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VERSITA

Axenic cultivation and comparative phospholipase A_2 activity of *Giardia duodenalis* in a serum-free medium

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Abstract

Mammalian serum is essential for the growth of *Giardia duodenalis* cultivated under axenic conditions. Unfortunately, some factors present in bovine serum used as supplement in the culture medium may inhibit protozoal growth and activity. TYI-33-PACSR is a TYI medium supplemented with a serum replacement (PACSR) made up of Earle´s amino acid solution, Diamond´s vitamin-tween 80 mixtures and LCR (a lipid-cholesterol – rich mixture). PACSR was previously used in the culture media for axenic cultivation of *Entamoeba histolytica* and *Trichomonas vaginalis*. The main objective of this work was to demonstrate that TYI-33-PACSR is useful for axenic cultivation of *G. duodenalis*. Additionally, the activity of phospholipase A_2 (PLA A_2) in the sub-cellular vesicular fraction (P30) of *G. duodenalis* grown in TYI-S-33 and TYI-33-PACSR was compared. All strains of *Giardia* grown in TYI-33-PACSR reached relative cellular densities of 91 to 95% compared to controls growing in serum-supplemented TYI-S-33 medium. Additionally, PLA A_2 activity was similar in the P30 sub-cellular fraction obtained from trophozoites growing in TYI-S-33 and TYI-33-PACSR. Thus, TYI-33-PACSR could be useful in analyzing the biological properties of *G. duodenalis* in the absence of serum.

Keywords

Giardia duodenalis, PACSR, culture media, mammalian serum, phospholipase A_2 activity

Introduction

Giardia duodenalis (syn. *Giardia lamblia* and *Giardia intestinalis*), an intestinal protozoan that causes giardiasis in humans and most mammals (Adam 2001). This protozoan is also a very common enteric parasite of domestic animals, including livestock, dogs, and cats (Thompson 2004). Thus, giardiasis is considered a zoonotic disease (Feng and Xiao 2011). Approximately 200 million people are affected by giardiasis in tropical and subtropical countries (Thompson and Monis 2004). Some recent research data suggest that humans are infected by two different species of *Giardia* with assemblages A and B, isolates WB and GS represents them, respectively. The WB and GS isolates can be considered as separate species according to the phylogenetic species concept because they group into different assemblages in genotyping studies (Jerlström-Hultqvist *et al*. 2010) *G. duodenalis* trophozoites grow efficiently *in vitro* in TYI-S-33 medium axenic cultivation containing 5–10% of mammalian (usually bovine) serum, L-cysteine (0.2 mg/ml), ascorbic acid (0.02 mg/ml) and bovine bile (0.05 mg/ml) (as recommended by Keister 1983 and Adam 2001).

Mammalian serum is considered essential for the growth of trophozoites of *G. duodenalis* and other protozoa, such as like *Entamoeba histolytica* and *Trichomonas vaginalis* (Clark and Diamond 2002). These protozoa survive for only a few hours in culture media without serum. It is not known which factors present in serum enable efficient growth of *G. duodenalis* trophozoites. Some factors, however, such as Cohn fraction III, Cohn fraction IV–1 and insulin-like growth factors, which have been isolated from bovine serum (Lujan *et al*. 1994a, b), have been shown to effect short-term stimulation

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of *Giardia* metabolism (Lujan *et al*. 1994b) and improve *in vitro* growth of this protozoan parasite in complex culture media.

Certain molecules in the mammalian serum, however, can have inhibiting effects on parasite growth; they can also modify biological activities and inhibit metabolic activity (Sissons *et al*. 2006).

Additionally, *G. duodenalis* trophozoites in culture undergo antigenic variation; the presence of these serum specific antibodies in culture may exert a potent selective pressure, reducing the antigenic variation of *G. duodenalis* trophozoites (Nash 1992).

A solution to this problem would be to use a mammalian serum-free medium for axenic cultivation of *G. duodenalis*. Several research groups have attempted to grow *G. duodenalis* trophozoites in serum-free media (Bifulco and Schaefer 1992, Gillin *et al*. 1986).

We have previously described a serum replacement for axenic cultivation: basal TYI or PEHP media supplemented with PACSR supported the growth of *Entamoeba histolytica* (Mata-Cárdenas *et al*. 1996) and *Trichomonas vaginalis* during a long term of sub-cultivation (Mata-Cárdenas *et al*. 1998).

G. duodenalis, like other parasites such as *T. vaginalis* (Vargas-Villarreal *et al*. 2003, 2005) and *E. histolytica* (Vargas-Villarreal *et al*. 1995), has phospholipase A_2 activity in the sub-cellular fraction P30 (Vargas-Villarreal *et al*. 2007); it has been demonstrated that the PLA A_2 activity encountered in this cellular fraction plays an important role in the virulence of some intestinal parasites (Castro-Garza and Said-Fernandez 1992).

Some parasites such as *Entamoeba histolytica* lose virulence factors when they grown in axenic culture media (González-Garza *et al*. 2000). It can happen with the *G. duodenalis* trophozoites, therefore design an experiment to demonstrate that the trophozoites can grow in a serum-free culture medium and preserving the PLA activity which is considered a virulence factor (Castro-Garza and Said-Fernandez 1992). The main objective of this study was to demonstrate the effects of long-term sub-cultivation of the three strains of *G. duodenalis* trophozoites in two different culture media, TYI-S-33 and TYI-33-PACSR, on growth and PLA A_2 activity.

Materials and methods

Parasites

Three *G. duodenalis* strains, 0980-IMSS, 3-IMSS and WB all with assemblages A, were used in this study. The parasites were maintained under axenic conditions by serial sub-cultivation (Cedillo-Rivera *et al*. 1991) in TYI-S-33 medium containing 10% (v/v) bovine serum, 0.05 mg/ml bovine bile, 0.02 mg/ml ascorbic acid (as recommended by Keister 1983), and Diamond´s vitamin-tween 80 mixture (Diamond *et al*. 1978).

Cultivation methods

Three screw-capped borosilicate tubes containing 5.5 ml of TYI-S-33 were inoculated with 1 X 10^3 trophozoites/ml and incubated at 36.5°C for 72 h. Each tube was observed with an inverted microscope, and the one containing protozoa with the highest density and mobility was cooled in ice water for 10 min before quantification of the density of trophozoites with a hemocytometer (Mata-Cárdenas *et al*. 1998). Trophozoites were inoculated, as above, into 3 tubes containing fresh culture medium. This procedure was repeated systematically every 72 hours to maintain the reference strain (Mata-Cárdenas *et al*. 1998, Vargas-Villarreal *et al*. 2007).

Giardia duodenalis *TYI-S-33-PACSR*

The PACSR was prepared as described elsewhere (Mata-Cárdenas *et al*. 1996). Briefly, 90 mL of Earle´s amino acid solution (Eagle 1959) was mixed with 2.5 ml of Diamond´s vitamin-tween 80 mixture (JHR, Biosciences, Lenexa, Kansas). The medium was divided into 9-ml aliquots and stored at 4°C immediately before each experiment; an aliquot of this solution was mixed with 1 ml of LCR (a lipid-cholesterol – rich mixture; Sigma Chemical Co., St Louis, Missouri) to obtain the PACSR working solution. Culture tubes containing 5 ml TYI-33 were combined with 0.5 ml PACSR and immediately inoculated and incubated as described above. Trophozoites were sub-cultivated 20 times in TYI-33-PACSR and TYI-S-33 before the start of the study.

The growth of three *G. duodenalis* strains incubated in TYI-S-33 or TYI-33-PACSR was determined in triplicate by 8 independent assays every 24 h over the course of 72 h (Mata-Cárdenas *et al*. 1998).

Mass cultures

One litre spinner flasks (Bellco Glass Inc., Vineland, New Jersey) containing 600 ml TYI-S-33 supplemented with 10% (v/v) bovine serum, bovine bile, ascorbic acid, and Diamond's vitamin-Tween 80 mixture (Diamond *et al*. 1978) were inoculated up to reaching a final concentration of 1×10^3 trophozoites/ml and incubated at 36.5°C for 96 h. The cultures were chilled in ice water for 10 min and centrifuged at 1,000 *g* for 15 min at 4°C. Trophozoites were washed twice with 10 volumes of phosphate-buffered saline (PBS; pH 7.0) and processed immediately.

Subcellular fraction P30

A pellet of fresh trophozoites was washed twice and resuspended in two volumes of Hank's balanced salt solution (BSS; 0.7 mM $CaCl_2$, 5.5 mM glucose, 120 mM NaCl, 5.3 mM KCl, 1.7 mM $MgSO_4$, 1 mM Trizma-base; pH 8.0). The cells were then disrupted with a motor-driven Elvehjem-Potter Teflon/glass homogenizer (Vargas-Villarreal *et al*. 2010). The total

extract was centrifuged at 30,000 *g* for 15 min at 4°C. The resulting supernatant (S30) was stored until use, at which point the pellet (P30) was resuspended in 1 volume of BSS, divided into 200-µl aliquots, and stored at –70°C. Immediately before starting each experiment, aliquots of P30 were thawed at room temperature and diluted with BSS to adjust the protein concentrations to 200 mg/ml.

Phospholipase A_2 assays

PLA A_2 activity was determined as previously described (Vargas-Villarreal *et al*. 2007) with minor modifications. In brief, 1, 2 dipalmitoyl-[2-palmitoyl-1-^{14}C]-PC ([2-^{14}C-PA]-PC [58 mCi/mmol]) was used to detect PLA A_2 activity. This radioactive substrate was purchased from New England Nuclear (Boston, Massachusetts). The substrate (4 µCi per assay) was mixed with 1.0 ml 100 mM Trizma-base (pH 8.0), 2 mM Ca^{2+}, 0.2% Triton X-100, and 0.27 mM phosphatidylcholine. The mixtures were sonicated with an Ultratip Labsonic System (Lab-Line Instrument Inc., Melrose Park, Illinois) at 40 W for 60 s. The emulsions were divided into 0.5-ml aliquots and stored at –70° C until use. The assays were performed in 15 × 15 mm borosilicate test tubes containing 20 µl of one of the substrate mixtures previously described and 20 µl of a solution containing various concentrations of proteins (0–200 µg/ml) from the P30 fraction of the three strains. After 1 h of incubation at 36.5° C in a water bath, phospholipid hydrolysis was stopped by the addition of 25 µl of a solution containing 1 mg/ml palmitic acid (PA), 1.0 mg/ml egg yolk lysophosphatidylcholine (LPC), and 0.75 mg/ml egg yolk phosphatidylcholine (PC) in n-butanol containing 5% trichloroacetic acid (final volume = 45 µl).

The radioactivity in PC and PA after exposure to P30 was determined using thin-layer chromatography (TLC), as follows. After the reaction was stopped, 45 µl of the reaction solution was transferred to the center of a 20 cm × 20 cm silica gel plate (0.25 mm thickness, 60 meshes; Merck, Darmstadt, Germany) and placed into a TLC developing tank with a solvent system consisting of chloroform, methanol, acetic acid and water in the ratio of 140:40:16:8 by volume. Lipid spots were developed by exposing the TLC plates to iodine vapor (Skipski and Barclay 1969).

The appearance and relative migration (R_f) coefficients of PC and PA spots were compared with those of the following standards (Sigma Chemical Co., St. Louis, Missouri): for PC, 0.43 and for PA, 0.93. The lipid spots were transferred to vials containing 5 ml scintillation liquid (Biodegradable Counting Scintillant, Amersham Corporation, Arlington Heights, Illinois), and radioactivity was determined as for relatively unquenched samples with a model 3255 Tri-Carb liquid scintillation spectrometer equipped with an external standard source (^{226}Ra) assembly (Packard Instrument Company Inc., Downers Grove, IL, USA). The masses of the products of PLA A_2 activity were estimated from the pmoles of the radiolabels.

Protein determination

Protein concentration was determined using Lowry assays (Lowry *et al*. 1951).

Statistics

All experiments were replicated in triplicate in 8 independent experiments. The growth curves were calculated using the 2-tailed linear correlation simple method. The results are presented as mean ± SE, and statistical significance (p) was compared by using 1-way analysis of variance test. The linearity of the increase in [^{14}C]-PA was assessed by linear regression with 95% confidence intervals, both test were using the Statistical Package for Social Scientific (PASW for Windows, Standard Version 18.0, Chicago, Illinois).

Results

Three strains of *G. duodenalis* showed no growth at 12 h of incubation in TYI-33 without bovine serum (data not shown). In contrast, both TYI-S-33 and TYI-33-PACSR media supported vigorous growth of *G. duodenalis* with similar yields at 72 h of incubation from initial concentration of 1 × 10^3 trophozoites/ml. The culture yield from the 0989: IMSS strain was 9% lower in TYI-33-PACSR than in TYI-S-33 (Table I). The cell densities reached by 3-IMSS in TYI-33-PACSR were 5%

Table I. Densities of *Giardia duodenalis* growth in TYI-S-33 or TYI medium with PACSR added

		Final density*	
Strain	**Medium**	**Absolute**	**Relative**
0989: IMSS	TYI-S-33	1,178 ± 283	1.00
	TYI-33-PACSR	1,075 ± 428	0.91§
IMSS-3	TYI-S-33	1,181 ± 336	1.00
	TYI-33-PACSR	1,127 ± 558	0.95§
WB	TYI-S-33	1,178 ± 277	1.00
	TYI-33-PACSR	1,093 ± 468	0.92§

*Trophozoites × 10^3 per ml, ± standard error of 8 triplicate experiments. § P>0.05.

lower than in the TYI-S-33; the final density of the WB strain was 8% lower in TYI-33-PACSR than in TYI-S-33. A comparison of culture yields across strains showed no significant difference between TYI-S-33 and TYI-33-PACSR media (P>0.05). In addition, the cultures maintained in both TYI-S-33 and TYI-33-PACSR media grew exponentially, without a lag phase (data not shown).

All of the P30 preparations obtained from trophozoites grown in TYI-S-33 and TYI-33-PACSR media showed PLA A_2 activity. Furthermore, the product of the PLA A_2 pathway, [14C]-palmitic acid was increased as a function of the concentration of proteins. The picomolar concentration of [14C]-palmitic acid as product of PLA A_2 activity obtained from the P30 fraction of the 3-IMSS strain grown in TYI-S-33 was, respectively, 1.06 and 1.04 times higher than those from the P30 fraction of 0989 and WB strains ($p = 0.09$ and 0.36); PLA A_2 activity in the P30 fraction of 3-IMSS grown in TYI-33-PACSR was also higher than PLA A_2 activity in 0989 and WB (1.08 and 1.14 times, respectively; $p = 0.45$ and 0.001). Notably, PLA A_2 activity was similar in all strains cultured in TYI-S-33 and those cultured in TYI-33-PACSR ($p = 0.46$, 0.16 and 0.12).

Discussion

Culture media for parasites and bacteria must provide the nutrients and essential factors for the microorganisms in conditions that allow them to survive and to produce virulence factors. The use of culture media permits studies of these parasites in conditions similar to those that the microorganism would find in his natural habitat.

Bovine serum is an essential factor for the growth of *G. duodenalis* that is added to the traditional culture media for this parasite; nevertheless, bovine serum also has factors that have been demonstrated to inhibit the activity of the parasite (Gillin and Reiner 1982). We have developed a bovine serum substitute that supports parasite growth without the inhibiting effects of factors present in bovine serum. This substitute, which we call PACSR, has already proved useful as an additive in the culture media for the trophozoites of amoebae (Mata-Cárdenas *et al*. 1996).

In this study, we demonstrate that trophozoites of *G. duodenalis* cannot grow without bovine serum and die after 12-hour incubation in media without bovine serum. *G. duodenalis* trophozoites, however, grow adequately in a culture media supplemented with bovine serum and in one supplemented with PACSR. Additionally, we demonstrate that trophozoites of *G. duodenalis* strains 0980-IMSS, 3-IMSS, and WB growing in TYI-S-33 have similar PLA A_2 activity to those growing in TYI-33-PACSR. The PLA A_2 activity of strain 3-IMSS growing in TYI-33-PACSR was higher than that observed in any experimental strain grown in TYI-S-33.

In previous studies (Castro-Garza and Said-Fernández 1992), it has been demonstrated that the P30 sub-cellular fraction plays an important role in the virulence of some intestinal parasites, such as amoebas. It is possible that in *G. duodenalis*, the PLA A_2 activity observed in the P30 sub-cellular fraction plays an important role as a virulence factor. Nevertheless, it is necessary to demonstrate that trophozoites of *G. duodenalis* growing in TYI-33-PACSR do not lose this pathogenic activity.

We are confident that our isolate has PLA activity in the P30 fractions because activity in each assay was similar to positive controls of phospholipases from *Crotalus adamantius*. While the negative controls containing only culture media, only cells and other non P30 fractions were placed for each trial and none of them showed any trace of activity of PLA.

As the genome of *Giardia* shows no known phospholipases sequences we can say that we have isolated from *Entamoeba* trophozoites enzymes with phospholipase activity and whose sequence was not in the genome of entamoebas. This is possible because sequences of enzymes with phospholipase activity from parasites are different to the already described phospholipases sequences.

Our outcomes supports that TYI-33-PACSR culture media is an option for cultivation of *G. duodenalis* trophozoites in a culture media without bovine serum and conserved PLA A_2 activity.

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