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The tegumental surface membranes of *Schistosoma mansoni* are enriched in parasite-specific phospholipid species

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ABSTRACT

The complex surface structure of adult *Schistosoma mansoni*, the tegument, is essential for survival of the parasite. This tegument is syncytial and is covered by two closely-apposed lipid bilayers that form the interactive surface with the host. In order to identify parasite-specific phospholipids present in the tegument, the species compositions of the major glycerophospholipid classes, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine and phosphatidylinositol, including lysophospholipid species, were analysed in adult *S. mansoni* worms, isolated tegumental membranes and hamster blood cells. It was shown that there are large differences in species composition in all four phospholipid classes between the membranes of *S. mansoni* and those of the host blood cells. The species compositions of phosphatidylserine and phosphatidylcholine were strikingly different in the tegument compared with the whole worm. The tegumental membranes are especially enriched in lysophospholipids, predominantly eicosenoic acid (20:1)-containing lyso-phosphatidylserine and lyso-phosphatidylethanolamine species. Furthermore, the tegument was strongly enriched in phosphatidylcholine that contained 5-octadecenoic acid, an unusual fatty acid that is not present in the host. As we have shown previously that lysophospholipids from schistosomes affect the parasite–host interaction, excretion of these tegument-specific phospholipid species was examined in vitro and in vivo. Our experiments demonstrated that these lysophospholipids are not significantly secreted during in vitro incubations and are not detectable in peripheral blood of infected hosts. However, these analyses demonstrated a substantial decrease in PI content of blood plasma from schistosome-infected hamsters, which might indicate that schistosomes influence exosome formation by the host.

1. Introduction

Schistosoma mansoni is a parasitic worm that causes schistosomiasis, a disease that afflicts over 200 million people and numerous animals in many rural areas in tropical countries (Gryseels, 2012). Adult *S. mansoni* worms are able to maintain themselves for many years in the blood vessels of their mammalian hosts (Gryseels et al., 2006). Despite their relatively large size (1 cm long with a diameter of 0.5 mm) compared with veins of their host, and their abundant exposure to immune cells present in the blood of the host, the parasite apparently prevents clot formation and an adequate immune response in its host (Pearce and MacDonald, 2002; Maizels, 2005; Mebius et al., 2013). Although the underlying molecular mechanisms involved in long-term parasite survival are not yet completely understood, the tegument surface structure of schistosomes is of crucial importance for parasite survival and modulation of the host response (Skelly and Wilson, 2006; Han et al., 2009).

The tegument of flatworms is a unique structure in nature that forms the interactive surface with the host. The tegument of schistosomes is syncytial and is covered by two closely-apposed lipid bilayers, constituting an inner plasma membrane and an outer membranocalyx (McLaren and Hockley, 1977; Brugger et al., 1997; Skelly and Wilson, 2006). Multiple studies have characterised the protein composition of these tegumental surface membranes and demonstrated that these membranes contain a specific set of proteins comprising nutrient transporters, structural membrane proteins, several proteins derived from the host and many

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proteins with yet unknown functions (van Balkom et al., 2005; Braschi et al., 2006a; Castro-Borges et al., 2011). The tegument of *S. mansoni* is also enriched in proteins that share no sequence similarity to any sequence present in databases of species other than schistosomes, demonstrating that the unique tegumental structures comprise multiple unique proteins that are likely to fulfil schistosome-specific functions involved in parasite survival (van Balkom et al., 2005; Braschi et al., 2006a; Skelly and Wilson, 2006).

Apart from proteins, the membranes of the tegument contain many distinct lipids and it was shown that eicosenoic acid (20:1), a rare species in the mammalian host, is a major component of the phospholipids of the tegumental membrane (Allan et al., 1987). The few early studies on the lipid compositions of the tegumental membranes provided little information on species compositions and were restricted by the limited technical possibilities at the time (Allan et al., 1987; Rogers and McLaren, 1987). More recent studies on tegumental membranes of schistosomes using MS techniques showed that the species composition of the phospholipids in the tegumental membranes differ not only substantially from those of blood cells of the host but also from those of whole adult worms (Brouwers et al., 1998b; van Hellemond et al., 2006). To date, the diacyl-phospholipid species composition of the two most abundant phospholipid classes in membranes of the tegument of schistosomes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE), has been characterised (Brouwers et al., 1998b). However, the species compositions of the less abundant phospholipid classes such as phosphatidylserine (PS) and phosphatidylinositol (PI) have not yet been characterised in the tegument. These phospholipids are of interest as they are known to function as precursors for the production of several potent signalling molecules. Furthermore, schistosome lyso-PS (monoa cyl-phosphatidylserine) species have been shown to activate Toll-Like Receptor 2 (TLR2) and affect dendritic cells of the host in such a manner that mature dendritic cells induce the development of IL-10 producing regulatory T cells, a process known to result in down-regulation of the immune response (van der Kleij et al., 2002). The present study provides a comprehensive analysis of the species compositions of all major glycerophospholipid classes, including PS, PI and lysophospholipids, in isolated membranes of whole adult *S. mansoni* worms, isolated tegumental membranes and membranes of host blood cells. In order to identify parasite-specific lipids, the phospholipid species compositions in whole adult worms were compared with those of host blood cells. Furthermore, to identify phospholipid species enriched in the tegumental membranes, the phospholipid species compositions of tegumental membranes were compared with those of whole adult worms. Finally, the possible excretion into the environment of schistosome-specific phospholipids enriched in the tegumental membranes was investigated both in vitro and in infected hamsters.

2. Materials and methods

2.1. Chemicals and materials

All solvents used were from Lab-Scan (Dublin, Ireland) and were of HPLC grade. Ammonium acetate, serine and Silica-60 for column chromatography were purchased from Merck (Darmstadt, Germany). Hydrochloric acid was purchased from Baker (St. Louis, MO, USA) and CM-52 was obtained from Serva (Heidelberg, Germany).

2.2. Isolation of tegument, membranes of host blood cells and lipids in host blood

Hamsters were infected with *S. mansoni* by bathing six hamsters for 1 h in a 1 cm deep layer of water containing 600 cercariae per hamster. Adult *S. mansoni* parasites were obtained by perfusion via the heart with 0.9% (w/v) NaCl of ether- or isofluorane-anaesthetised hamsters 45–49 days p.i., yielding approximately 100 adult worm pairs per hamster. Infections of hamsters and parasite isolation procedures were approved by Erasmus University, Rotterdam, The Netherlands (licence EUR1860-11709). Approximately 80–90% of the adult schistosomes were paired couples, with 10–20% single males and hardly any single females. Isolated adult *S. mansoni* worms were washed in Hanks Balanced Salt Solution (HBSS) (Sambrook et al., 1989) before tegumental membranes were isolated by a freeze–thaw method according to Roberts et al. (1983) as described by Brouwers et al. (1999). Briefly, adult worms in HBSS were plunged, drop by drop, into liquid nitrogen. After thawing on ice, worms were extensively washed with ice-cold Tris-buffered saline (20 mM Tris–HCl, pH 7.4, 0.9% (w/v) NaCl, 1 mM of the protease inhibitor phenylmethylsulfonyl fluoride). The tegumental membrane complex was removed from the worms by applying 10 vortex pulses of 1 s. The supernatant, containing the tegumental membranes, was passed over a fine stainless-steel mesh and the filtrate was centrifuged at 5000*g* for 30 min at 4 °C. The isolated tegumental membrane pellets were frozen at −20 °C until further use. It should be understood that the vast majority of the tegumental membranes will be derived from males, due to the size difference and the masking of the surface of females by their male partners.

Blood was drawn from anaesthetised hamsters by heart punctures. To determine the phospholipid composition of host blood cells, blood from non-infected hamsters was diluted (1:5, v/v) in water in order to lyse the red blood cells by hypotonic shock, shortly before lipid extraction. To analyse the phospholipids possibly excreted by schistosomes while living in the blood of the host, blood plasma from infected hamsters was prepared by centrifugation for 10 min at 1200*g* of citrated blood collected 45–48 days p.i. with *S. mansoni*. As a control, plasma from non-infected hamsters was obtained by the same procedure.

2.3. Incubation of adult worms and lipid extraction from culture supernatant and hamster blood plasma

Adult *S. mansoni* worms were isolated by perfusion with warm (37 °C) 0.9% (w/v) NaCl and were then incubated for 2 h in S100 medium, which contained 20 mM Hepes (pH 7.4), 85 mM NaCl, 5.4 mM KCl, 0.7 mM Na_2HPO_4, 1 mM $MgSO_4$, 1.5 mM $CaCl_2$, 25 mM $NaHCO_3$, 100 mM glucose and 20% heat-inactivated human serum. After incubation, the adult worms were removed from the medium and ice-cold trichloric acid (TCA) was added to the culture supernatant after which the proteins were allowed to precipitate on ice for 30 min. The culture supernatant was subsequently spun down at 18,000*g* for 10 min. Lipids from hamster blood plasma and from incubation media were extracted according to the method of Bligh and Dyer (1959).

2.4. Lipid extraction

Lipids from schistosomal fractions and whole blood were extracted from the biological samples according to the method of Bligh and Dyer (1959), with the minor modification that 6 M HCl (0.1% v/v) were added to the second chloroform wash to increase recovery of acidic phospholipids. Neutral lipids were removed from the extracted lipids by fractionation on a 3 ml silica column prepared from 0.063 to 0.200 mm Silica 60. Lipid extracts were dissolved in chloroform and loaded on top of the silica column. Subsequently, neutral lipids were eluted with acetone (four volumes) after which phospholipids and free fatty acids were eluted with methanol (four volumes). This latter fraction was dried under

nitrogen and stored at −20 °C until HPLC–MS analysis of the phospholipids.

2.5. Quantification and identification of phospholipid species composition

Isolated phospholipid samples were analysed by an HPLC–MS method as previously described (Retra et al., 2008a). In short, phospholipids were separated by HPLC, using a Synergi 4 μm MAX-RP 18A column (250 × 3 mm) (Phenomenex, CA, USA). Elution was performed within 55 min with a decreasing linear gradient of water in methanol/acetonitrile, with serine and ammonium acetate used as additives. Mass spectrometry was performed using electrospray ionisation on a Sciex 4000QTRAP (Applied Biosystems, Nieuwerkerk aan de IJssel, the Netherlands). Samples were analysed in a multiple reaction mode (MRM) or by neutral loss scanning or precursor scanning. Data analysis was performed according to the manufacturers' protocols with Markerview version 1.0 software (Sciex, Toronto, Canada).

3. Results

This study aimed to provide a comprehensive characterisation of the glycerophospholipid and lysophospholipid species compositions of the tegumental membranes of adult *S. mansoni* worms, in order to identify parasite and tegument-specific phospholipids that might be involved in host–parasite interactions. We used a universal HPLC–MS method for the identification of the molecular species of the four most abundant phospholipid classes (PC, PE, PS and PI) in homogenates of whole adult schistosomes, isolated tegumental membranes of adult schistosomes and blood cells of the host. The applied method separates isobaric species of all phospholipid classes prior to identification and quantification by MS and, therefore, allows detection and relative quantification of phospholipid species including lysophospholipids (Retra et al., 2008a).

Tegumental membranes were isolated by a well-established freeze–thaw method originally developed by Roberts et al. (1983). A modified version of this method has been used in our laboratory to characterise the proteome and the species compositions of the phospholipids PE and PC in the tegumental membranes of adult schistosomes (Brouwers et al., 1998a,b, 1999; van Balkom et al., 2005).

Results from the current analysis of the species compositions of the analysed phospholipids in membranes of whole schistosomes (Table 1 and Supplementary Tables S1–S3) are in general in agreement with our earlier study and the most abundant species of PS, PE, PC and PI in schistosomes were again found to be PS 18:0/22:4, PE 18:0/22:4, PC 16:0/20:1 and PI 18:0/22:4 (Retra et al., 2008a). The present study did not focus on ether-linked PE species because the positive ion mode now used is less suitable

Table 1
Molecular species of diacyl-phosphatidylserine. Molecular species were determined in homogenates of whole adult *Schistosoma mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood cells. Phosphatidylserine species were quantified by multiple reaction mode with $(M-H)^-$ for MS1 and $(M-H-87)^-$ for MS2. Percentages shown are the average percentages of the total phosphatidylserine species ± S.D., of species comprising more than 1% of the total phosphatidylserine. The three most abundant species in each membrane preparation are printed in bold and species that comprise more than 2% of the total phosphatidylserine and are enriched at least twofold in tegument compared with lipids in whole worms are underlined in red. Species marked with an asterisk (*) comprise less than 1% of the total phosphatidylserine in that membrane preparation.

m/z	Rt (min)	Acyl chains	Component	Whole worms ($n = 5$) % ± S.D.	Tegument ($n = 3$) % ± S.D.	Hamster blood cells ($n = 5$) % ± S.D.
760	24.7	16:0/18:1	Diacyl PS	*	*	1.0 ± 0.1
772	29.7	16:0/20:1	Plasmalogen PS	1.9 ± 0.2	5.2 ± 1.4	*
782	21.7	16:0/20:4	Diacyl PS	*	*	1.8 ± 0.2
784	22.7	18:1/18:2	Diacyl PS	*	*	1.3 ± 0.1
786	25.1	18:0/18:2	Diacyl PS	2.6 ± 0.2	1.5 ± 0.1	2.5 ± 0.1
788	28.5	18:0/18:1	Diacyl PS	5.3 ± 0.5	3.2 ± 0.5	2.4 ± 0.3
796	22.8	17:0/20:4	Diacyl PS	*	*	1.9 ± 0.1
798	31.4	[a]		1.6 ± 0.2	3.8 ± 1.2	*
800	35.8	18:0/20:1[b]	Plasmalogen PS	3.6 ± 0.6	10.1 ± 2.9	*
802	27.6	[a]		*	2.0 ± 0.7	*
804	29.6	38 :0 $	Alkyl, acyl PS	*	7.1 ± 0.7	*
806	19.9	18:2/20:4	Diacyl PS	*	*	2.4 ± 0.2
808	22.0	18:1 / 20:4	diacyl PS	*	*	14.8 ± 0.6
810	24.1	18:0/20:4	Diacyl PS	5.4 ± 0.3	2.3 ± 0.3	36.0 ± 2.7
812	25.6	18:0/20:3	Diacyl PS	2.0 ± 0.1	*	4.8 ± 0.3
814	28.1	18:0 / 20:2	Diacyl PS	1.9 ± 0.1	1.3 ± 0.2	*
816	32.1	18:0/20:1	Diacyl PS	3.5 ± 0.4	5.4 ± 0.3	*
818	32.1	18:0/20:0	Diacyl PS	*	1.8 ± 0.1	*
822	29.0	[a]		1.3 ± 0.2	1.4 ± 0.2	*
824	25.3	19:0/20:4	Diacyl PS	*	*	1.1 ± 0.1
830	19.2	20:4/20:4	Diacyl PS	*	*	4.4 ± 0.5
830	29.8	[a]		*	4.1 ± 0.5	*
832	22.0	40:7	Diacyl PS	*	*	3.0 ± 0.1
832	35.0	40:0	Alkyl, acyl PS	*	9.7 ± 1.4	*
834	22.0	18:1/20:5	Diacyl PS	*	*	1.9 ± 0.1
834	23.2	18:0/22:6	Diacyl PS	7.6 ± 1.3	4.2 ± 0.2	3.5 ± 0.3
836	24.1	18:1/22:4	diacyl PS	*	*	1.9 ± 0.1
836	25.2	18:0/22:5	Diacyl PS	5.2 ± 0.5	3.0 ± 0.9	1.2 ± 0.1
838	26.1	18:0/22:4	Diacyl PS	34.3 ± 1.8	16.5 ± 2.2	2.8 ± 0.2
840	28.9	[a]		5.3 ± 0.2	2.6 ± 0.4	*
854	27.9	[a]		*	1.6 ± 0.5	*
864	27.0	[a]		1.7 ± 0.2	*	*
866	29.4	20:0/22:4	Diacyl PS	1.1 ± 0.1	*	*
866	30.0	18:0/24:4	Diacyl PS	1.4 ± 0.1	*	*

[a] Not identified.
[b] Tentative identification based on *m/z*, retention time (Rt) and head group fragmentation as full product spectra could not be obtained.

for detection of those PE phospholipids (Brugger et al., 1997). Therefore, this study could not confirm the previously observed abundant presence of ether-linked PE species in schistosomal membranes nor the specific enrichment of ether-linked PE species in tegumental membranes, such as 16:0/20:1 plasmalogen-PE (Brouwers et al., 1998b).

3.1. PS species analysis

The HPLC-elution pattern of PS species derived from tegumental membranes differed drastically from that of membranes of whole worms and that of membranes from blood cells, as the tegumental membranes clearly contain much more lyso-PS and etherlipid-PS (Fig. 1B). Identification and quantification of the PS species demonstrated that schistosome membranes comprise many distinct PS species which are hardly present in the membranes of blood cells, such as PS 18:0/22:4, PS 18:0/20:1 and several etherlipids (Table 1). PS species containing a fatty acid of 22 or more carbon atoms were abundantly present in schistosome membranes compared with membranes of blood cells, 57% versus 9%, respectively (Table 1). On the other hand, species with 22 or more carbon atoms are much less present in the tegument compared with membranes of whole worms and comprise only 28% of the PS species in the tegument (Table 1).

In order to identify the PS species most different in abundance between membranes of adult worms, the tegument and blood cells, a discriminant analysis was performed. This statistical method identifies the components that differ most between groups of samples and thus contribute most to the distinction between the samples. The discriminant analysis identified lyso-PS 20:1 ($m/z = 550$), lyso-PS 22:4 ($m/z = 572$) and the PS species with an m/z ratio of 772, 800, 804, 830 and 832 as the PS species most abundant in the tegumental membranes (Supplementary Fig. S1). At least four of these five PS species contain ether-linked acyl chains (Table 1). Therefore, the PS species composition in the tegumental membranes is specifically enriched in ether-linked PS species (Fig. 1), which is similar to the already reported enrichment of ether-linked PE species in the tegument (Brouwers et al., 1998b).

3.2. PE species analysis

Analysis of the species compositions of PE showed striking differences between PE in blood cells and in lipids of schistosomes (Supplementary Table S1). The three most abundant species in PE of hamster blood cells (18:1/20:4, 18:1/18:2 and 16:0/18:1) are not even amongst the top 10 of the most abundant PE species in worms. The reverse is also true: the most abundant PE species in schistosomes (18:0/22:4, 18%) ranks as number 15 in PE species of hamster blood cells and is only present in trace amounts (1.2%).

In contrast to the large difference in PS species composition between whole worms and the tegument, there were no significant differences observed in PE species of the tegument compared with the whole worm (Supplementary Table S1).

3.3. PC species analysis

The species composition of PC in schistosomes was clearly different from that of hamster blood cells (Supplementary Table S2). For example, the number one in PC of the worms with 14% was 16:0/20:1, while that species represented less than 1% of the total PC in hamster blood cells. The most abundant PC species in hamster blood cells was 16:0/18:2 (20%), while this species ranked fifth and represented only 5% of the total PC species in the worms.

Also striking, however, are the differences in PC species composition between worms and their tegumental membranes. The tegument is twofold enriched in 16:0/16:0 diacyl-PC, the most abundant PC species in these membranes (Supplementary Table S2). The most remarkable difference between tegument and worms is the gross enrichment in 16:0/18:1 PC, where the 18:1 fatty acid is very unusual, as we showed earlier that it contains the double bond at C_5 instead of the common C_9 position (Brouwers et al., 1998c). In tegumental membranes this 16:0/18:1 $\Delta 5$ PC represented 12% of the total PC, while worm PC contained only 2%. This is in agreement with our earlier study where we showed that this rare PC species is not detectable in blood of the host and is, apart from dipalmitoyl PC, the most abundant PC species in the tegument of schistosomes (Brouwers et al., 1998c).

3.4. PI species analysis

Analysis of the species composition of PI revealed that large differences also existed between the PI species composition of blood cells and that of the schistosome membranes (Supplementary Table S3). Blood cells contain predominantly PI 18:0/20:4 and PI 18:0/18:2, together representing half of the PI species in hamster blood cells. On the other hand, membranes of whole worms as well as tegumental membranes contained, in addition to PI 18:0/20:4, a large amount of PI 18:0/18:1(21% and 27% in worms and tegument, respectively), which in hamster blood cells represents only 2% of the total PI species (Supplementary Table S3).

Compared with the differences in species compositions of PC and PS, the differences we observed in PI species composition were remarkably small between whole worms and tegumental membranes. PI species are well known to generate potent signalling molecules, but the absence of specific enrichment of certain PI species in the tegument suggests that specific PI species are not involved in tegument-specific functions.

3.5. Analysis of lysophospholipids

Apart from the analysis of the diacyl-phospholipid species, we also investigated the presence of monoacyl-phospholipid (lyso-PL) species of the four above-mentioned phospholipid classes, as it had been reported that lyso-PC as well as lyso-PS species are important in host–parasite interactions in schistosomiasis (Furlong and Caulfield, 1989; van der Kleij et al., 2002; Magalhaes et al., 2010). Lysophospholipids and diacyl-phospholipids do not ionise with the same efficiency and therefore these phospholipids are not detected with the same efficiency by MS. For this reason, the molar ratio between lysophospholipids and diacyl-phosp holipids cannot be estimated by MS unless careful calibration is performed with a large array of standards which are not commercially available. However, in order to determine whether one of the three investigated membrane fractions contains a relatively large proportion of lysophospholipid species, the ratio of the detected signals of lysophospholipid species over that of diacyl-phosphol ipid species can be compared between these fractions. Table 2 shows the ratio of detected signals derived from lysophospholipid species to that of diacyl-phospholipids for each phospholipid class in the three analysed membrane preparations. These results are in line with earlier observations that the schistosomula of *S. mansoni* can produce lyso-PC (Golan et al., 1986), and we now show that in adult schistosomes the tegumental membranes, compared with whole worms, are enriched in lysophospholipid species, especially lyso-PS and lyso-PE.

Analysis of the lysophospholipid species of the distinct phospholipid classes demonstrated that in blood cells the total acyl-chain length and number of desaturations of the minor amounts of lyso-PC, lyso-PE and lyso-PS were very similar to each other, because over 75% of the lysophospholipid species in these classes comprised acyl chains with 16 or 18 carbon atoms and

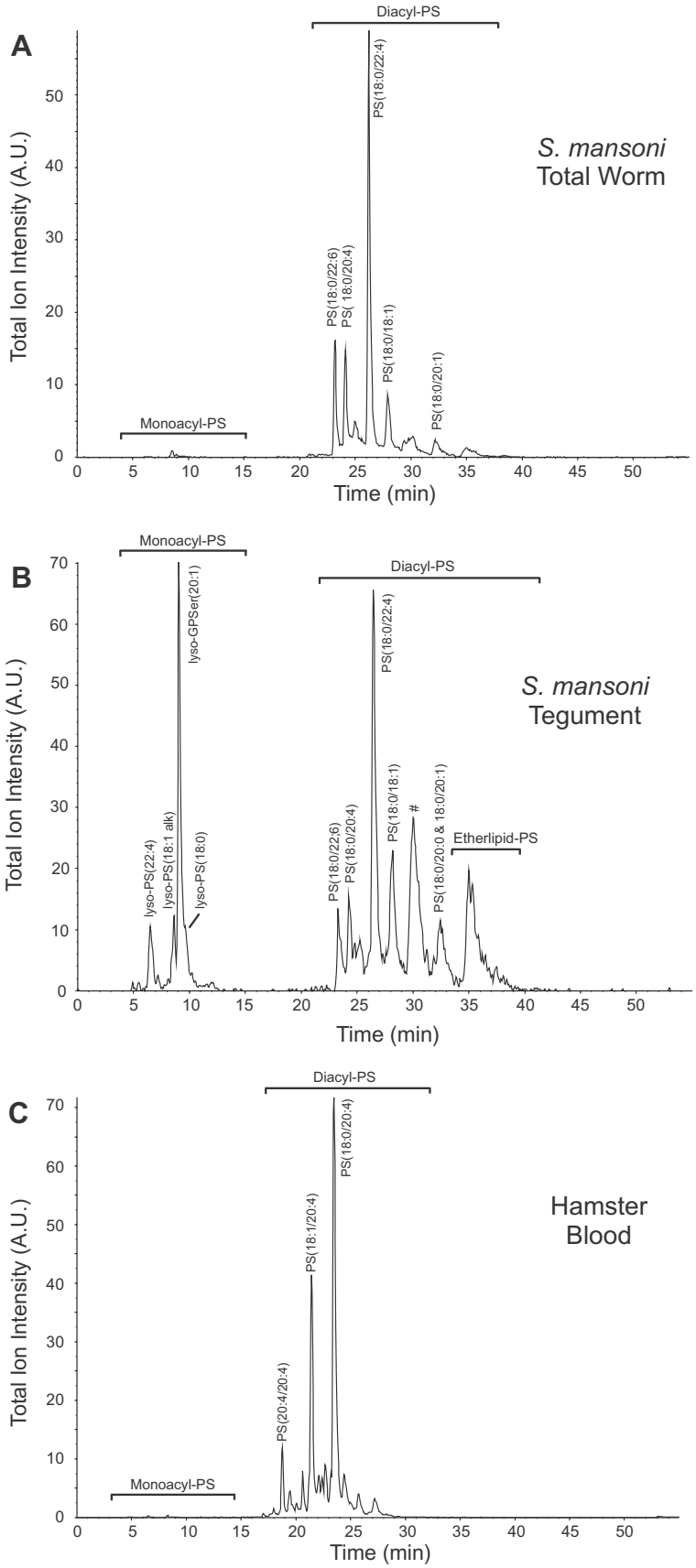

Fig. 1. Analysis of the phosphatidylserine species composition by HPLC–MS. Molecular species were determined in lipid extracts of homogenates of whole *Schistosoma mansoni* worms (A), isolated tegumental membranes from adult *S. mansoni* worms (B) and hamster blood cells (C). Phosphatidylserine species were detected and quantified by multiple reaction monitoring using $(M-H)^-$ for MS1 and $(M\text{-}H\text{-}87)^-$ for MS2. Shown is the total ion intensity in arbitrary units (A.U.) during elution. Peak labels correspond to those in Table 1.

Table 2
Ratio of lysophospholipids to diacyl-phospholipids. Molecular species were determined and quantified as described in the Section 2.5 in homogenates of adult *Schistosoma mansoni* worms, in isolated tegumental membranes from adult *S. mansoni* worms and in hamster blood cells. Ratios shown are the average ratios of all detected lysophospholipid species to that of all diacyl-phospholipids species ± S.D. of each phospholipid class in arbitrary units where the ratio in whole worms was set at 1.0.

	Whole worms ratio ± S.D. (n = 5)	Tegument ratio ± S.D. (n = 4)	Hamster blood cells ratio ± S.D. (n = 4)
Lyso-PS/diacyl-PS	1.0 ± 0.2	15.8 ± 4.0	2.3 ± 2.9
Lyso-PE/diacyl-PE	1.0 ± 0.3	7.2 ± 7.1	1.3 ± 0.4
Lyso-PC/diacyl-PC	1.0 ± 0.4	1.6 ± 0.9	1.4 ± 0.5
Lyso-PI/diacyl-PI	1.0 ± 0.1	3.4 ± 0.2	0.2 ± 0.1

PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; PI, phosphatidylinositol.

none or a single desaturation (not shown). The lyso-PI species composition in blood cells also contained a large amount of lyso-PI 20:4 in addition to the lyso-PI species with acyl chains of 16 and 18 carbon atoms (not shown). On the other hand, membranes of adult *S. mansoni* worms had a lyso-phospholipid species composition with species containing unsaturated acyl chains of 16 or 18 carbon atoms and substantial amounts (over 20%) of lyso-PS 20:1 and lyso-PE 20:1 (Table 3). Interestingly, these two lysophospholipid species were drastically enriched in the tegumental membranes compared with whole worms, and these species were by far the most abundant lyso-PS and lyso-PE species present in the tegumental membranes (Table 3). Eicosenoic acid (20:1) is synthesized by the parasite by chain elongation of oleic acid (18:1) and it is an abundantly present fatty acid in schistosomes while virtually absent in the host (Fripp et al., 1976; Brouwers et al., 1997, 1998a). The enrichment in the tegument of eicosenoic acid containing lyso-PS and lyso-PE is not observed in lyso-PC and lyso-PI, and the species composition of these lysophospholipid classes is dominated by species containing unsaturated acyl chains of 16 or 18 carbon atoms in lyso-PC and lyso-PI, respectively (Table 3). Therefore, no major differences exist in the lysophospholipid species composition of lyso-PC and lyso-PI between tegumental membranes and those whole worms, whereas the tegumental membranes are drastically enriched in lyso-PE and lyso-PS species containing eicosenoic acid (20:1).

3.6. Analysis of possible excretion of lysophospholipids by schistosomes

Due to the increased water solubility of lysophospholipids compared with diacyl-phospholipids, the specific enrichment of lysophospholipids in the tegumental membranes that form the outer surface of the parasite, and due to the suggested function of lysophospholipids in parasite–host interactions (van der Kleij et al., 2002; Retra et al., 2008b; Magalhaes et al., 2010), we investigated whether these lysophospholipids are excreted into their environment. Lysophospholipid secretion by schistosomes was examined in vitro by total phospholipid analysis of medium incubated with or without adult schistosomes. None of the schistosome-specific lysophospholipid or phospholipid species appeared to be present in the medium incubated with schistosomes, and no significant differences in species composition of PC, PE, PS and PI were detected between medium incubated with schistosomes and control medium (not shown).

The possible excretion of tegument-specific lysophospholipids was also investigated in vivo by analysis of total phospholipids present in blood plasma of infected and non-infected hamsters. Again, none of the schistosome-specific or tegument-specific lysophospholipids or diacyl-phospholipid species was increased in plasma derived from schistosome-infected hamsters compared with non-infected hamsters. These results demonstrate that the tegument-specific lysophospholipids are not significantly secreted into aqueous solutions with albumin and do not circulate in the blood of infected hosts. However, a substantial decrease in total PI content was detected in plasma from infected hamsters compared with non-infected hamsters (Table 4). All five of these PI species are common and abundant diacyl PI species in host blood cells (Supplementary Table S3).

4. Discussion

The tegumental surface membranes of adult schistosomes form a unique biological structure which is the site of interaction with the host (McLaren and Hockley, 1977). The tegumental surface membranes were shown to contain many schistosome-specific proteins and proteins important in host–parasite interactions (van Balkom et al., 2005; Braschi et al., 2006b; Skelly and Wilson, 2006). The tegumental surface membranes also comprise many schistosome-specific and tegument-specific lipids (Brouwers et al., 1998a,b,c). Since potent bio-active lipids interfering with the host–parasite interactions have been isolated from adult schistosomes (van der Kleij et al., 2002; Magalhaes et al., 2010), we performed a comprehensive study to identify schistosome-specific, as well as tegument-enriched, phospholipids.

In addition to the already reported tegument-specific enrichment of several PC and ether-linked PE species compared with whole worms (Brouwers et al., 1998a,b,c), our results showed that multiple PS species are also specifically enriched in the tegument. On the other hand, no tegument-specific PI species could be detected. The species composition of the most abundant phospholipid classes (PC and PS) in tegumental membranes differs drastically from that of whole worms. However, this phenomenon is not true for all phospholipid classes, as the species compositions of PI and PE in tegumental membranes did not differ from those of whole worms. Furthermore, the enrichment in the tegument of eicosenoic acid (20:1) containing lysophospholipids was only observed for PS and PE and not for PC and PI. These results, therefore, suggest that the tegument comprises mechanisms that facilitate the enrichment of certain phospholipids in the membranes of the tegument. The function of most of the identified tegument-specific phospholipids and lysophospholipids is not yet known, but the lyso-PS and lyso-PC species of schistosomes have been shown to activate TLR-2 on host immune cells, resulting in a down-regulation of the host immune response and eosinophil activation and recruitment, respectively (van der Kleij et al., 2002; Retra et al., 2008b; Magalhaes et al., 2010).

Interestingly, the various phospholipid species enriched in the tegument of the distinct phospholipids classes do not contain a similar set of acyl chains, as different species for PC and PS are enriched in the tegument (Table 1 and Supplementary Table S2). Hence, the tegumental membranes are enriched in specific phospholipid species that differ between the distinct phospholipid classes. It should be understood that the lipids were isolated from total membranes and it is not yet known to what extent these lipids are exposed to the host. It cannot be concluded a priori that these lipids play a significant role in host–parasite interactions, as at least some of them might only be involved in internal biochemistry of the worms.

Earlier observations on a different life cycle stage, schistosomula, clearly demonstrated that this stage exports homemade lysophospholipids (Furlong and Caulfield, 1989). It is not yet known whether adult schistosomes excrete the schistosome-specific and/or tegument-specific phospholipids. Detection of the possible excretion of these lipids is hampered by the biophysical properties of the lipids in question, as they contain relatively hydrophobic long-chain fatty acids and

Table 3
Molecular species of lysophospholipids. Molecular species were determined in lipid extracts of homogenates of whole *Schistosoma mansoni* worms and isolated tegumental membranes from *S. mansoni* worms. Lyso-phosphatidylserine species were quantified by multiple reaction mode with $(M-H)^-$ for MS1 and $(M-H\text{-}87)^-$ for MS2. Lyso-phosphatidylethanolamine species are quantified by multiple reaction mode with $(M+H)^+$ for MS1 and $(M+H\text{-}141)^+$ for MS2. Lyso-phosphatidylcholine species were quantified by multiple reaction mode with $(M+H)^+$ for MS1 and *m/z* 184 for MS2. Lyso-phosphatidylinositol species were quantified by multiple reaction mode with $(M-H)^-$ for MS1 and *m/z* 241 for MS2. Percentages shown are the average percentages of the total detection of lysophospholipid species ± S.D., of species comprising more than 1% of the total lysophospholipids. Species marked with an asterisk (*) comprise less than 1.0% of the total lysophospholipids in that membrane preparation. Species containing an ether linkage are indicated by 'alk' and the most abundant lysophospholipid species in each fraction is printed in bold. Species that are enriched at least twofold in tegument compared with whole worms are underlined in red.

	Lyso-PS (% ± S.D.)			Lyso-PE (% ± S.D.)			Lyso-PC (% ± S.D.)			Lyso-PI (% ± S.D.)		
Fatty acyl	*m/z*	Worms (*n* = 5)	Tegument (*n* = 5)	*m/z*	Worms (*n* = 5)	Tegument (*n* = 5)	*m/z*	Worms (*n* = 5)	Tegument (*n* = 5)	*m/z*	Worms (*n* = 5)	Tegument (*n* = 5)
16:0	496	1.5 ± 0.3	2.5 ± 1.1	454	7.1 ± 4.7	7.7 ± 3.3	496	**38.4 ± 4.0**	**47.8 ± 3.1**	571	1.8 ± 0.9	5.6 ± 1.1
18:1 alk	508	7.6 ± 2.3	4.1 ± 3.8	466	*	*	508	*	*	583	*	*
18:0 alk	510	2.1 ± 0.6	*	468	*	*	510	*	*	585	5.3 ± 0.6	2.6 ± 1.1
18:2	520	*	*	478	*	*	520	2.4 ± 1.0	2.6 ± 1.8	595	*	4.5 ± 0.8
18:1	522	1.3 ± 0.7	1.5 ± 0.4	480	8.3 ± 4.2	11.5 ± 2.2	522	9.1 ± 1.7	11.4 ± 3.1	597	3.2 ± 2.0	11.7 ± 3.6
18:0	524	**46.2 ± 4.4**	9.3 ± 4.2	482	**63.1 ± 8.5**	19.8 ± 9.5	524	27.5 ± 2.2	23.9 ± 5.9	599	**80.2 ± 4.3**	**62.8 ± 4.8**
20:4	544	*	*	502	*	*	544	2.6 ± 0.3	*	619	1.3 ± 0.4	3.1 ± 1.0
20:2	548	*	*	506	*	*	548	3.6 ± 1.2	3.1 ± 1.3	623	*	1.5 ± 0.9
20:1	550	24.0 ± 4.5	**62.6 ± 7.2**	508	21.6 ± 10.4	**57.1 ± 8.1**	550	9.0 ± 1.1	8.0 ± 3.6	625	*	2.0 ± 0.9
20:0	552	2.1 ± 0.4	2.6 ± 0.4	510	*	1.4 ± 2.2	552	2.3 ± 0.3	*	627	*	*
22:4	572	8.1 ± 1.4	10.1 ± 0.7	530	*	*	572	1.3 ± 0.7	*	647	*	*
22:1	578	*	1.3 ± 0.7	536	*	*	578	*	*	653	*	*
24:1	606	1.3 ± 0.2	*	564	*	*	606	*	*	681	5.2 ± 1.4	4.4 ± 1.2

Table 4
Phospholipid species with a significantly different abundance in blood plasma of *Schistosoma mansoni*-infected versus non-infected control hamsters. Molecular species of phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine and phosphatidylinositol were determined in blood plasma of hamsters infected with *S. mansoni* or non-infected control hamsters. Species were quantified by multiple reaction mode as described in Section 2.5 and the legends of Table 1 and Supplementary Tables S1–S3. The five phospholipid species are shown that differ most significantly, at least twofold in abundance in blood plasma of infected hamsters compared with non-infected control hamsters, and represent at least 0.4% of the total phospholipid signal.

m/z	PL class	Acyl chains	Ratio infected/control	*P* value
833	PI	34:2	0.35	$1.17\ 10^{-7}$
861	PI	36:2	0.37	$5.46\ 10^{-6}$
857	PI	36:4	0.38	$1.21\ 10^{-6}$
887	PI	38:3	0.40	$4.96\ 10^{-5}$
885	PI	38:4	0.42	$2.61\ 10^{-6}$

lysophospholipids that can be expected to partition quickly into nearby phospholipid membranes. In vivo, the tegument-specific lysophospholipids might spread quickly to nearby membrane structures, which would then allow receptor activation of host immune cells as reported in in vitro studies (van der Kleij et al., 2002; Retra et al., 2008b; Magalhaes et al., 2010). Knowing this, we nevertheless searched specifically for excretions of those schistosome-specific lipids by adult schistosomes. We could not, however, detect any significant secretion of these lipids by schistosomes, not during our in vitro incubations nor in the blood of infected hamsters. As adult worms do not extensively regurgitate their abdominal content during in vitro culturing (Planchart et al., 2007), it cannot be excluded that in real life specific phospholipids are usually excreted in the "vomit" of the adult worms.

Therefore, the in vivo mechanism by which these lysophospholipids might fulfil the effects observed in vitro on host immune cells remains to be resolved and probably requires sophisticated co-culture studies that are beyond the scope of this study. However, analysis of the phospholipid composition in blood plasma of infected and non-infected hamsters demonstrated another interesting difference, as a substantial decrease in PI content was observed in blood plasma from schistosome-infected hamsters. As phospholipids are not bound to albumin, because this protein binds predominantly fatty acids, these phospholipids probably constitute small vesicular structures such as exosomes. The substantial decrease in PI is peculiar, as this phospholipid is well known for its precursor function in signal transduction. Therefore, these results might indicate that the schistosome infection alters exosome formation by endothelial cells or by circulating immune cells. However, it is not yet known whether these differences are directly induced by the schistosomes, or the result of immunological reactions of the host and/or schistosome-induced pathology.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijpara.2015.03.011.

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