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RESEARCH ARTICLE

Ex vivo study of the importance of sphingolipid in *Leishmania* amastigote

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Abstract

Leishmania is the causative agent of leishmaniasis, a widely distributed disease. Amastigote forms of *Leishmania* are intracellular and reside within the macrophage of the vertebrate host. Previous studies showed that certain *Leishmania* species may scavenge host factors for survival, specially sphingolipids, the key element of the eukaryotic membranes. In this study we have investigated the survival of new world *L. mexicana* amastigotes in murine macrophage cell-line in the presence and absence of foetal bovine serum (FBS). Results showed that there was no significance in the infectivity of amastigotes and also the number of parasite per cell; such findings suggest that *L. mexicana* amastigotes have its own pathway of sphingolipid intake and can survive and infect the macrophage with no serum added. This differs from previous studies on the old world *Leishmania major* which has been found to scavenge host sphingolipid for surviving and infectivity.

Introduction:

The protozoan parasite *Leishmania* undergoes two distinct developmental stages and hosts during its life cycle, promastigotes that replicate in the mid-gut of sand flies, and amastigotes that reside within the phagolysosomes of vertebrate macrophages (Zahng, 2005).Sphingolipids are structural components of the eukaryotic plasma membrane that are involved, together with cholesterol, in the formation of lipid rafts; additionally, sphingolipid metabolites have been shown to modulate a wide variety of cellular events, including differentiation and apoptosis (Denny, et al., 2004). *Leishmania* promastigotes are covered with a variety of interrelated glycosylphosphatidyl-inositol(GPI)-anchored molecules, including lipophosphoglycan(LPG), glycosylinositolphospholipids (GIPLs), proteophosphoglycan(PPG) and GPI-anchored proteins, all of which have been implicated in various steps important to the infectious cycle (Ilgoutz and McConville, 2001). Previous studies showed that these molecules may play important roles in survival and pathogenesis of *Leishmania*, such importance includes survival in the sand fly host where *Leishmania* flagellated promastigotes differentiates from a replicating procyclic to a non-replicating infectious metacyclic form, while in mammalian host, the parasite is ingested by macrophages and differentiates into non-flagellated amastigotes form (Sacks and Kamhawi, 2001; Zhang, et al. 2005). In contrast of GPI-anchored molecules, the role of sphingolipid is still not fully known, early studies showed that sphingolipids including ceramides and sphingoid bases are important in general membrane functions and cell-to-cell recognition, regulation of cell growth, differentiation, intracellular signaling, apoptosis and modulation of immune response (Merrill,et al., 1993; Kaneshiro, et al., 1986). Unlike mammalian cells which synthesize sphingomyelinand glycosylsphingolipids to high abundance, the majority of sphingolipids (SLs) in *Leishmania* are unmodified inositol phosphorylceramide (IPC), a class of lipids mainly found in fungi and plants (Kaneshiro, et al., 1986; Spiegel and Milstien, 2002).

Sphingolipids are lipids of sphingosinecore with fatty acid chain and a polar alcohol as attachments. Ceramide is an unmodified sphingolipid that functions as a secondary messenger, found in the outer membrane of eukaryotes and plays an important role in signaling mechanisms (Simons and Ikonen, 1997).The first rate-limiting enzyme in the de novo sphingolipids biosynthesis is serine palmitoyltransferase (SPT), it is expressed in large number of species from bacteria to human and belongs to the family of transferases. SPT catalyses the condensation of L-serine and palmitoylCoA to form 3-ketodihydrosphinganine. Subsequently, Nacetylation of sphingoid base in the endoplasmic reticulum leads to the formation of ceramide. In some eukaryotes, such as yeast, plants and kinetoplastid protozoan

parasite *Leishmania*, the ceramide is catalyze into Inositol Phosphorylceramide (IPS)by the enzyme Inositol Phosphorylceramide synthase (IPS synthase); while in mammals, the ceramide is catalyze into Sphingomyelin (SM) by the enzyme sphingomyelin synthase (SMase) (Hanada, 2003, Naqiec, 1997).Recent studies showed that *Leishmania* species can maintain sphingolipidsin different pathways, for example, the biosynthesis if sphingoid base and ceramide (precursors of complex sphingolipid) is absent and non-essential for the proliferation of *L. major* amastigotes within phagolysosomes in model animal system, however, the intra-macrophage amastigotes synthesize the complex sphingolipid IPC de novo utilizing precursors from alternative, host sources and maintain this lipid species at equivalent cellular levels to promastigotes (Pratt, et al. 2013, Smith, et al. 2012), moreover, the visceral *L. donovani* shown to stimulate host macrophages which suggested that endogenous ceramide generation induced in host cells by the parasite was responsible for the impairment of the normal macrophage anti-microbial machinery (Dey, et al. 2007).

In this study, we have established an ex vivo infection of axenic culture *L. mexicana* amastigotes with murine macrophage cell line RAW264.7 under different conditions where cells cultured in serum –media and serum-free media to reduce the exogenous sphingolipid available to address the parasite infectivity in both conditions.

Materials and methods:

L. mexicana MYNC/BZ/M379 and murine macrophage cell line RAW264.7 were kindly provided by Dr. Paul Denny, Durham University, UK.

1- Media preparation: Schneider's Drosophila medium was purchased from Sigma-Aldrich and was prepared as described by manufacturer's protocol (Zilberstein and Shapria, 1994).

2- Axenic culture of *L. mexicana* promastigotes was cultured in Schneider's Drosophila medium at pH.7 supplemented with 15% HIFBS, 1% pen/strep.

3- Axenic metacyclogenisis was induced by transferring procyclic promastigotes to Schneider's Drosophila medium contains 20% HIFBS at pH.5.5. Promastigotes were seeded at 5×10^5 cell/ml and incubated at 26^{o}C for six days to produce infectious metacyclic promastigotes. Axenic amastigotes were induced by transferring metacyclic promastigotes to Schneider's Drosophila medium at pH.5.5 with 20% HIFBS and this culture was incubated at 32^{o}C for five days, parasite were became amastigotes (Bates, 1994).

4- Murine macrophage cell line RAW264.7 was cultured in Dulbecco Modified Eagle Medium, GIBCO® (DMEM) supplemented with 10% HIFBS, pH.7 at 37^{o}C, 5% CO_2.

5- Ex vivo infection of RAW264.7 macrophages with *L. mexicana* amastigotes: macrophage cells were cultured in 24-well tissue culture plate and seeded at 5×10^5cell/ml (DMEM with 10% HIFBS or with 1% Nutridoma) and incubated at 37^{o}C, humidified 5% CO_2, after 24 hours, the old medium was removed carefully from the wells and washed with DMEM before new media added. *L. mexicana* were then applied in a ratio of 10:1.Myriocin (potent inhibitor of SPT) was added in 25 and 50 µM, methanol was added for control, plates were incubated at 32^{o}C, 5%CO_2 for 48 hours.

6- Fluorescent microscopy: after 48 hours incubation, media was removed and each well was fixed with 37% formaldehyde, washed with PBS, then wells were stained with nucleic acid DAPI stain (4', 6-diamidino-2-phenylindole)(Bok, et al. 2009). Plates were seen under epi-fluorescent microscope in which random fields were examined to count 100 macrophages in each well to determine the number of infected macrophages and the total number of amastigotes in the field in order to measure the percentage of infection and the proliferation of the parasite within the host macrophage.

7- The t Test was used to determine the significant differences between test and control.

Results and discussion:

Plates of ex vivo infection using myriocin, in media with or without FBS, were examined under epi-fluorescent microscope; in each well 100 macrophages were counted in random fields to calculate the number of infected and non-infected cells, the total number of parasites in 100 macrophages and the number of parasites in infected macrophages; in addition, the mean of the number of parasite/cell was calculated, table-1.

Table-1: Ex vivo infection of RAW264.7 with *L. mexicana* amastigotes, proliferation, infectivity and number of parasite/cell (results are mean of triplicates).

PARAMETER	FBS-DMEM PLATE		FBS-FREE DMEM PLATE	
NUMBER OF INFECTED CELLS	100µM myriocin	49.25	100µM myriocin	46.75
	50µM myriocin	68.5	50µM myriocin	64.5
	5µl MeOH	49.75	5µl MeOH	52.25
NUMBER OF NON-INFECTED CELLS	100µM myriocin	50.75	100µM myriocin	53.25
	50µM myriocin	31.5	50µM myriocin	35.5
	5µl MeOH	50.25	5µl MeOH	47.75
NUMBER OF PARASITE/100 CELLS	100µM myriocin	125.25	100µM myriocin	101.75
	50µM myriocin	128.5	50µM myriocin	126
	5µl MeOH	99.5	5µl MeOH	115.25
PERCENTAGE OF INFECTIVITY	100µM myriocin	49.25%	100µM myriocin	46.75%
	50µM myriocin	68.5%	50µM myriocin	64.5%
	5µl MeOH	49.75%	5µl MeOH	52.25%
NUMBER OF PARASITE/CELL	100µM myriocin	2.553	100µM myriocin	2.17
	50µM myriocin	1.873	50µM myriocin	1.963
	5µl MeOH	2.002	5µl MeOH	2.21

In the second plate, we have used media-free serum to reduce the available exogenous sphingolipid to the macrophage RAW264.7 and intracellular amastigotes. The results showed that there was no significant difference between test and control in the plates of serum media or serum-free media in both the percentage of infectivity and the number of parasite/cell, figure1 and 2.

This indicates that host (macrophage) sphingolipid biosynthesis and exogenous sphingolipid are not pivotal to the invasion and proliferation of the parasite in RAW264.7 promastigotes, showed much lower infection rates than cells treated with methanol as a control and there were significantly decreased infection rates, with a significant reduction in parasite number per infected cell at 2 hr. Our findings may indicate one of the differences of the de novo SLs pathways between the New World *L. mexicana* and Old World *L. major*. Previous study (Zhang, 2003) on the old world *L. major* promastigotes proved that SL-deficient spt2$^-$were undergo metacyclogenesis normally but they had poorly infectivity to macrophages as amastigotes. Another study (Zhang, 2009) revealed an essential role of host SL degradation in *L. major* virulence, in which mutant ISCL (Inositol phosphosphingolipid phospholipase C-Like

Protein) *Leishmania* lost the activity of SMase (which hydrolyze sphingomyelin) yet there were no obvious defects in growth or differentiation of the parasite in vitro.

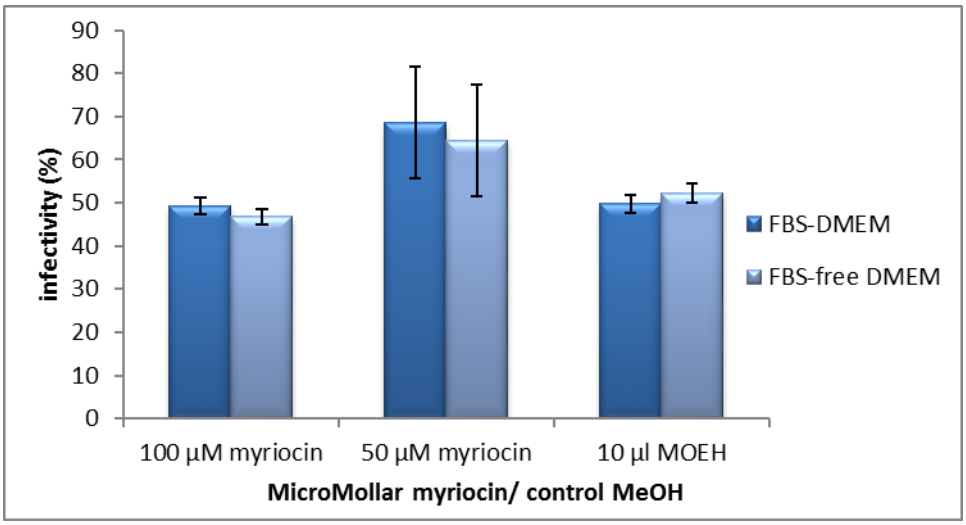

Figure-1: Percentage of infectivity in RAW264.7 infected with *L. mexicana* amastigotes, data are compared between cells cultured in DMEM with or without FBS.

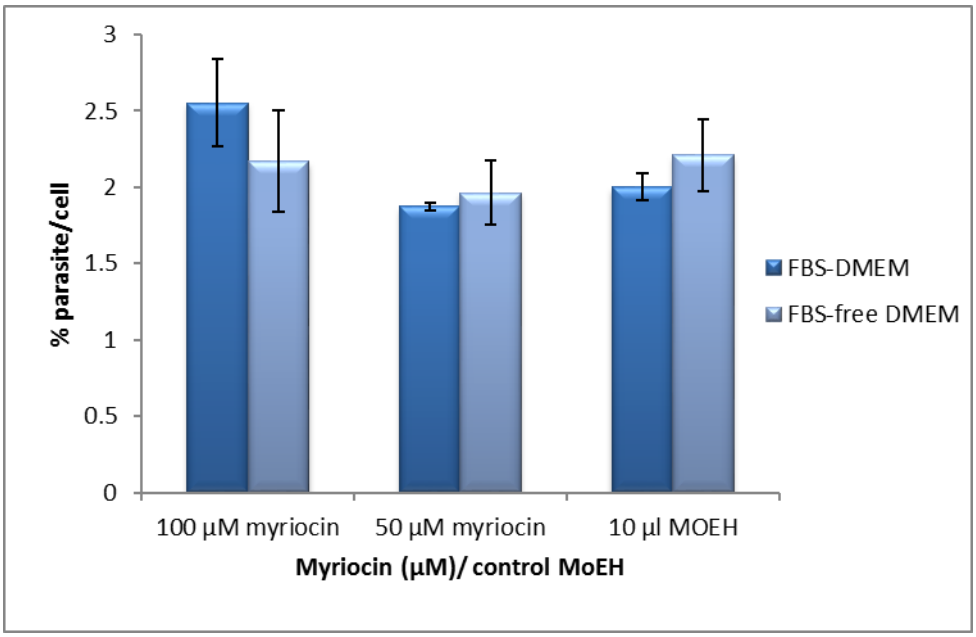

Figure-2: Number of parasite/cell in RAW264.7 infected with *L. mexicana* amastigotes, data are compared between cells cultured in DMEM with or without FBS.

Furthermore, it has been found that genetic ablation of one SPT subunit, *L.majorLCB2*, yields viable null parasites that can no longer synthesize ceramide and SL de novo(Denny, et al, 2004).Another study on the African Trypanosomes, found that myriocin treatment had no visible effect on the Endoplasmic reticulum-to-surface-transport of GPI-anchored variant surface glycoprotein (VSG), however, the SLs synthesis was essential and required for endocytosis in blood-stream trypanosomes (Sutterwala, et al. 2007). Study on the visceral leishmaniasis, *L. donovani*, showed that subunit which encodes the SPT in the RNAi screening was important for the uptake of the parasite by S2 phagocytic cells, while specific inhibitor studies demonstrated that SPT activity is required for

efficient phagocytosis of *L. donovani* in mammalian macrophages (Naqiec, et al., 2012), this study also showed that myriocin-treated RAW264.7 macrophages infected with late stationary *L. donovani*.

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