Available online at www.sciencedirect.com
SCIENCE DIRECT®
Experimental Parasitology 110 (2005) 318–321
Experimental Parasitology
www.elsevier.com/locate/yexpr

Entamoeba invadens: In vitro axenic encystation with a serum substitute

M.P. Barrón-González [a], L. Villarreal-Treviño [b], J.A. Verduzco-Martínez [a], B.D. Mata-Cárdenas [c], M.R. Morales-Vallarta [a,*]

[a] *Departamento de Biología Celular y Genética, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León; Av. Pedro de Alba y Manuel L. Barragán S/N, Ciudad Universitaria, C.P. 66451, A.P. 67-F, San Nicolás de los Garza, Nuevo León, Mexico*
[b] *Departamento de Microbiología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León; Av. Pedro de Alba y Manuel L. Barragá n S/N, Ciudad Universitaria, C.P. 66451, A.P. 67-F, San Nicol ás de los Garza, Nuevo León, Mexico*
[c] *Laboratorio de Bioquímica y Fisiología Celular del Centro de Investigaciones Biomédicas del Noreste del Instituto Mexicano del Seguro Social; Administración de correos No.4, Apartado Postal 20 Col. Independencia, C.P. 64720, Monterrey, Nuevo León, Mexico*

Received 8 January 2005; received in revised form 18 March 2005; accepted 18 March 2005
Available online 27 April 2005

Abstract

The current media for axenic cultivation of *Entamoeba histolytica* and *Entamoeba invadens* are supplemented with bovine or equine serum, which provides several essential nutrients to amoebas. Serum has also been considered an essential component in encystation media for *E. invadens*. A substitute of serum, PACSR has been described as an alternative for growth of *E. histolytica* and also maintains growth of *E. invadens*. When PACSR was used instead of serum for encystation of *E. invadens* the efficiency was the same as for serum. Our present data show that PACSR can support the growth and induction of encystation of *E. invadens* strain IP-1.

Index Descriptors and Abbreviations: PACSR, protozoa axenic cultivation serum replacement; ANOVA, multifactorial analysis of variance; SD, standard deviation; TYI, tripticase, yeast, and iron; IU, international units; LCR, lipid cholesterol rich; NEFAs, non-esterified fatty acids

Keywords: *Entamoeba invadens*; Encystations; Serum; PACSR

1. Introduction

Amebiasis is the third commonest parasitic cause of death globally and is a major health problem in several developing countries (Stanley, 2003; Trol et al., 1997) and is acquired by ingestion of *Entamoeba histolytica* cysts. Although cysts are the infective stage, have not been induced in vitro, due to the absence of a medium or method that supports the in vitro mass encystation of this species. This has been a barrier to research into control methods for this phase of *E. histolytica* life cycle. On the other hand, *Entamoeba invadens*, a reptiles parasite that produces the same symptoms as *E. histolytica* in humans can be encysted axenically in vitro (Das et al., 1980; García et al., 1995; Morales et al., 1997; Rengpien and Bailey, 1975; Vázquezdelara and Arroyo, 1984) and has been traditionally used as an alternative model for the study of the cyst and encystation of *Entamoeba* (Bailey and Rengpien, 1980; Cervantes and Martínez, 1980). Serum provides essential nutrients for axenic cultivation of *E. histolytica*, but its use masks the effects of certain cytotoxic activities (Laushbaug et al., 1981; Mata et al., 1996; Said et al., 1988) and fetal bovine serum is not acceptable, as fetuin is toxic to the *E. histolytica* (Clark and Diamond, 2002). Several trials have been carried out to obtain a serum-free medium that supports

* Corresponding author. Fax: +181 8376 2813.
E-mail addresses: mvmario@hotmail.com, mv_mario2000@yahoo.com.mx (M.R. Morales-Vallarta).

0014-4894/$ - see front matter © 2005 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2005.03.021

trophozoite growth for a long time when maintained in axenic culture in vitro. A few cell lines have been serially propagated in a protein-free medium (Eagle, 1959). A semi-defined medium, PDM-805, without serum has been described (Diamond and Cunnick, 1991), but this is not satisfactory for *E. invadens* growth (Mata et al., 1997). Recently it has been reported that several combinations of non-esterified fatty acids (NEFAs) sustained low *Plasmodium falciparum* growth. Furthermore, various phospholipids and lysophospholipids were tested, lysophosphatidylcholine containing C-18 unsaturated fatty acids was found to sustain the complete development of the *P. falciparum*, while several other lysophospholipids could partially support growth of this parasite (Asahi et al., 2005). Bovine or equine serum usually has been an essential component in encystation media for *E. invadens* (Myers and Morgan, 1971). It has been reported that *E. histolytica* can be grown in absence of serum when this is replaced by an aminoacids and lipids mixture (PACSR) (Mata et al., 1996), we have observed that *E. invadens* grows in a similar manner to *E. histolytica* when PACSR is used. In this work we demonstrate that PACSR can also support the mass encystation of *E. invadens* when the serum is substituted by PACSR in both the growth and encystation media.

2. Material and methods

2.1. Cultivation of trophozoites

(a) *In TYI-S-33*. Trophozoites (2×10^4/mL) of *E. invadens* IP-1 strain were cultivated in 16×150 mm borosilicate screw capped tubes containing 10 mL of TYI-S-33 medium (Diamond et al., 1978), 1.0 mL serum and 0.1 mL antibiotic mixture (400 IU penicillin and 4 mg streptomycin) and incubated at 25 °C for 72 h.
(b) *In TYI-PACSR*. The same procedure was applied but the serum was replaced with PACSR.

2.2. Encystation method

The encystation method for *E. invadens* (Morales et al., 1997) consists of two phases, both CO_2 gassed. The first phase is a growth with the complete medium, and then a glucose-free second phase medium trigger the encystation phase.

(a) *First phase (growth phase)*. Encystation of *E. invadens* strain IP-1 was induced with trophozoites from cultures previously grown with serum (TYI-S-33) and also with trophozoites previously grown with a serum substitute (TYI-PACSR). For encystation of trophozoites previously grown with serum, in the first phase (growth phase), 12 tubes of 18×150 mm, each with 10 ml of TYI-S-33 medium (Diamond et al., 1978) were inoculated with 2×10^4 trophozoites/mL with added antibiotic (400 IU penicillin and 4 mg streptomycin). The tubes were then CO_2 gassed (99.9% purity) for 2 min with a flux of 0.2 L/min. CO_2 was bubbled into the culture medium through a sterile Pasteur pipette with a cotton plug, tubes were capped and incubated at 25 °C for 5 days.
(b) *Second phase (encystation phase)*. The 12 tubes were cooled to 0–4 °C for 20 min and then centrifuged at 900 rpm for 5 min. The contents of three tubes were concentrated into a single one tube (6×10^6 trophozoites), and the cells were washed with serum-free medium. The resultant four tubes were prepared as follows: two tubes with 10 mL of glucose-free TYI-PACSR medium; a third tube with glucose-free TYI-S-33 medium as a positive control, and a serum-free and glucose-free TYI-33 medium as a negative control as the fourth tube. The antibiotic mixture described above was added to all tubes, newly gassed and incubated at 25 °C during 96 h. The efficiency of encystation was determined and the cysts were analyzed by transmission electron microscopy and optical microscopy.

Encystation of trophozoites previously grown with serum substitute (TYI-PACSR) were treated in the same way. All experiments were repeated three times.

2.3. Encystation efficiency

After encystation, assay tubes were cooled at 0–4 °C for 20 min, the cells were counted, and then tubes were centrifuged at 900 rpm for 5 min, medium was removed and cells were treated with 1% Triton X-100 for 10 min and then washed with distilled water. Cysts were counted and the encystation efficiency was calculated (cysts × 100/cysts + trophozoites).

2.4. Viability assay

Resistant cysts to 1% Triton X-100 were incubated at 25 °C for 4 days in TYI-S-33 medium or TYI-PACSR medium and a new population emerged.

2.5. Microscopy

For electron microscopy the specimens were fixed in 2.5% glutharaldehyde with 0.2% phosphate buffer, pH 7.2; post-fixed in 1% osmium tetroxide in 0.2 M phosphate buffer, pH 7.2; dehydrated in ethyl alcohol; and then embedded in epoxy resin (Medcast). Thin sections were stained with uranyl acetate and lead citrate and observed with a Zeiss EM-109 electron microscope. Semi-fine sections were stained with toluidine blue and observed with a Zeiss light microscope. For fluorescence studies, cysts were treated with 0.1% calcofluor M2R and observed with a Zeiss fluorescence microscope.