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Induction of virulence factors, apoptosis, and cytokines in precision-cut hamster liver slices infected with *Entamoeba histolytica*

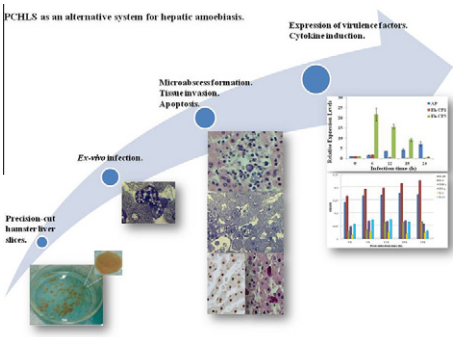
Pilar Carranza-Rosales [a,*], María Guadalupe Santiago-Mauricio [a], Nancy Elena Guzmán-Delgado [b], Javier Vargas-Villarreal [a], Gerardo Lozano-Garza [a], Ezequiel Viveros-Valdez [c], Rocío Ortiz-López [c], Javier Morán-Martínez [d], A. Jay Gandolfi [e]

[a] *Centro de Investigación Biomédica del Noreste, Instituto Mexicano del Seguro Social, Administración de Correos No. 4, Apartado Postal 020, Colonia Independencia, Monterrey, NL, CP 64720, Mexico*
[b] *Unidad Médica de Alta Especialidad # 34, Instituto Mexicano del Seguro Social, Monterrey, NL, CP 64730, Mexico*
[c] *Universidad Autónoma de Nuevo León, Monterrey, NL, CP 64460, Mexico*
[d] *Facultad de Medicina, Universidad Autónoma de Coahuila, Torreón, Coahuila, CP 66451, Mexico*
[e] *Department of Pharmacology and Toxicology, College of Pharmacy, University of Arizona, 1723 E, Mabel Street, Tucson, AZ 85724, USA*

HIGHLIGHTS

- An alternative model for the study of hepatic amoebiasis is demonstrated.
- The model is based in the *ex vivo* infection of precision-cut hamster liver slices.
- Infected slices show histopathological lesions found in animal models.
- Cysteine proteinases 1, and 5, and amoebapore are expressed in infected liver slices.
- In this system *Entamoeba histolytica* induces inflammatory cytokines, apoptosis, and necrosis.

GRAPHICAL ABSTRACT



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ABSTRACT

Precision-cut liver slices (PCLS) are mainly used to evaluate hepatotoxicity and metabolism of chemicals, as well as to study mechanisms of liver damage and repair. However, recently they have been used as a system to study amoebic infections. The aim of this study was to validate this model as an alternative for experimental amoebic liver absess (ALA) in animals. To do this, the PCLS was analyzed for the expression of amoebapore and cysteine proteinases 1 and 5, three of the most studied virulence factors of *Entamoeba histolytica*, as well as the induction of apoptosis and cytokines production in response to the *ex vivo* infection. PCHLS were prepared with the Brendel-Vitron tissue slicer and then, infected with 200,000 trophozoites of *E. histolytica*. Samples were taken at 0, 6, 12, 18, and 24 h and compared to control non-infected slices. Morphological studies were performed in order to verify the infection; while apoptosis was studied by TUNEL and PAS techniques. The expression of cysteine proteinases (1 and 5), and amoebapore, was analyzed by real-time PCR. By using ELISA assays, the production of cytokines was also studied. PCHLS were found to be a reproducible infection system, and *E. histolytica* caused the expression of cysteine proteinases and amoebapore in infected slices. At the same time, trophozoites induce release of cytokines and apoptotic death of the hepatocytes close to them. PCHLS represent a new and suitable alternative model to study the pathogenesis of hepatic amoebiasis.

* Corresponding author. Fax: +52 81 8190 4035.
E-mail address: pilarcarranza@cibinmty.net (P. Carranza-Rosales).

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1. Introduction

Amebiasis, a disease caused by the protozoan parasite *Entamoeba histolytica*, is estimated to result in 50 million cases of colitis and liver abscess and up to 100 thousand deaths each year (Ali et al., 2008). It is a cosmopolitan disease of high prevalence in Africa, Asia, India, South America and Mexico. Although these countries have improved their living conditions and level of sanitation, the disease is still a major public health problem. In Mexico, amebiasis currently ranks eighth among the 10 leading causes of general disease (CENAVECE, 2010). Following exposure *E. histolytica* inhabits the large intestine, where it causes intestinal amebiasis. However, in approximately 1% of cases, trophozoites disrupt the intestinal mucosa and spread to other organs, causing various forms of extraintestinal amebiasis, of these, amebic liver abscess (ALA) is the most common (Bernal Redondo, 2001; WHO, 1997; Stanley, 2003). The pathogenesis of ALA is very complex and involves host, and parasite factors, as well as micro-environmental conditions (Cook, 1990). The ability of amoebae to destroy host tissue and survive in the liver is accompanied by a strong adaptive response and regulation of proteins, such as amebic virulence factors (Bruchhaus et al., 2002). The most studied virulence factors of *E. histolytica* are the adhesion molecule Gal/Gal NAC lectin (Gilchrist and Petri, 1999), cysteine proteinases (Que and Reed, 2000; Bruchhaus et al., 2003), amoebapore protein (Leippe, 1997; Leippe et al., 2005), and lipophosphoglycan molecules (Moody-Haupt et al., 2000). Because of its important role in the pathogenesis of ALA in humans and in animals, in this work cysteine proteinases 1 and 5 (EhCP1 and EhCP5) and amoebapore were monitored.

Cysteine proteinases (CP) are present in many organisms. They have been isolated from axenic cultures of *E. histolytica* and have the property to degrade collagen, fibrinogen, elastin and laminin, extracellular matrix elements that trophozoites have to break through in order to cause invasive disease (Keene et al., 1986; Luaces and Barrett, 1988). These proteins are involved in the disruption of cellular monolayers (Keene et al., 1990; Lauwaet et al., 2004). Its inhibition with antisense codons decreases amebic phagocytosis, inflammation of the intestine, and the formation of ALA. It has also been proposed that CP contribute to create the anaerobic environment that trophozoites require to grow *in vivo* during ALA development (Olivos-García et al., 2004; Pérez Tamayo et al., 2006).

Amoebapore proteins from *E. histolytica* are also involved in the formation of ALA (Bracha et al., 2003; Zhang et al., 2004). They cause lysis of bacteria and eukaryotic cells (Leippe, 1997; Leippe et al., 2005). Its cytolytic capacity and participation in apoptosis induction has been demonstrated (Berninghaus and Leippe, 1997; Andra et al., 2003). *In vivo*, it has been found that when hamsters are inoculated with trophozoites expressing low levels of these proteins, virulence is reduced; this fact supports its importance in the development of ALA (Bracha et al., 2003). In another context, it has been shown that *E. histolytica* induces apoptosis, both, in human cells, and during the development of ALA in hamsters and mice (Huston et al., 2003; Boettner et al., 2008; Blazquez et al., 2007). It has also been found that the death of hepatocytes and immune cells during amebic invasion is not only due to the cytolytic activity of the trophozoites, but also because of an apoptotic process (Pelosof et al., 2006). With regard to the immune response during ALA development, an intense acute inflammatory reaction characterized by the presence of trophozoites, abundant polymorphonuclear cells (PMN), and some eosinophils, have been observed (Tsutsumi et al., 1984; Ventura-Juárez et al., 2002). In individuals predisposed to this disease, like other parasites, *E. histolytica* can spread to other organs and induce an immune cellular response (Murray, 1998). In fact, elevated levels of IL-1, IL-4, IL-6, IL-10, TGF-β, and TNF-α in patient with ALA have been found (García-Zepeda et al., 2007). Among cytokines, IL-6 is an important mediator in the synthesis of acute phase proteins in hepatocytes. During the experimental induction of ALA in IL-6 deficient mice, they develop a marked inflammatory response mediated primarily by eosinophils. These results suggest that IL-6 could be involved in resistance against the development of ALA (Tilg et al., 1992, 1994) and exerts a differential control in the recruitment and activation of leukocytes during infection (Berninghausen and Leippe, 1997).

For the study of ALA, the hamster animal model is the most used; however, systematic studies require the use of 50 to more than 100 animals (Tsutsumi et al., 1984; Tsutsumi and Martínez-Palomo, 1988). A practical alternative to reduce the number of animals for ALA studies is the use of precision-cut hamster liver slices (PCHLS), which have the advantage of being intermediate between *in vitro* and *in vivo* systems; they are simple, low cost, and reproducible. Besides of this, tissue slices maintain its metabolic, histological, and structural characteristics, and importantly, they uses, less than one third of the animals needed for experiments.

Recently, our group has demonstrated that this system can be used as an *ex vivo* model of infection by pathogenic amoebae (Carranza-Rosales et al., 2010). Therefore, the goal of the present research was to characterize PCHLS as an alternative model for amebic liver infection. To do this, the expression of *E. histolytica* virulence factors, induction of immune response, and apoptosis at short times of infection in PCHLS were studied. Morphologic studies were also performed in order to confirm the presence of lesions we previously described (Carranza-Rosales et al., 2010).

2. Material and methods

2.1. Parasites

Highly virulent trophozoites of *E. histolytica* strain HM1-IMSS were maintained axenically in TYI-S-33 medium. The inoculum was prepared from 72 h amoebic cultures in logarithmic phase of growth. Virulence of this strain was previously tested by inoculating trophozoites directly into the hamster liver and confirmation of ALA production after one week. This virulent strain was used in all the infection experiments.

2.2. PCHLS preparation

Precision-cut hamster liver slices were prepared from 2 months old male Syrian golden hamster *(Mesocricetus auratus)*. Hamsters were sacrificed with an overdose of sodium pentobarbital (6 mg/100 g) and treated following institutional and international guidelines for humanitarian care of animals used in experimental work. Once the hamster was unconscious, the liver was quickly removed and placed into ice-cold Krebs-Henseleit buffer (KB buffer). The hepatic lobes were separated with a scalpel and cored into 10 mm diameter cylinders. The cores were then sliced in oxygenated KB buffer (4 °C, 95:5 O_2:CO_2) into 250–300 μm thick slices, using the Brendel Vitron tissue slicer (Vitron, Tucson, AZ, USA). In order to optimize the obtained livers, 4 mm precision-cut tissue chips were prepared from the original 10 mm liver slices, as previously described in detail by Catania et al. (2007). Tissue chips were gently placed onto 24-well polystyrene microplates with 1 ml per well of DMEM/F12 medium and pre-incubated 1 h at 37 °C on a cell culture incubator in order to stabilize the tissue slices because of the mechanical stress they suffered during the slicing process. After this pre-incubation, the slices were infected with *E. histolytica* trophozoites as described below. The maintenance and care of experimental animals complied with the Instituto Mexicano del Seguro Social and Ley General de Salud (México) regulations, as