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MORPHOMETRIC STUDY OF THE HEPATIC LESIONS EXPERIMENTALLY INDUCED IN HAMSTERS BY *ENTAMOEBA DISPAR* AND *E. HISTOLYTICA*

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Summary:

Evolution of experimental hepatic lesions produced in hamsters with *Entamoeba histolytica* and *E. dispar* was evaluated quantitatively and qualitatively through morphometry and immunohistochemistry. Animals infected with *E. dispar* developed hepatic lesions quantitatively and qualitatively similar to those produced by *E. histolytica* on the first three days of infection. On the 6th and 8th days of infection, *E. histolytica* produced larger tissue damage than *E. dispar*. A gradual decrease was observed in the number of trophozoites along the infection. A negative correlation was observed between the reduced number of trophozoites and the larger area of necrosis in both groups, confirming the importance of trophozoites killed in the lesion genesis. Regarding the genetic similarity between *E. histolytica* and *E. dispar*, comparison strategy between lesions produced by these species may culminate in identifying virulence factors of *E. histolytica*.

KEY WORDS : *Entamoeba histolytica*, *Entamoeba dispar*, pathogenesis, hepatic necrosis.

Résumé : ÉTUDE MORPHOMÉTRIQUE DES LESIONS HÉPATIQUES INDUITES EXPÉRIMENTALEMENT CHEZ LES HAMSTERS PAR *ENTAMOEBA DISPAR* ET *E. HISTOLYTICA*

L'évolution des lésions hépatiques expérimentalement produites chez les hamsters par Entamoeba histolytica *et* E. dispar *a été évaluée quantitativement et qualitativement par morphométrie et immuno-histochimie. Les animaux infectés par* E. dispar *ont développé des lésions hépatiques semblables à celles produites par* E. histolytica *pendant les trois premiers jours d'infection. Au 6ème jour d'infection,* Entamoeba histolytica *provoque une destruction plus importante des tissus qu'*E. dispar. *Une diminution progressive du nombre de trophozoïtes a été observée dans les deux groupes d'animaux au cours de l'infection. Une corrélation négative a été observée entre la réduction du nombre de trophozoïtes et l'augmentation de la surface nécrosée dans les deux groupes, confirmant le rôle des trophozoïtes détruits dans la genèse des lésions. En considérant, la proximité génétique entre* E. histolytica *et* E. dispar, *la comparaison des lésions produites par ces espèces peut aboutir à l'identification de facteurs de virulence.*

MOTS CLÉS : Entamoeba histolytica, Entamoeba dispar*; pathogénèse, nécrose hépatique.*

Entamoeba histolytica is a protozoan parasite capable of penetrating and destroying the intestinal mucosa and leading to the amoebic dysentery. Since then, trophozoites may reach the liver through blood circulation. Amoebic liver abscesses are the most frequent extraintestinal form of amoebiasis. *E. histolytica* lytic capacity has been corroborated using a wide range of *in vivo* and *in vitro* experimental models. The main types of amoeba products that have been suggested as cell destruction-inducing agents are: adhesins, amoebapores, phospholipase A, phosphatases, collagenases and cysteine proteinases (Leippe *et al*., 1993; Lopez-Vancell *et al*., 2000; Que & Reed, 2000; Aguirre-Garcia *et al*., 2003). Pathogenesis of amoebiasis has not been totally understood, however.

Since the 1980s, data have been collected supporting Brumpt's hypothesis about the existence of two different organisms involved in amoebiasis. Biochemical, immunological and genetic data were still being collected up to 1993, when a new description of *E histolytica* was published, separating it as a pathogenic, invasive form, causing symptomatic forms, *Entamoeba histolytica* and, a non-pathogenic form, *Entamoeba dispar* (Diamond & Clark, 1993).

E. dispar is a species closely related to *E. histolytica*. Virulence-related genes, present in *E. histolytica* are changed or even absent in *E. dispar*, suggesting a common ancestral (Clark & Diamond, 1997; Mcfarlane *et al*., 2005). Although this, *E. dispar* was able to infect experimental animals and produce lesions apparently similar to those of *E. histolytica* (Costa *et al*., 2000; Gomes *et al*., 2000; Furst *et al*., 2002). This fact has stimulated us to evaluate experimental lesions produced by *E. dispar* compared to those produced by *E. histolytica*. In this context, the pathogenicity of these two types of amoebas were compared through a quan-

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titative study of the necrotic area and the number of immunohistochemically labeled trophozoites in different periods of infection.

MATERIALS AND METHODS

Maintenance and growth of trophozoites

E. dispar MCR and *E. histolytica* EGG strains were used in this study. MCR strain was isolated from an asymptomatic carrier with negative serology for *E. histolytica*. EGG strain was isolated from a patient with dysenteric colitis, hepatic abscess and positive serology. These samples were identified as *E. dispar* and *E. histolytica* by zymodeme and PCR (Martinez *et al*., 1996; Gomes *et al*., 1999).
Trophozoites from each strain were thawed in water bath and maintained in Pavlova medium at 37° C, with passages at each every three days.

Inoculation

Two groups of 25 hamsters each – *Mesocricetus auratus* – were inoculated via the intrahepatic route (in the left lobe) with *E. dispar* and *E. histolytica*. Animals were previously anesthetized with 79 mg/kg of sodium pentobarbital. All procedures were conducted in accordance to the Brazilian College of Animal Experimentation. Inocula consisted of 100,000 trophozoites in 0.1 ml of saline. As control to evaluate the possibility of bacteria induced lesions, three animals were inoculated with the flora of MCR strain and other three ones with EGG strain.

Necropsy and histopathology

Animals were observed daily and groups of five animals were killed by cervical dislocation and necropsied on the 1st, 2nd, 3rd, 6th and 8th day after inoculation. All animals inoculated with the flora were necropsied on the 2nd day after infection. The liver was removed in order to obtain a 2 mm thick slice, always in the same position, with a free edge towards the hepatic hilus. Liver slices were collected and fixed in 10 % buffered formaldehyde pH 7.2. After processing in alcohol and xylol, fragments were included in paraffin and 4 µm thick sections were obtained and stained by haematoxylin and eosin (H&E).

Immunohistochemistry

From the same tissue fragments, streptavidin-peroxidase immunohistochemical reactions were performed in order to detect trophozoites. Sections were treated with 3.5 % PBS/H_2O_2 solution in order to block endogenous peroxidase. Unspecific binding was blocked by goat serum diluted 1:50. Sections were incubated with polyclonal anti-*E. dispar* serum or anti-*E. histolytica* serum diluted 1:2000, followed by biotinylated goat IgG diluted 1:100 (Zymed Laboratories Inc., San Francisco, Calif.) and streptavidin diluted 1:100 (Zymed Laboratories Inc.). Color was detected using a 0.05 % diaminobenzidine solution and 0.2 % H_2O_2 and sections were counterstained with diluted Harris's Haematoxylin. Primary antiserum was substituted by PBS in some sections for negative control purposes.

Quantitative analysis of necrosis and trophozoites

All necrotic areas, as well as trophozoites, identified by immunohistochemistry were quantified with the use of the KS300 software coupled to a Carl Zeiss image analyzer (Carl Zeiss, Oberkochen, Germany). In order to evaluate necrosis, sections were thoroughly analyzed and all images of normal and destroyed hepatic tissues were digitalized through 4 × or 10 × objectives and a JVC TK-1270/RGB microcamera. All necrotic areas were delimited with a cursor, areas in µm² were automatically calculated and data were sent to a chart. The methodology employed to digitalize, to establish conditions to measure and to obtain results is described by Caliari (1997). Results were evaluated by Kruskal-Wallis statistical test.
From the sections labeled with antibodies against trophozoites, 30 random images were digitalized through 40 × objective (achieving a total of 16×10^5 µm² of analyzed hepatic tissue). All trophozoites identified by immunohistochemistry were counted using the same image analyzer. A probable correlation between the lesion area and the number of trophozoites was evaluated by the Spearman test.

RESULTS

Histopathological analyses

Amoebic hepatic lesions were detected in 100 % of the animals inoculated with *E. dispar* and *E. histolytica*. Necrosis zone, well delimited by cell debris gathering and by normal hepatic parenchyma, was observed in both groups of animals (Fig. 1a & b). Inflammatory infiltrate was discrete or moderate, constituted by scarce neutrophils and macrophages and did not completely surround the necrotic zone. A large number of trophozoites was found especially on the border of necrosis and inside granulomas. Epithelioid granulomas were observed mainly in animals inoculated with *E. dispar* (data not show).

Animals inoculated with MCR and EGG floras

Neither macro nor microscopic lesions were observed in any animals.

QUANTITATIVE ANALYSIS OF THE NECROTIC ZONES INDUCED BY *E. DISPAR* AND BY *E. HISTOLYTICA*

On the first day of infection, the mean of the necrotic area did not vary between the two animal groups (Table I). In contrast, on the 6th and 8th day of infection, E. *histolytica* produced visibly larger necrotic areas. On the 8th day of infection with *E. dispar*, necrosis was not observed in two animals. In these animals, the destroyed tissue was completely substituted by granulation tissue (endothelial cells, fibroblasts, macrophages and collagen fibers), fibrosis and some granulomas. In the other animals of this group, the induced necrosis was larger than that observed on the first three days of infection. In general, more intense lesions were observed in most animals inoculated with *E. histolytica* and in one animal on the 2nd day of infection with *E. dispar*. In spite of that, statistical analysis showed that there was no difference in the two studied groups (Kruskal-Wallis: $p > 0.05$). Figures 2 and 3 show the percentage of necrotic area produced by *E. dispar* and *E. histolytica* trophozoites in relation to normal hepatic tissue.

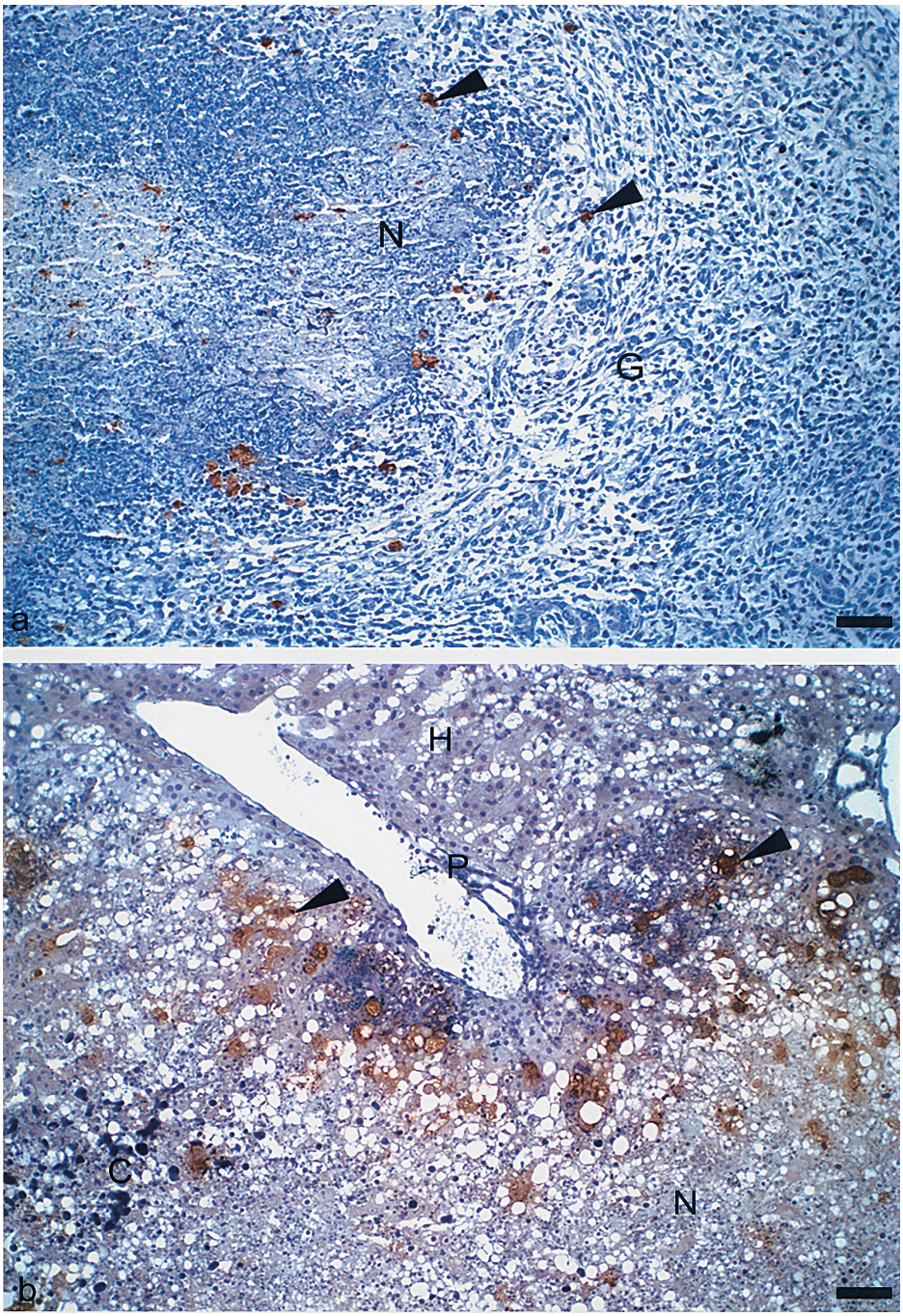

Fig. 1. – (a) Livers of hamsters inoculated with *E. histolytica* (3rd day of infection). Necrosis (N); positive trophozoites of *E. histolytica* identified for the immunohistochemistry (arrowheads); granulation tissue (G); (b) Livers of hamsters inoculated with *E. dispar* (3rd day of infection). Degenerated hepatic tissue (H); portal tract (P); necrosis (N); calcification zones (C); several immunohistochemically positive *E. dispar* trophozoites (arrowheads). Haematoxylin counterstained. Bar = 100 µm

Amoeba	Hepatic necrotic area (10^6 um^2) after infection (days)				
	1°	2°	3°	6°	8°
E. dispar	0,2 ± 0,06	52,8 ± 117,3	0,9 ± 0,9	90,6 ± 156,7	69,9 ± 136,2
E. histolytica	0,5 ± 0,2	1,3 ± 1,3	3,2 ± 2,1	253,7 ± 48,3	307,3 ± 61,2
	n = 5	n = 5	n = 5	n = 5	n = 5

No statistical difference was found between *E. dispar* and *E. histolytica* groups as measured by Kruskal-Wallis test ($p > 0.05$).

Table I. – Means of hepatic necrotic area (10^6 µm^2) induced by *E. dispar* and *E. histolytica* in different periods of the infection.

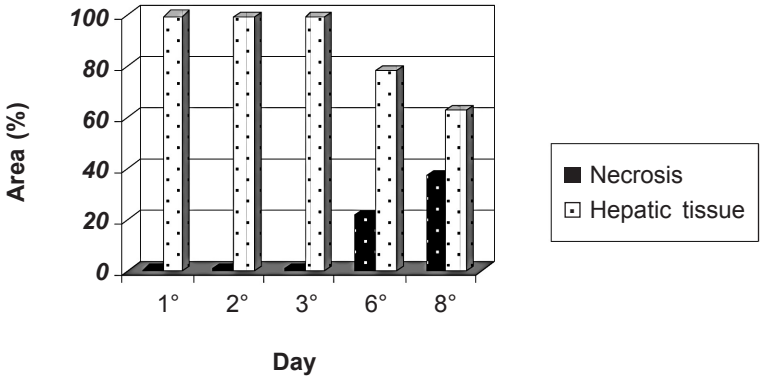

Fig. 2. – Area of necrosis and of normal hepatic tissue in hamsters (n = 5 per group) inoculated with *E. dispar*.

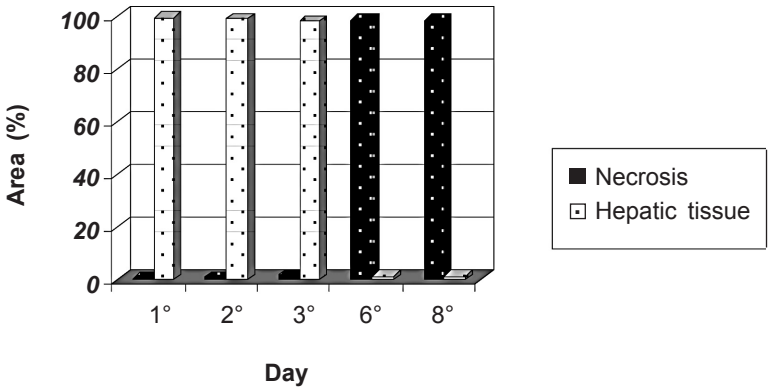

Fig. 3. – Area of necrosis and of normal hepatic tissue in hamsters (n = 5 per group) inoculated with *E. histolytica*.

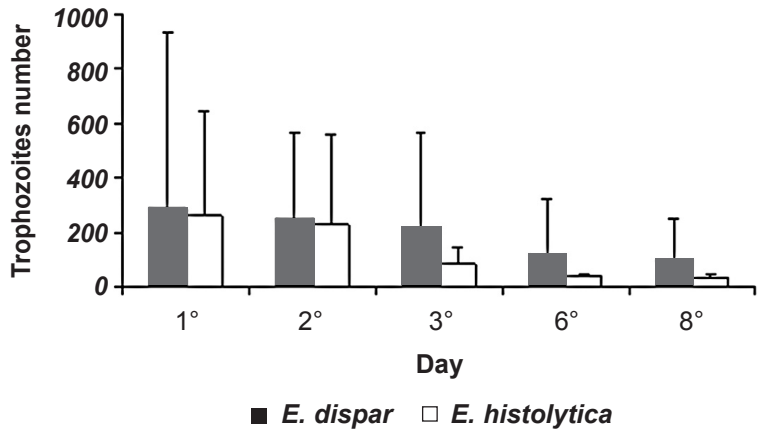

Fig. 4. – Number of *E. dispar* and *E. histolytica* trophozoites in hepatic necrosis in different periods of infection (n = 5 per group). No statistical difference was found between *E. dispar* and E. *histolytica* groups as measured by Kruskal-Wallis test ($p > 0.05$).

QUANTITATIVE ANALYSIS OF *E. DISPAR* AND *E. HISTOLYTICA* TROPHOZOITES

A larger number of trophozoites was observed in animals inoculated with *E. dispar* (220 ± 341, 122 ± 199.3 and 105 ± 144.6, 3rd, 6th and 8th days respectively) than in those inoculated with *E. histolytica* (87 ± 59, 42 ± 8 and 36 ± 14.7, 3rd, 6th and 8th days respectively), although no statistical difference could be verified (Kruskal-Wallis: $p > 0.05$) (Fig. 4). The number of counted trophozoites decreased with time in both experimental groups.

A correlation was shown between the increase of necrotic area and the reduction of the number of trophozoites in the two groups of animals using Spearman's test (*E. histolytica*: r = - 0.6984); (*E. dispar*: r = - 0.4549).

DISCUSSION

The ability of *E. histolytica* to lyse tissues results in multiple necrotic areas mainly in the intestine and in the liver. There are many molecules involved in the tissue destruction by amoebas (Leippe *et al*., 1993; Lopez-vancell *et al*., 2000; Que & Reed, 2000; Aguirre-Garcia *et al*., 2002), but amoebiasis pathogenesis is still not totally understood. In this context, the development of new strategies for the study of amoebic lesions may identify other functions of *E. histolytica* virulence, adding data to better understand the disease. The comparative study of lesions experimentally induced by other amoebas may be considered as strategy. Some *E. dispar* strains, species closely related to *E. histolytica*, may produce lesion in experimental animals (Costa *et al*., 2000; Gomes *et al*., 2000; Furst *et al*., 2002). The comparison of these lesions to those produced by *E. histolytica* was made in this work. Hamster was used as experimental animal model as it satisfactorily reproduces hepatic amoebic lesions (Tsutsumi *et al*., 1984).

Out of 25 animals inoculated with 100,000 *E. dispar* trophozoites, hepatic necrosis was observed in virtually all animals. Such lesions appeared within 24 hours after inoculation, were very similar to those induced by *E. histolytica,* and, in general, presented increased intensity along inflammatory kinetics. A bigger susceptibility in one animal on the 2nd day of infection seems to have been the responsible for the genesis of a great area of necrosis.

These results differ from what has been observed by other authors (Espinosa-Cantellano *et al*., 1998).

Although necrotic zones, in general, had their size increased significantly on the 6th and 8th days after inoculation, a proportional increase was not observed in the number of trophozoites. On the contrary, in both *E. dispar* and *E. histolytica* the number of trophozoites was significantly reduced. The decrease in the number of trophozoites during inflammatory kinetics suggests that trophozoites self-destruction could promote the consequent release of toxic products that would contribute to the increase of necrosis. In fact, on the 6th and 8th days of infection, *E. histolytica* produced a larger tissue destruction and damaged hepatic tissue presented a smaller number of trophozoites, in contrast with what was observed in animals inoculated with *E. dispar*. The hypothesis that destroyed leukocytes would be important agents for the development of hepatic necrosis is also discussed (Tsutsumi *et al*., 1984). However, the depletion of neutrophils in mice inoculated with *E. histolytica* trophozoites did not affect the frequency of ulcerative lesions in the large intestine (Rivero-Nava *et al*., 2002). On the other hand, the products of trophozoites contribute to amplify the inflammatory reaction. Zhang *et al.* (2003) showed that TNF-α is a crucial mediator in amoebic intestinal inflammation induced in rats with combined immunodeficiency. The authors have observed that *E. histolytica* cysteine-proteinases activate IL-1β precursor, amplifying inflammation and the damages to the host tissues.

The absence of necrosis induced by inoculation of the flora that accompanies the two species of amoeba, excludes the possibility that lesions were produced by bacteria. These results stimulate the use of *E. dispar* as a non-pathogenic amoeba model for *in vivo* comparative studies with *E. histolytica*. The use of other approaches of this model may revert to a better understanding of amoebiasis physiopathology.

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