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A pcDNA-*Ehcpadh* vaccine against *Entamoeba histolytica* elicits a protective Th1-like response in hamster liver

Máximo B. Martínez[a], Mario A. Rodríguez[b], Guillermina García-Rivera[b], Tomás Sánchez[b], Rogelio Hernández-Pando[c], Diana Aguilar[c], Esther Orozco[b,*]

[a] *Posgrado en Ciencias Genómicas, Universidad Autónoma de la Ciudad de México, San Lorenzo 290, Colonia Del Valle, México D.F., 03100, Mexico*
[b] *Departamento de Infectómica y Patogénesis Molecular, CINVESTAV-IPN, Av. Instituto Politécnico Nacional # 2508, Colonia San Pedro Zacatenco, A.P. 14-740, México D.F., 07000, Mexico*
[c] *Departamento de Patología, Instituto Nacional de Ciencias Médicas y Nutrición, Salvador Zubirán, Calle Vasco de Quiroga 15, Tlalpan, México D.F., 14000, Mexico*

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ABSTRACT

DNA vaccines are promising tools to fight parasitic diseases, including amoebiasis caused by the protozoan *Entamoeba histolytica*. Here we studied the immunogenicity and protective efficacy of a DNA vaccine against this parasite composed by the EhCPADH surface complex encoding genes (*Ehcp112* and *Ehadh112*). EhCPADH is formed by an adhesin (EhADH112) and a cysteine proteinase (EhCP112), both involved in the parasite virulence. We evaluated transcription, protein expression, immunological response and protection against hepatic amoebiasis in hamsters intradermally and intramuscularly immunized with a mixture of *pcDNA-Ehadh112* and *pcDNA-Ehcp112* plasmids. RT-PCR and immunohistochemical assays showed that both antigens were differentially expressed in spleen and liver of immunized animals. No significant antibody immune response was induced by either route. However, intradermally inoculated hamsters presented a robust Th1-like immune response, characterized by high levels of INF-γ and TNF-α cytokines, detected in the liver of animals challenged with virulent trophozoites. Animals showed significant protection against amoebiasis manifested by a higher survival rate and a significant prevention of liver abscess formation. We conclude that a refinement of this DNA vaccine could be a good choice to control hepatic amoebiasis.

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1. Introduction

Entamoeba histolytica is a worldwide distributed protozoan responsible of human amoebiasis. Although there is effective chemotherapy against amoebiasis, in many countries this parasitosis has a high prevalence [1–3]. Throughout the world, 40 million individuals are affected by amoebiasis, and around 100,000 die due to this parasite every year [4].

Different experimental approaches have been developed [5–10] against amoebiasis including DNA vaccines [11–13], but up to now, an efficient vaccine is not available. Passive antibodies transferring to SCID mice suggested that specific antibodies are important effectors in the infection control [14,15]. Nevertheless, other experiments indicated that the presence of specific IgG in serum correlates with an increased susceptibility to infection by *E. histolytica* [16,17]. Thus, the role of systemic humoral response to invasive amoebiasis is yet unclear.

On the other hand, several studies have shown that Th1 immune response is involved in host defense against *E. histolytica* infection [18]. In support of this hypothesis, there are results showing that in mice, Th2 cytokines depletion decreases the severity of colitis caused by amoebae [19], and that innate immunity is important to control amoebiasis [20,21]. However, we still do not know certainly which branch of the immune response has the central role in protection against *E. histolytica* infection.

In addition, many studies have indicated that the type of immune response and the level of protection are influenced by the route of inoculation [22]. Furthermore, experimental variables, such as antigen nature, antigen dose, booster times and inoculation routes, among others, need to be tested for each antigen.

EhCPADH is a surface and vacuolar heterodimeric *E. histolytica* complex formed by a cysteine protease (EhCP112, 446 amino acids) and an adhesin (EhADH112, 687 amino acids) [23], involved in adhesion, cytopathic effect and phagocytosis [23–26]. Recombinant EhCP112 polypeptide (rEhCP112) is recognized by human antibodies from patients with amoebiasis [27]. Furthermore, rEhCP112 digests *in vitro* gelatin, type I collagen, fibronectin and haemoglobin. In addition, it binds to red blood cells and destroys MDCK cell monolayers [27]. Whereas, the EhADH112 protein has a cell adhesion domain at its carboxy-terminal [23,25], and a Bro-1 domain at its amino-terminal region [28]. Thus, this protein could have a double function in trophozoites [28]. Interestingly, the subcutaneous

* Corresponding author. Tel.: +52 55 57473800x5642.
E-mail address: esther@cinvestav.mx (E. Orozco).

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immunization with a recombinant peptide containing the last 243 amino acids of the EhADH112 carboxy-terminus (rEhADH243) as well as DNA vaccination of hamsters with a mixture of *Ehcp112* and *Ehadh112* genes decreased hepatic abscesses formation [25,13].

To get further insight on the protective effect of EhCP112 and EhADH112 against amoebiasis, here we investigated first whether vaccination with the gene mixture results in transcription and protein expression in animal tissues. Additionally, we evaluated the immune response induced in vaccinated animals before and after challenge with virulent trophozoites. Humoral response was poor, but intradermal vaccination induced an efficient cell mediated immune response. A Th1-like response characterized by a high level production of INF-γ and TNF-α cytokines in the liver of immunized and challenged animals, correlated with protection against liver abscesses formation.

2. Materials and methods

2.1. E. histolytica cultures

Trophozoites of strain HM1:IMSS were previously passed through hamster liver to enhance their virulence. Then, they were axenically grown in TYI-S-33 medium [29] at 37 °C until they reached mid-log-phase (72 h). Trophozoites harvested during log-phase were used in all experiments.

2.2. DNA plasmids and plasmid preparations

For DNA immunization we used the pcDNA-Ehcp112 plasmid expressing the full-length *Ehcp112* gene and the pcDNA-Ehadh112 construct [13], coding for 586 amino acids at the carboxy-terminus of EhADH112. Plasmids were purified using the QIAGEN endotoxin-free plasmid maxi kits (QIAGEN, USA), according to the manufacturer's instructions. Contamination with bacterial endotoxin was discarded by Limulus amoebocyte lysate (LAL) assay using the ToxinSensorTM kit (Gen Script, USA), according to the manufacturer's instructions.

2.3. Recombinant amoebic antigens

Recombinant EhCP112 (rEhCP112) and EhADH243 (rEhADH243) proteins fused to a 6× histidine tag [25,27] were expressed in *E. coli* BL21 (DE3) pLys S strain (Invitrogen, USA). The recombinant proteins were purified to homogeneity by affinity chromatography on Ni-NTA resin [25,27]. Protein concentration was determined by Bradford assay [30] using bovine serum albumin as standard.

2.4. Immunization of hamsters with plasmid mixture and challenge with trophozoites

Groups of 8–10 male Syrian golden hamsters (*Mesocricetus auratus*), anesthetized with 45 mg/kg of sodium pentobarbital (Anestesal, Pfizer Mexico), were immunized by intradermal injection (id) at the base of the tail or in both tibialis anterior muscle (intramuscular route, im) with 100 μl of PBS containing a mixture of *pcDNA-Ehcp112*/*pcDNA-Ehadh112* (100/100 μg) plasmids or with 200 μg of pcDNA3 plasmid, used as a control. Animals were immunized on days 0 and 20 and sacrificed under anesthesia at 18 h, days 7 and 21 and spleen and liver were extracted for examination.

For virulence experiments, groups of twice immunized animals (8 per group) were intraportally challenged with 250,000 HM1:IMSS virulent trophozoites 20 days after the second booster (day 40) and animals were bled from the retro-orbital plexus on days 0, 40 and 47, under anesthesia. Seven days after challenge (day 47), animals were sacrificed under anesthesia and liver damage was calculated as the weight of abscesses formed divided by the weigh of complete liver before injured areas were removed. Other group of challenged animals were maintained with water and food *ad libitum* and monitored for survival during 60 days. After this time, animals were sacrificed under anesthesia. In all experiments we used non-immunized hamsters as control.

2.5. Expression of Ehcp112 and Ehadh112 transcripts in immunized hamsters

Fragments of tissues from the inoculation site (skin or muscle), spleen and liver of animals sacrificed at 18 h and 7 and 21 days after DNA vaccination were analyzed for gene transcripts. Total RNA was extracted from frozen tissues kept at −70 °C using Trizol reagent (Gibco BRL, USA) and alcohol precipitation, followed by DNase I treatment (Gibco BLR, USA) to avoid genomic and plasmid contaminations.

Total RNA (1 μg) was reverse transcribed using a QuantiTect Reverse Transcription Kit (QIAGEN, USA) in a final reaction volume of 20 μl according to the manufacturer's recommendations. For gene expression analysis, quantitative real-time PCR was accomplished with the ABI prism 7300 sequence detection systems (Applied Biosystems, USA) using 1 μl/well of cDNA in 96-well optical reaction plates with optical caps and 24 μl/well of a mix containing 12.5 μl of SYBR green PCR master mix (Applied Biosystems) and specific primers (300 nM) (Table 1), in a final reaction volume of 25 μl [31]. Amplification conditions were 10 min at 95 °C, 40 cycles of 30 s at 95 °C, 30 s at 55 °C, and 30 s of data collection at 70 °C. As an internal control, a fragment of 91 bp of the hamster hypoxanthine phosphoribosyltransferase (hprt) transcript was amplified using specific primers (Table 1). For gene quantification we included in each assay (by triplicate) a standard curve of eight 1/10 serial dilution points of *Ehcp112*, *Ehadh112* or hprt cDNA, and a non-template control. PCR products were subjected to a dissociation curve analysis to confirm the specificity of the reactions. The levels of *Ehcp112* and *Ehadh112* mRNA were normalized using hprt mRNA production in simultaneous reaction.

2.6. Detection of EhCP112 and EhADH112 in tissues from immunized hamsters by immunohistochemistry

Animals were sacrificed under anesthesia at 18 h and at 7 and 21 days after DNA vaccination. Liver, spleen, skin and muscle of immunized animals were excised, fixed in ethanol and embedded in paraffin. Then, we prepared 4 μm sections of each tissue. After deparaffinization, sections were treated with 10% hydrogen

Table 1
Nucleotide sequences of primers used in RT-PCR for amplification of specific transcripts.

Gene		Primers
Ehcp112	Forward	5′-CTGCACAACGTTTTCTTGATGTTA-3′
	Reverse	5′-CTCGAGGATGACCACCACAA-3′
Ehadh112	Forward	5′-AAGCTGCTGGTGCATTCCA-3′
	Reverse	5′-ATCACCACTACCTGCTGCACATA-3′
hprt	Forward	5′-AGATCCACTCCCATAACTGTAGATTTTAT-3′
	Reverse	5′-CATCCGCACCATTAATTTTTAAGTC-3′
INF-γ	Forward	5′-GGCCATCCAGAGGAGCATAG-3′
	Reverse	5′-CCATGCTGCTGTTGAAGAAGTTAG-3′
TNF-α	Forward	5′-AACTCCAGCCGGTGCCTAT-3′
	Reverse	5′-GTTCAGCAGGCAGAAGAGGATT-3′
IL-4	Forward	5′-CCACGGAGAAAG ACCTCATCTG-3′
	Reverse	5′-GGGTCACCTCATGTTGG AAATAA-3′
TGF-β	Forward	5′-GCGGCAGCTGTACATCGA-3′
	Reverse	5′-GGCTCGTGAATCCACTTCCA-3′

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