (Silica gel 60, 10 × 10 cm; EM Science, Cherry Hill, N.J.). These were developed in chloroform/methanol/water (65:25:4, by vol.) and visualized using orcinol, a reagent specific for carbohydrate residues (Svennerholm 1956).

The acidic glycolipid fraction was isolated by DEAE-Sephadex column chromatography (Ledeen et al. 1973). Purified total glycolipid extract was applied to a column of DEAE-Sephadex in chloroform/methanol/water (30:60:8, by vol.), and the neutral glycolipids were rapidly eluted with this solvent. The acidic fraction was then eluted with chloroform/methanol/0.8 M aqueous sodium acetate (30:60:8, by vol.) and, subsequently, desalted by a combination of dialysis at 5° C and Sep-Pak C18 chromatography (Waters, Milford, Mass.; Katz et al. 1985), dried under nitrogen, and resuspended in chloroform/methanol (1:1, v/v). Samples containing 7.5 µg sialic acid as estimated by a thiobarbituric acid assay (Warren 1959; corresponding to 400 µl purified total lipid) were spotted onto HPTLC plates. The plates were developed in chloroform/methanol/water (60:35:8, by vol.) and visualized using a resorcinol reagent specific for sialic acid residues (Svennerholm 1957).

Neutral lipid and phospholipid analysis

For analysis of neutral lipids and phospholipids, thin-layer plates (Silica gel GHLF Uniplates, 10 × 10 cm; Analtech, Newark, Del.) were spotted with 50 µl purified total lipid extract in chloroform. For neutral lipid analysis, TLC plates were developed in hexane/diethyl ether/formic acid (80:20:2, by vol.), and the lipids were visualized by charring with 50% sulfuric acid in ethanol or by using an acidic ferric chloride spray for steroids (Lowry 1968). Neutral lipids were further fractionated and identified by Bond Elut amino column chromatography (Analytichem International, Harbor City, Calif.) using a system of stepwise elution with a series of organic solvents (Kaluzny et al. 1985). The fractions were dried under nitrogen and resuspended in chloroform to their original concentrations in the total lipid extract. The fractions were then analyzed by TLC as described above. By combining TLC analysis with amino column chromatography, lipids of the *O. gibsoni* neutral lipid fraction could be identified. Identifications were made only in cases in which the migration on TLC plates, the elution of the individual lipid components from amino columns, and the staining properties mirrored those of authentic lipid standards.

For phospholipid analysis, TLC plates were developed in two directions. The first development was carried out in chloroform/methanol/28% ammonium hydroxide (65:25:4, by vol.; solvent A). Plates were thoroughly dried under nitrogen gas and then developed in the second direction with chloroform/acetone/methanol/acetic acid/water (30:40:10:10:1, by vol.; solvent B). Phospholipids were identified by staining with a molybdenum blue spray reagent (Sigma; Dittmer and Lester 1964) specific for phosphate groups and by comigration with phospholipid standards. Those containing free amino groups were identified using a ninhydrin reagent (Sigma; McCaldin 1960).

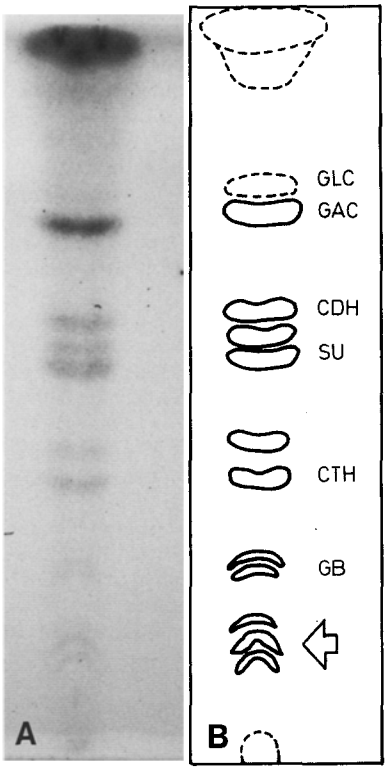

Fig. 1 A, B. HPTLC analysis of adult *Onchocerca gibsoni* glycolipids prepurified by Florisil column chromatography. **A** Chromatogram developed using chloroform/methanol/water (65:25:4, by vol.) and visualized with orcinol reagent. **B** Diagram of the chromatogram with abbreviations indicating the migration of standards relative to *O. gibsoni* glycolipids. The *arrow* indicates the prominent resorcinol-positive triplet. *GLC*, Glucosylceramide; *GAC*, galactosylceramide; *CDH*, ceramide dihexoside; *SU*, sulfatide; *CTH*, ceramide trihexoside; *GB*, globoside

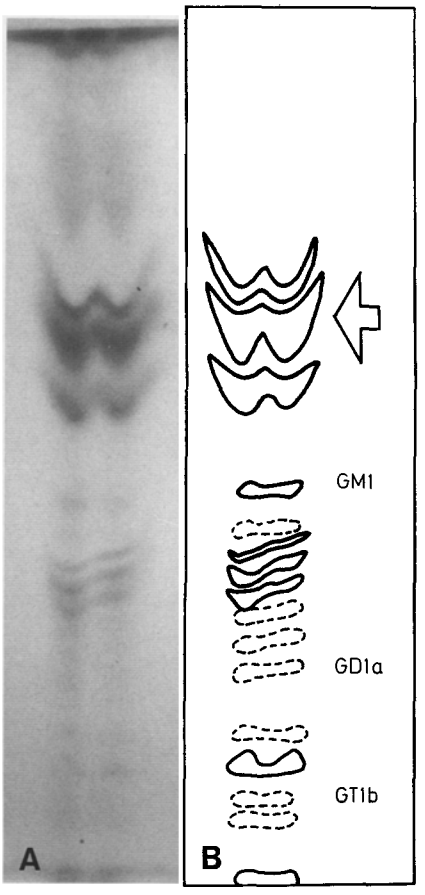

Fig. 2 A, B. HPTLC analysis of the acidic glycolipid content of adult *Onchocerca gibsoni* isolated by DEAE-Sephadex column chromatography. **A** Chromatogram developed using chloroform/methanol/water (60:35:8, by vol.) and visualized with resorcinol reagent. **B** Diagram of the chromatogram indicating the migration of ganglioside standards relative to *O. gibsoni* acidic glycolipids. The *arrow* indicates the prominent triplet. *GM1*, Standard containing one sialic acid residue; *GD1a*, standard containing two residues; *GT1b*, standard containing three residues

Results

HPTLC analysis of adult Onchocerca gibsoni glycolipids

The results of HPTLC analysis of the total glycolipid fraction isolated from adult tissue are shown in Fig. 1. The *O. gibsoni* adult tissue was found to possess glycolipids that migrated in the areas corresponding to the cerebroside standards glucosylceramide and galactosylceramide as well as the ceramide dihexoside, sulfatide, ceramide trihexoside, and globoside standards. Also present was a triplet of glycolipids that contained sialic acid as determined by reaction with the resorcinol reagent. This triplet migrated below the globoside standard but above the selected bovine ganglioside standards, which did not migrate significantly from the origin in the chloroform/methanol/water (65:25:4, by vol.) solvent system used. The material that migrated at the solvent front in Fig. 1 did not stain with orcinol and may represent contaminating neutral lipids. This material did not interfere with visualization of the glycolipid fraction.

To improve the resolution of the acidic glycolipids, these were isolated by DEAE-Sephadex ion-exchange chromatography. HPTLC analysis was then performed using chloroform/methanol/water (60:35:8, by vol.), a solvent system in which the majority of known gangliosides migrate significantly from the origin. The migration patterns of the resorcinol-positive glycolipid bands relative to those of ganglioside standards containing one (GM1), two (GD1a) or three (GT1b) sialic acid residues are illustrated in Fig. 2. *O. gibsoni* adult worms contained at least 12 sialic acid-containing glycolipids in addition to the prominent, rapidly migrating triplet.

Analysis of adult O. gibsoni neutral lipids

Triacylglycerols, cholesterol, cholesterol esters, and free fatty acids were found to be major components of the neutral lipids of *O. gibsoni* adults, whereas 1,2- and 1,3-diacylglycerols and monoacylglycerols were identified as minor components (Figs. 3, 4). Separation on amino columns aided in visualization of the relatively minor 1,3-diacylglycerol and monoacylglycerol components (Fig. 4), which were otherwise obscured by the cholester-

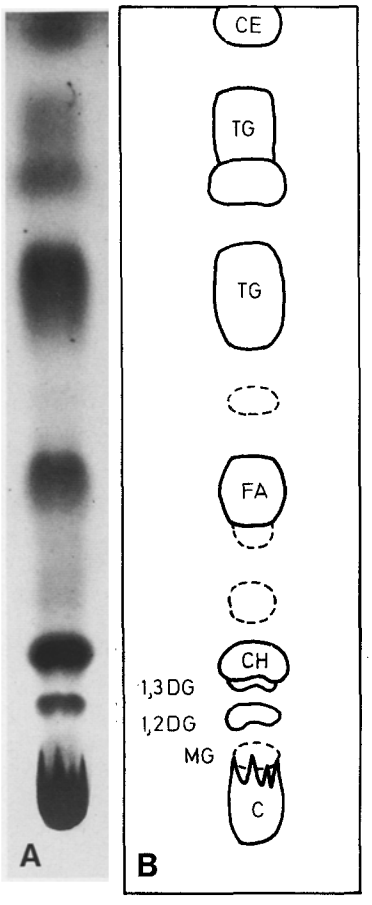

Fig. 3A, B. TLC analysis of neutral lipids extracted from *Onchocerca gibsoni* adult tissue. **A** Chromatogram developed using hexane/diethyl ether/formic acid (80:20:2, by vol.) and visualized by charring. **B** Diagram of the chromatogram. Lipids were identified on the basis of their migration on thin-layer chromatograms and their amino column elution patterns relative to standards (see Fig. 4). *CE*, Cholesterol esters; *TG*, triacylglycerols; *FA*, free fatty acids; *CH*, cholesterol; *DG*, diacylglycerols; *MG*, monoacylglycerols; *C*, complex lipids

ol and complex lipids (phospholipids and glycolipids), respectively (Fig. 3). Although some complex lipids eluted from the amino column with the monoacylglycerols, they were present in a sufficiently reduced quantity and remained at or near their origin on TLC so as not to obscure the monoacylglycerols. Lipids of the amino-column fatty acid fraction migrated somewhat faster than did the same spots in the total lipid fraction. It is not known whether this reflected an alteration in these lipids during amino column chromatography or their interaction with other lipids when the total lipids were applied as a mixture. The identity of the spot that migrated just above the triacylglycerol standard on thin-layer chromatograms but eluted from the amino column with the fatty acid fraction is not known. The material that migrated between the fatty acid and the cholesterol standards also remains unidentified; this material eluted from the amino columns partially with the triacylglycerol fraction and partially with the sterol fraction.

Analysis of adult O. gibsoni phospholipids using two-dimensional TLC

Phosphatidylethanolamine and phosphatidylcholine were the predominant phospholipids (Fig. 5). Phosphatidylserine, phosphatidylinositol, sphingomyelin, lysophosphatidylcholine, and lysophosphatidylethanolamine made up a significant proportion of the adult worm phospholipids, whereas phosphatidic acid and cardiolipin were detected only in trace amounts. The presence of amino groups in the phosphatidylserine, phosphatidylethanolamine and lysophosphatidylethanolamine bands was confirmed by staining of thin-layer chromatograms with the ninhydrin reagent (results not shown). There were a few molybdenum blue-positive TLC spots that did not comigrate in the area of the phospholipid standards; these unidentified phospholipids did not stain with ninhydrin.

Discussion

In the present study, the lipids of *Onchocerca gibsoni* adult worms were fractionated into glycolipids, neutral lipids, and phospholipids and analyzed by HPTLC and liquid column chromatography. Despite the possible effects of parasite lipids on the course of infection, little information on the lipid composition of parasitic helminths of the genus *Onchocerca* was available prior to this study.

Adult *O. gibsoni* were shown to possess an extensive repertoire of glycolipids. Neutral glycolipids comigrated in the areas corresponding to the cerebroside, ceramide di- and trihexoside, sulfatide, and globoside standards (Fig. 1). The reaction of the acidic glycolipids with resorcinol reagent (Svennerholm 1957) and their behavior during DEAE-Sephadex column chromatography and on HPTLC plates relative to that of the glycolipid standards suggest that these molecules are probably gangliosides, glycosphingolipids that contain sialic acid residues. In all, 15 resorcinol-positive bands were observed (Fig. 2). Such a complex composition of gangliosides is typically found in nervous tissue of mammalian hosts, which makes up a small percentage of nematode tissues. The complexity of this *O. gibsoni* lipid fraction is of particular interest because of the variety of interactions between gangliosides and the immune system (Feizi and Childs 1985; Hakomori and Kannagi 1983; Hannun and Bell 1989; Watanabe et al. 1980).

Sterols and sterol esters were found to be major components of the neutral lipid fraction of adult *O. gibsoni* (Figs. 3, 4). These were identified as cholesterol and cholesterol esters on the basis of their comigration with authentic standards, their reaction with a ferric chloride spray (Lowry 1968), their behavior during amino column chromatography (Kaluzny et al. 1985), and the observation that cholesterol is certainly the major, if not the only, sterol of virtually every parasitic helminth investigated to date (Barrett 1981; Furlong 1989; von Brand 1973). Triacylglycerols and free fatty acids also made up a large proportion of the neutral lipids despite

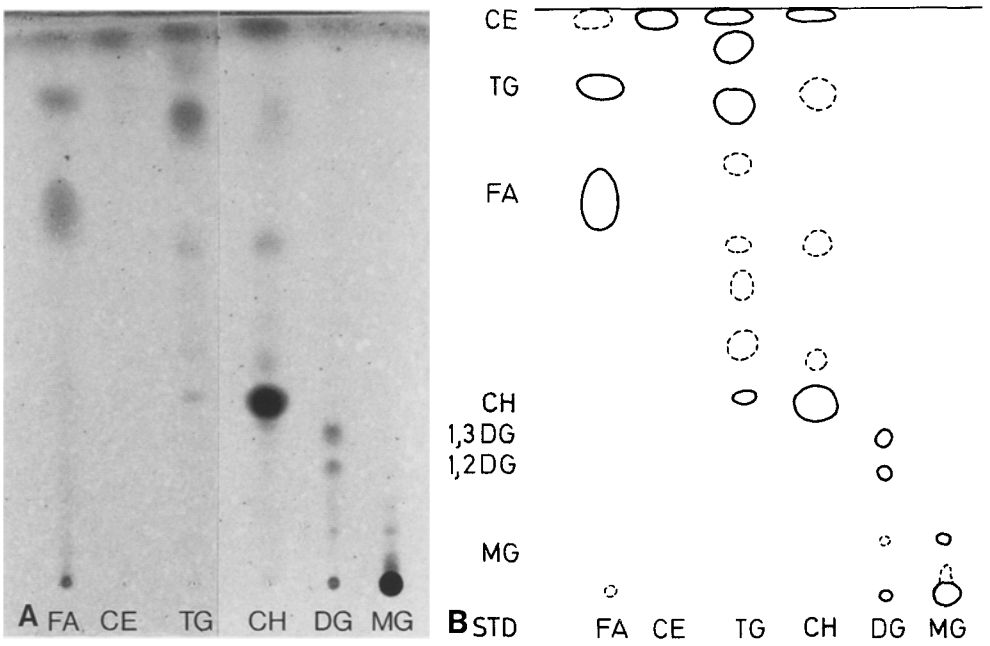

Fig. 4 A, B. Analysis of adult *Onchocerca gibsoni* neutral lipids by amino column chromatography. **A** Neutral lipid fractions were eluted from the amino columns with the following solvents: 2% acetic acid in diethyl ether (fatty acids, *FA*), hexane (cholesterol esters, *CE*), 1% diethyl ether/10% methylene chloride in hexane (triacylglycerols, *TG*), 5% ethyl acetate in hexane (cholesterol, *CH*), 15% ethyl acetate in hexane (diacylglycerols, *DG*), and 2:1 (v/v) chloroform/methanol (monoacylglycerols, *MG*). The thin-layer chromatogram was developed and visualized as indicated in Fig. 3. **B** Diagram with additional abbreviations indicating the migration of standards (*STD*) on the chromatogram relative to the *Onchocerca* neutral lipids

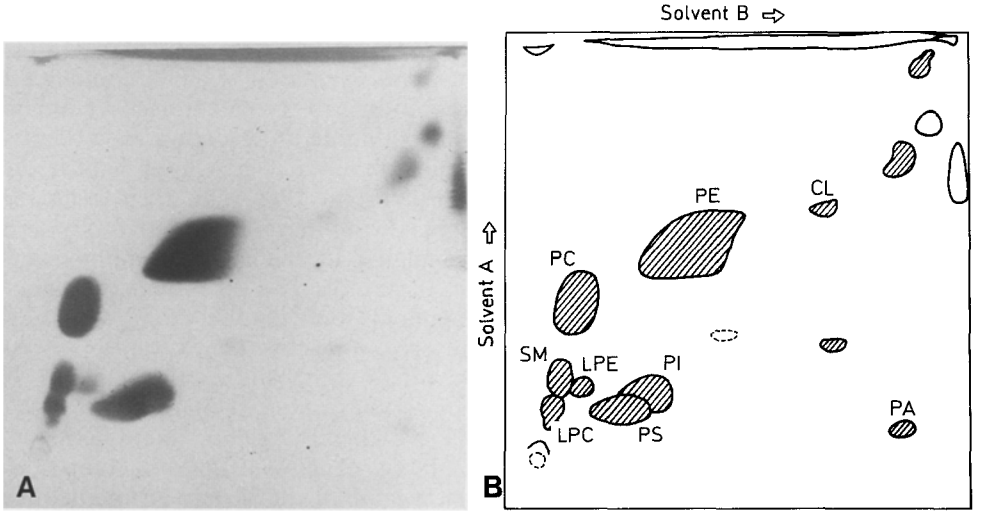

Fig. 5 A, B. Two-dimensional TLC analysis of adult *Onchocerca gibsoni* phospholipids. **A** Chromatogram of phospholipids developed first with solvent A (chloroform/methanol/ammonium hydroxide; 65:25:4, by vol.) and then, in the second direction, with solvent B (chloroform/acetone/methanol/acetic acid/water; 30:40:10:10:1, by vol.) and subsequently visualized by reaction with molybdenum blue reagent followed by charring. **B** Diagram of the chromatogram, indicating the identity of *O. gibsoni* phospholipids as determined by comigration with phospholipid standards (not shown). Phospholipids are indicated by *shading*. *PE*, Phosphatidylethanolamine; *PC*, phosphatidylcholine; *PS*, phosphatidylserine; *PI*, phosphatidylinositol; *SM*, sphingomyelin; *LPC*, lysophosphatidylcholine; *LPE*, lysophosphatidylethanolamine; *PA*, phosphatidic acid; *CL*, cardiolipin

the fact that parasitic helminths, unlike most animals and plants, appear to derive little, if any, energy from lipid oxidation (Barrett 1981; von Brand 1973). Diacylglycerols and, especially, monoacylglycerols were minor components.

Phosphatidylethanolamine and phosphatidylcholine were the two predominant phospholipids (Fig. 5). Phosphatidylserine and phosphatidylinositol were also major components, as were sphingomyelin and the lysophosphoglycerides (lysophosphatidylcholine and lysophosphatidylethanolamine), whereas phosphatidic acid and cardiolipin were detected only in trace amounts. The

neutral lipid and phospholipid composition of *O. gibsoni* resembles that previously reported for other parasitic helminths (Barrett 1981; von Brand 1973). Lysophosphoglycerides are typically present in animal tissues, including those of free-living helminths, in very low or trace amounts (<1% of total lipid; Barrett 1981). However, in *O. gibsoni* adults, as has been reported for other parasitic helminths (Barrett 1981; von Brand 1973), much larger amounts of these lipids were found. Because lysophosphoglycerides are intermediates in phosphoglyceride catabolism, this observation may reflect differences in the lipid metabolism of host and parasitic helminth tissues. Both lysophosphoglycerides and free fatty acids are disruptive to cell membranes (Corr et al. 1987; Golan et al. 1988; Gregson 1989; Kagan 1989; Ungemach 1989; Weltzien 1979) and could be involved in the pathology of onchocerciasis. Such a role has been associated with lysophosphatidylcholine released by schistosomula (Furlong and Caulfield 1989; Golan et al. 1986).

Lipids of *Onchocerca* species undoubtedly serve a variety of functions analogous to those required by host cells. However, unlike their vertebrate hosts, parasitic helminths typically lack a functional B-oxidation pathway for lipid catabolism, often possess high levels of free fatty acids and lysophosphoglycerides, are incapable of synthesizing sterols de novo, are severely limited in their ability to synthesize long-chain fatty acids de novo, and have in some instances been reported to use sterols as sex attractants (Barrett 1981; Haseeb and Fried 1988). Obviously, significant differences in lipid metabolism and function exist between host tissues and those of parasitic helminths. Therefore, *Onchocerca* lipids, their receptors, and the intermediates and enzymes of their metabolic pathways are good candidates to serve as targets for chemotherapeutic agents. *Onchocerca* lipids, especially those at the parasite surface, could have a role in vaccine development as well. Neutral glycolipids, gangliosides, and even certain phospholipids are targets of immune effectors in several disease systems (Costello and Green 1988; Feizi and Childs 1985; Hakomori and Kannagi 1983). Gangliosides have been shown to enhance the antigenic activity of membrane proteins by influencing their configurations (Watanabe et al. 1980) and could be important components of protein-liposome vaccine preparations.

Conversely, *Onchocerca* lipids could play a role in the host immune dysfunction that occurs following maturation of filarial parasites (Kwa and Mak 1987). Adult filarial worms have been reported to release lipid into surrounding host tissues, and macrophages in onchocercal nodules contain high levels of lipid droplets (Mac Kenzie et al. 1987). Glycosphingolipids and sphingolipid breakdown products as well as exogenous neutral lipids and phospholipids can depress the responsiveness of immune effectors (Hannun and Bell 1989; Traill and Wick 1984). Changes in lipid content during development have been associated with stages of other parasitic helminths becoming refractory to immune effectors (Billecocq 1987; Rumjanek and McLaren 1981) but are correlated in some parasitic nematode infections with the onset of protective host immune responses (Barrett 1981). Clearly, the possible influences of *Onchocerca* lipids on the course of infection should be taken into account in the design of strategies for the immunoprophylactic or chemotherapeutic control of onchocerciasis.

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