Revista Latinoamericana de Microbiología (1999) 41:205-210
Asociación Latinoamericana de Microbiología

Cellular Immune Response of Intracecally Inoculated Mongolian Gerbils with *Entamoeba histolytica* Trophozoites

FERNANDO ALBA-HURTADO,[1]* FERNANDO DÍAZ-OTERO,[3] GUILLERMO VALDIVIA,[1] ROCIO REYES,[3] VÍCTOR TSUTSUMI[2] AND GUSTAVO ACOSTA[3]

Unidad de Investigación Multidisciplinaria, Departamento de Ciencias Biológicas, Facultad de Estudios Superiores. Cuautitlán, Universidad Nacional Autónoma de México. Km. 2.5 Carretera Cuautitlán- Teoloyucan, Cuautitlán Izcalli, Edo. Mex. C. P. 54700. A.P. 25 México.[1] *Departamento de Patología Experimental, Centro de Investigación y de Estudios Avanzados, I.P.N., México.*[2] *Hospital Juárez de México. México*[3]

*Corresponding author

ABSTRACT. We analyzed the local and systemic cellular immune response in mongolian gerbils inoculated with *Entamoeba histolytica*. Two groups were intracecally inoculated with *E. histolytica* trophozoites and two groups were used as controls. A inoculated groups and a control groups were sacrificed on days 10 and 30 post inoculation (p.i.), the spleen and mesenteric lymph nodes (MLNs) lymphocytes (Ly) were isolated and incubated *in vitro* with 2 different amebic antigens. The proliferative Ly response of inoculated groups was greater than the Ly mitogenic response seen in control groups, at day 10 p.i. as well as day 30 p.i. (α=0.05). Ly response of MLNs was greater in comparison to those of the spleen (α=0.05). In other four groups, intradermal reactions with a antigen were used to demonstrated delayed hypersensitivity in gerbils after being inoculated with *E. histolytica* trophozoites. The percentage of volume increase of the plantar pad swelling were measured. Groups inoculated presented greater increases (α=0.05) than groups controls. The evidence presented herein demonstrates that the presence of *E. histolytica* trophozoites in cecum induced a local and systemic cellular immune response.
Key Words: *Entamoeba histolytica*, Cellular Immune Response, Gerbils.

RESUMEN. Se analizó la respuesta inmune local y sistémica de tipo celular en jerbos inoculados con *Entamoeba histolytica*. Dos grupos fueron inoculados intracecalmente con trofozoitos de *E. histolytica* y dos grupos fueron usados como grupos control. Los jerbos de un grupo inoculado y un grupo control fueron sacrificados a los 10 y 30 días post-inoculación, linfocitos de bazo y nódulos linfáticos mesentéricos se aislaron e incubaron con dos diferentes extractos amibianos. La respuesta proliferativa de los linfocitos aislados de los grupos inoculados fue mayor que la respuesta mitogénica observada en los grupos control (α=0.05). La respuesta en nódulos linfáticos mesentéricos fue mayor que en bazo (α=0.05). En otros cuatro grupos, se utilizaron reacciones intradérmicas para evaluar hipersensibilidad retardada en jerbos inoculados con trofozoitos de *E. histolytica*. Se midió el porcentaje de incremento del volumen plantar. Los grupos inoculados presentaron un mayor incremento que los grupos control (α=0.05). En este trabajo demostramos que la presencia de trofozoitos de *E. histolytica* en ciego estimula tanto en forma local como sistémica una respuesta inmune celular.
Palabras Clave: *Entamoeba histolytica*, Respuesta Inmunológica Celular, Gerbos.

INTRODUCTION

Although amebiasis is a very frequent infection in developing countries, the invasive forms of the disease (mainly amebic colitis and amebic liver abscess) are proportionately less commonly seen.[16] The condition that trigger tissue invasion is possibly related with the rupture of the equilibrium between virulence factors of the parasite and the local and systemic immune responses of the host.[11,17]

Several laboratory animals have been used to study mechanisms of amebic pathogenicity and host immune responses. Hamsters, and more recently gerbils are the most commonly used animals for experimental hepatic amebiasis.[15,20] Studies on these animal models have shown that cell mediated immune mechanisms can limit or prevent hepatic abscesses.[15] Chadee and Meerovitch have reported the production of characteristic intestinal amebic lesions in gerbils inoculated intracecally with axenic and monoxenic trophozoites of *Entamoeba histolytica*. Recently, Shibayama and cols. (1997) reported also the production of typical intestinal amebic lesions in gerbils, how-

ever these lesions were temporal and healed spontaneously at 96 h after inoculation.[19] Stimulation of the immune system in these animals can be suspected based on the fact that there is a mucosal penetration by amebas or at least, a diffusion of amebic antigens in the cecal mucosa after intracecal inoculation of virulent trophozoites of *E. histolytica*.

Presently we analysed the local and systemic cellular immune response in mongolian gerbils after an experimental intestinal inoculation of *E. histolytica* trophozoites.

MATERIALS AND METHODS

Animals. Male gerbils (*Meriones unguiculatus*) of 55 to 65 days of age and 60 to 80 g of weight were used. Animals were feed with mouse commercial food and sterile water *ad libitum*.

Amebic strain. Trophozoites of *E. histolytica* strain HM1-IMSS cultured in an axenic medium BI-S-33 for 72 h were used.[7] **Amebic antigens.** Trophozoites were washed twice with sterile phosphate buffer solution (PBS). Total amebic extract (TE) was obtained by rupturing the trophozoites by three freeze-thaw cycles at -20 °C and 15 °C. The membrane fraction (P-15) was obtained by centrifuging the TE at 14 000 x g for 15 min. The supernatant was discarded and the remaining sediment was the fraction utilized.[21] Protein content of the amebic extracts was determined by Lowry method.[12] All amebic extracts were prepared at the same day of immunization.

Surgical procedure. Each animal was inoculated intracecally with 5 X 10^5 trophozoites, according to the technique described by Chadee & Meerovitch (1985).[3]

Lymphocyte isolation from spleen and mesenteric lymph nodes (MLN). The gerbils were sacrificed by chloroform inhalation. In a sterile environment, MLN and spleen were entirely excised and placed separately in small Petri dishes containing RPMI-1640 medium at 4°C. The organs were mechanically disrupted with dissecting forceps and cell suspensions were washed twice with cold RPMI-1640. The last suspension was made in 4 ml of cold RPMI-1640 supplemented with 5% bovine fetal serum (BFS) inactivated for 30 minutes at 56 °C with mercaptoethanol (5 X 10^{-5}M). Final lymphocyte concentration was 2 X 10^6 lymphocytes per ml.

Blastoid transformation technique. Spleen and MLS lymphocytes were isolated from 14 normal gerbils. In each well of culture microplates, 2 x 10^5 lymphocytes were placed with 100 µl of RPMI-1640 supplemented with BFS. Posteriorly by triplicate, 0, 1, 5, 20, 50 and 100 µg of amebic antigen proteins (TE or P-15), dissolved in 100 µl of RPMI-1640 supplemented with BFS were added to each well. Concanavaline A was used as a positive control.

Cells were incubated for 5 days at 37 °C with 5 % of CO_2 and 100 % humidity. Eighteen hours before finishing the incubation period, 1 µCi of ^{3}H-thymidine was added by each well (specific activity 6.7 Ci/mol NEN, Boston Mass., U.S.A.). At the end of incubation period, cells were fixed in glass fiber filters by means of a microharvester. 5 and 10% trichloroacetic acid, and 70% methanol were passed through the filter.[8] Finally the ^{3}H-thymidine uptake was measured in a scintillation counter. Lectures were expressed in counts per minute (CPM).

Delayed hypersensitivity skin test (DHST). The plantar pad test was used for intradermalreaction studies. Ten µg of membrane antigen (P-15) were injected intradermally in the plantar pad of the left hind foot. Same volume of sterile saline (SS) was injected in the right foot of all animals. Using a microcalibrator, measurements of plantar pad swelling were done 24 h before and after the challenge with amebic antigen. Increase in percentage of the plantar pad swelling (PI) was obtained with the following formula.

$$PI = \frac{Xt24 - XtO}{Xt0} \times 100$$

where Xt24 is the lecture at 24 h after the challenge with antigen or SS, and Xt0 is the lecture before the challenge.

Experimental protocol to determine blastoid transformation of the intracecally inoculated gerbils with *E. histolytica* trophozoites. Fifteen days before intracecal inoculation with *E. histolytica*, all animals were treated (intragastric catheter) with metronidazole (Flagyl) for 3 days, at a dose of 200 mg/Kg. A total of 28 gerbils were divided in 4 groups. Groups I and III were intracecally inoculated with 5 X 10^5 trophozoites. Groups II and IV were used as control groups and were not inoculated with amebas.

Animals from groups I and II were sacrificed at 10 days, lymphocytes were isolated from spleen and MLN treated with the 2 different amebic antigens (TE and P-15) at six concentrations. Blastoid transformation was determined as mentioned previously. The animals from groups III and IV were sacrificed at 30 days and lymphocytes were processed similarly as groups I and II.

Experimental protocol to determine intradermoreactions in gerbils inoculated intracecally with *E. histolytica* trophozoites. Forty animals were divided in 4 groups. All inoculations were done intradermally according to the conditions mentioned before. Group A: noninoculated but challenged with amebic protein (P15). Group B:inoculated 10 days before with amebas and challenged with amebic protein. Group C: inoculated 30 days before with amebas and challenged with bovine serum albumin. Group D: inoculated 30 days before with amebas and challenged with amebic protein.

Statistical analysis. Results from the 4 groups were statistically analysed by simple variance, factorial variance and multiple comparison test (minimum significant difference).

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RESULTS

Mitogenic effect: dose response of two amebic fractions (TE and P-15) on the ^{3}H-thymidine incorporation by lymphocytes from spleen and MLN of normal gerbils. The ^{3}H-thymidine incorporation by lymphocytes (from MLN and spleen) without TE always was statistically lower (α=0.05) than those incubated with different concentrations of TE. The highest stimulation on spleen lymphocytes was obtained with 1 µg of TE and with 5 µg on MLN lymphocytes. However, no statistical differences (α=0.05) were observed in the kinetics of dose-response stimulation between spleen lymphocytes and MLN lymphocytes for TE.

In a similar manner, when MLN lymphocytes were cultivated free of P-15, ^{3}H-thymidine uptake was significantly smaller as compared to any concentration of P-15 utilized. The optimum stimulation for both populations of lymphocytes was 5 µg (Table 1).

^{3}H-thymidine uptake by lymphocytes of gerbils inoculated 10 and 30 days previously with 5 X 10^5 *E. histolytica* trophozoites. The highest ^{3}H-thymidine uptake by all lymphocytes was observed at a concentration of 5 µg protein. Table 2 shows the thymidine uptake by lymphocytes from the experimental groups stimulated with 5 µg of amebic proteins. With both antigens (TE and P-15) and both lymphocyte populations (spleen and MLN), the intracecally inoculated groups presented greater responses (α= 0.05) than the controls. Statistically, a greater response (α=0.05) was noticed in MLN than spleen lymphocytes. Moreover, P-15 (membrane antigen) elicited a greater stimulation than TE. (Fig. 1).

Delayed hipersensitivity skin test with P-15 in gerbils inoculated 10 and 30 days before with 5 x 10^5 *E. histolytica* trophozoites. The percentage of swelling increment of the 4 groups of gerbils is shown in fig. 2. Groups b and d presented greater increases (α=0.05) than in groups a and c. No statistical differences were found between groups b and d.

DISCUSSION

The intestinal mucosa constitutes the first line of defense against many microorganisms. The immune system associated to the intestinal mucosa originates a local hu-

Table 1. ^{3}H-thymidine uptake in Mesenteric lymph node (MLN) and spleen lymphocytes of normal gerbils incubated with two amebic fractions, expresed by counts per minute (cpm).

	Protein µg/well	Total extract mean/s.d.	Membranal fraction mean/s.d.
Lymphocytes MLN	0 5*	3150/1399 14078/3301	3582/1303 18065/2795
Lymphocytes Spleen	0 5*	3442/1438 13342/2733	3496/1498 12995/2733

The mean represents the average of 14 animals tested by triplicate. * The protein concentration which caused the greater stimulation in cultures.

Table 2: 3H-thymidine uptake in mesenteric lymph node and spleen lymphocytes incubated with 5 µg of total extrac (T. E.)or membranal fraction (P-15) amebic of gerbils inoculated with *E. histolytica* 10 and 30 days before.

		Lymphocytes			
		Mesenteric node		Spleen	
Amebic fraction	Group	10 days	30 days	10 days	30 days
T. E.	Inoculated	21481/3750	33752/5300	19018/4264	21312/5042
	Control	13466/3290	14659/3292	12469/3300	12221/2143
P-15	Inoculated	27488/3016	36970/5371	15378/2606	26851/4190
	Control	14373/2447	16504/2240	11726/3101	12130/2085

Control groups= animals not inoculated and tested at the same time that the inoculated group.

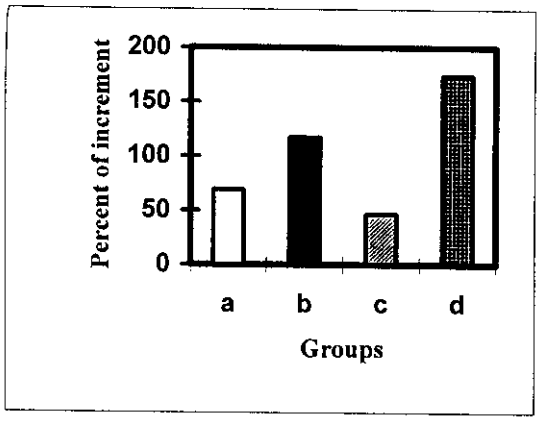

Fig. 1. ^{3}H-thymidine uptake by spleen (S) and mesenteric lymph node (MLN) lymphocytes when incubated with amebic total extract (TE) and amebic membrane fraction (P-15) of gerbils inoculated intracecally 10 and 30 days before with *E. histolytica* trophozoites. The inoculated groups presented statistical differences with its control group (a=0.05).
CPM = counts per minute.

moral or cellular responses that can be independent from the systemic immune response. The use of mongolian gerbils in experimental intestinal amebiasis opens the possibility of studying the effect of an intestinal infection on the cellular immune response.

In the present study we confirmed a mitogenic activity of amebic extracts (TE and P-15) on spleen and MLN lymphocytes of healthy gerbils. Such activity was also shown in previous studies with human T lymphocytes[6,18] and with spleen[5] and MLN lymphocytes[8] from normal mice.

It has been suggested that the mitogenic activity produced by different extracts of *E. histolytica* can be an evasive mechanism of the immune response, since various populations of T lymphocytes with different functions (suppresive and helper) could be activated in a polyclonal form and restricting in turn way the development of specific populations.[8]

The recognotion to amebic antigens by MLN and spleen lymphocytes of gerbils intraceacally inoculated 10 days before with *E. histolytica* trophozoites, suggests an induction of a primary response to amebic infection. In preliminary studies we have confirmed the cecal colonization by *E. histolytica* in gerbils. Although trophozoites were isolated and cultured from cecum until the 9th day after inoculation, lesiones were not observed. Other group of animals inoculated with monoxenic cultured *E. histo-*

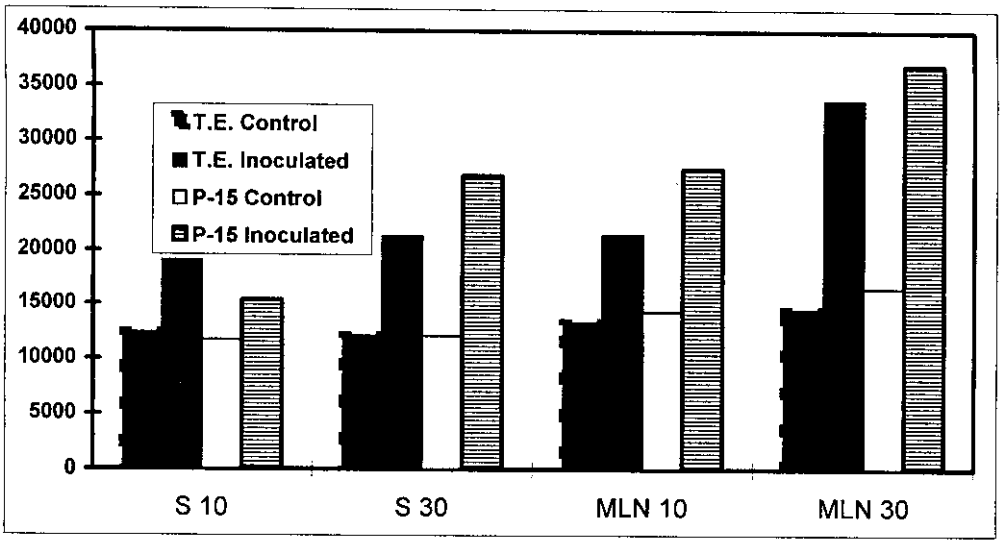

Fig. 2. Delayed skin test hypersensitivity: Evaluation by the increase of the plantar pad volume in groups of gerbils inoculated intracecally 10 and 30 days before with *E. histolytica* trophozoites. Group a) gerbils not inoculated and challenged intradermically with 10 µg of P-15; Group b) gerbils inoculated 10 days before and challenged intraderminally with 10 µg of P-15; Group c) gerbils inoculated 30 days before and challenged with 10 µg of bovine serum albumin; Group d) gerbils inoculated 30 days before and challenged with 10 µg of P-15 fraction. Each group consisted in 10 animals.

lytica trophozoites displayed a temporary self-limited cecal lesions.[19] Chadee and Meerovittch (1985a) have detected trophozoites in the cecal mucosa of gerbils until 12 days after the intracecal inoculation with *E. histolytica*.[3] These previous data could suggest that the immune response observed in gerbils infected intracecally 30 days before, is due to an immunologic memory developed during the presence of amebic antigens in this experimental model. Furthermore, the degree of this response was higher than in the primary response.

The higher immune response produced by the membrane fraction (P-15) is due probably to the fact that *E. histolytica* antigens are more abundant and immunogenic in the membrane fraction (P-15), which are constituted by both internal and external membranes.[2,14]

The first contact of *E. histolytica* with the intestinal mucosa produces apparently a local immune response characterized by a homing of MLN lymphocytes toward the site of host-parasite interaction.[1] Migration of these lymphocytes to the spleen seems to be in a minor grade. The higher *in vitro* response observed in MLN lymphocytes than in spleen of infected gerbils is in agreement with this previous observation. Moreover, have been described histologic changes in the MLN and spleen of gerbils infected intracecally with *E. histolytica*.[4] Their studies showed an increased in MLN size and hyperplasia of lymphoid follicules, whereas the changes observed in spleen were of lesser degree.

Besides the stimulation of local immune system by the initial contact of amebas into the intestinal mucosa, amebic antigens could possibly reach the general circulation producing also a systemic immune response. Although in lesser degree, our studies have shown that lymphocytes from spleen are stimulated in a greater intensity as compared to controls, suggesting a systemic response. However, this systemic response is better sustained by the delayed skin hypersentivity tests performed in gerbils. IDR has been used to measure the delayed hypersensitivity response in amebiasis.[9,10,13] The inoculation of antigen (P-15) in the plantar pad of gerbils infected cecally with *E. histolytica* trophozoites, provoked an increase in swelling greater than that observed when antigen at the same doses was given to non-infected animals or in infected but challenged intradermally with bovine serum albumin.

The evidence presented herein demonstrates that the presence of *E. histolytica* trophozoites in the gerbils cecum stimulates a local immune response of cellular type and a systemic response characterized by delayed hypersensitivity. Therefore, it is evident that this response observed may have an important role in a natural infection.

ACKNOWLEDGEMENTS

This work was supported in part by the National Council of Science and Technology of Mexico (Conacyt). We thank Ms Alicia Ramirez-Rosales for her valuable technical assistance.

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