

INFLUENCE OF BACTERIA UPON CYTOPATHIC EFFECT AND ERYTHROPHAGOCYTOSIS OF DIFFERENT AXENIC STRAINS OF *ENTAMOEBIA HISTOLYTICA*

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SUMMARY

At this moment, the duality of species suggested for *E. histolytica* is being considered for discussion. In order to contribute to settling this question, we investigated the possibility of conversion of avirulent ameba to virulent ones, as well as, the possibility of increasing virulence of virulent strains, by means of association with bacteria. Five strains of *E. histolytica* were employed, two of them regarded as avirulent and three virulent ones. Amebas were associated with the bacteria *Escherichia coli* 055 and 0115, previously demonstrated as capable to modify the pathogenic behavior of *E. histolytica*. Changes in virulence of amebas were assessed by cytopathic effect upon cultured mammal cells and erythrophagocytosis.

The virulence of pathogenic strains was significantly increased after bacteria association in opposition to what was observed for nonpathogenic ones, which were not influenced by bacteria association.

KEYWORDS: . Ameba-bacteria interaction; Cytopathic effect; Erythrophagocytosis; Virulence.

INTRODUCTION

Entamoeba histolytica is a cosmopolitan parasite which infects about 10% of the world's population¹⁹. In most infected people, the so called asymptomatic carriers, a harmless host-parasite pathogen relationship is established. Occasionally, however, the amebas invade the intestinal wall, producing ulcers and may reach the liver, via portal vein producing amebic liver abscess. The incidence of invasive amebiasis varies from region to region. This fact may be related to various factors involving the host, the parasite and the environment where the parasite lives. Some hypotheses have been proposed to explain the striking differences in the invasive capability of amebas such as the unicist, dualist and pluralist theories. Recently, some investigators have demonstrated biochemical differences between pathogenic and nonpathogenic strains of *E.*

histolytica^{5, 13, 14, 15}, reinforcing the dualist theory⁴ which admits that *E. histolytica* represents, in fact, two distinct species morphologically similar. On the other hand, in some reports^{9, 10, 18}, these biochemical differences have been attributed to environmental factors such as culture conditions whether axenic, monoxenic or polyxenic. MIRELMAN et al.^{9, 10} observed changes in the virulence of amebas by means of axenization or modification in the associated bacterial flora. Since 1937, the influence of bacterial flora on the virulence of ameba has been studied^{1, 2, 3, 12, 20, 21}. There is consensus that bacteria may act increase the virulence of ameba. However, little is known about the possibility of conversion of commensal and harmless nonpathogenic ameba into aggressive ameba, capable of invading tissues.

In this study we verified the behavior of five axenic strains of *E. histolytica* two of them regarded as avirulent in respect to virulence, assessed by cytopathic effect and erythrophagocytosis, before and after association with bacteria.

MATERIAL AND METHODS

Strains of *E. histolytica*

Five strains of *E. histolytica* were used. Strain's ICB-CSP, ICB-462, ICB-32 and ICB-RPS (further referred to as CSP, 462, 32 and RPS) were isolated by SILVA et al. ¹⁶. Strain HM1-IMSS (further referred to as HM1) was isolated in Mexico by DE LA TORRE et al. ⁷. Strain's CSP and HM1 were isolated from patients with amebic dysentery. At soon after axenization, these strains produced hepatic lesions in high proportions of inoculated hamsters. After long maintenance in axenic conditions, these strains showed gradually decrease in virulence for hamsters. At the moment of this study, they were not able to infect hamsters. Strains 462, 32 and RPS were isolated from cysts of asymptomatic carriers. Strain 462 is of unstable virulence and at moment of this study was capable to infect and produce hepatic lesions in about 25% of inoculated hamsters. Strains 32 and RPS in axenic conditions never infected nor produced lesions in hamster liver.

All the strains of *E. histolytica* utilized were grown axenically for 72 h in TYI-S-33 medium ⁶.

Bacteria

Escherichia coli 055 and 0115 were kindly provided by Dr. Mirelman (Weizmann Institute of Sciences, Israel) and maintained in Lignières medium. These bacteria were studied by BRACHA and MIRELMAN ² who observed to be serotypes of *E. coli* capable to increase the virulence of strains HM1, HK-9 and NIH-200 of *E. histolytica*.

Interaction ameba and bacteria

Cultures of *E. histolytica* in logarithmic phase of growth were (72 hours old) concentrated by centrifugation at 150xg. The number of amebas was determined by counting to in Neubauer's chamber. Association was performed for 1h with an initial bacteria ameba ratio of 1000:1.

Virulence determination

Cytopathic effect upon cultured mammal cells

The determination of destruction of cultured

mammal cell was conducted according to the method described by MIRELMAN & BRACHA ⁸. Briefly, VERO cells cultured in Dulbecco's modified Eagle's medium (DMEM) were shown into wells polystyrene plaques to grow to confluence reaching approximately 2.5×10^5 cells per well. The trophozoites of *E. histolytica* were counted and adjusted to a concentration of 2.5×10^5 amebas/mL, resuspended in DMEM without serum and added 1 mL per well. The cells were incubated for 1 hour at 37°C and 5% de CO₂ with the axenic trophozoites and with that interacted with bacteria. Afterwards the plaques were submitted to the following treatments: a) icebath for 10 minutes for releasing of adhered trophozoites, b) washing twice with cold saline, c) fixing with 4% formaldehyde for 10 minutes and d) washing twice with cold saline. The cells remaining in the plaque were stained with a solution of 0.01 M sodium borate buffer pH 8.7 containing 0.1% methylene blue. The dye retained by cells in the plaque was extracted with 1 mL of 0.1 M HCl and the absorbance of the HCl solution determined at 660 nm. Controls were performed with cells which did not interact with amebas and the color extracted in this condition was considered to be 0% of destruction. Controls for the effect of bacteria upon cells were also performed. The experiments were carried out in duplicate and repeated at least 3 times.

Erythrophagocytosis

The erythrophagocytosis assay was carried out as described by TRISSL et al. ¹⁷. Trophozoites axenic or previously associated with bacteria for 1h, adjusted to a concentration of 1.0×10^6 per milliliter in phosphate buffered saline pH 7.2 (PBS). Afterwards 0.4 mL of this suspension, containing 4.0×10^5 trophozoites were incubated with 0.4 mL of a PBS suspension, containing 40×10^7 per milliliter in phosphate buffered saline pH 7.2 (PBS). Afterwards, 0.4 mL of this suspension containing 4.0×10^7 human erythrocytes (1:100 ameba: erythrocytes ratio), at 37°C for 20 minutes being the interaction interrupted by the addition of 1 mL of distilled water. The tubes were centrifuged at 150xg for 3 minutes being the trophozoites and the uptake erythrocytes fixed for 30 minutes in PBS containing 2.5% glutaraldehyde at room temperature. The excess of glutaraldehyde was washed with PBS and the uptake erythrocytes contrasted with PBS containing diaminobenzidine 25 mg% for 30 minutes at room temperature. After contrast reaction, the trophozoites were washed with PBS and the uptake erythrocytes

counted in 100 randomly selected trophozoites. Results are expressed as the mean number of erythrocytes per ameba. The result represents the mean of 3 experiments carried out in duplicate.

RESULTS

Cytopathic Effect

As can be observed in the Fig. 1, the virulent axenic strains HM1, CSP and 462 showed significantly higher cytopathic effect as compared to avirulent strains RPS and 32 as determined by the capability to destroy the VERO cells. The previous incubation of the trophozoites of the virulent strains HM1, CSP and 462 of *E. histolytica* with the bacteria *E. coli* 055 caused a significant increase of the monolayer cultured VERO cells destruction rate. On the other hand, for the avirulent strains 32 and RPS, no significant effect upon destruction rate of VERO cells was observed after association with these bacteria. Similar results were observed for the association of *E. histolytica* with *E. coli* 0115. No effect upon the VERO cells in cultivation was observed by *E. coli* 055 or 0115.

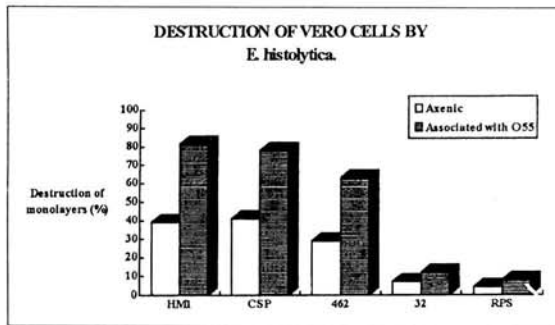


Fig. 1 - Destruction of monolayer cultured VERO cells by *Entamoeba histolytica* in the absence (□) and presence (■) of *Escherichia coli* 055. The trophozoites were previously incubated with bacteria for 1 hour. The relationship bacteria/ameba was 1000:1.

Phagocytosis of Human Erythrocytes

The virulent axenic strains HM1, CSP and 462 showed more efficient in the phagocytosis of human erythrocytes than the avirulent strains 32 and RPS (Fig. 2). As observed for the destruction of the VERO cells, the erythrophagocytosis rate was higher after incubation with bacteria for the virulent strains but no significant difference was observed for the avirulent strains. The Fig. 2 show the results obtained for the association of *E. histolytica* with *E. coli* 0115. Similar result was observed for the association with *E. coli* 055.

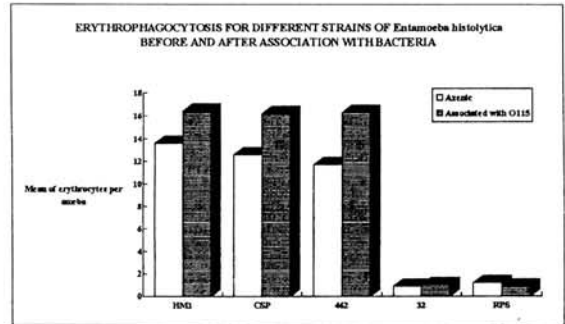


Fig. 2 - Erythrophagocytosis for different strains of *Entamoeba histolytica* before (□) and after (■) association with *Escherichia coli* 0115. The amebas were previously incubated with bacteria for 1 hour. The relationship bacteria/ameba was 1000:1. The trophozoites associated with bacteria were incubated at 37°C with erythrocytes for 20 minutes.

DISCUSSION

Our results show that the association with bacteria *E. coli* 055 or 0115 caused a significant increase of both erythrophagocytosis and the capability of destruction of mammal cells, observed for the three different virulent strains CSP, HM1 and 462. On the other hand, for the avirulent strains 32 and RPS no significant difference was observed for these parameters after association with bacteria. These results strongly suggest that live bacteria augment the virulence of amebas, in agreement with the results of other investigators^{1, 2, 3, 12, 21}. However, at same conditions, these bacteria were not able to change an avirulent ameba to virulent.

Within recent years a great deal of attention has been placed on the virulence of amebas and the interaction bacteria-ameba. However several points remain obscure and therefore speculative. Thus, little is known about how the bacteria interfere with the virulence of ameba mainly related to the avirulent strains isolated from asymptomatic carriers, with the possible conversion to virulent. Our results unequivocally show the existence of virulent and avirulent forms which may support the theory of duality of species as proposed by BRUMPT⁴. However, we can not rule out the possibility of a clustering of virulent and avirulent forms in the heterogeneity of strains as postulated by OROZCO et al.¹¹. We believe to be necessary the inclusion of a higher number of axenic avirulent strains to permit a more wide study for we can speculate about the biology of ameba and to bring into the theory's pluralist or the dualist of Brumpt.

RESUMO

Influência de bactérias sobre o efeito citopático e a eritrofagocitose de diferentes espécies axênicas de *Entamoeba histolytica*.

Atualmente, tem se discutido muito a dualidade da *Entamoeba histolytica*. Na tentativa de contribuir no esclarecimento desta questão, investigamos a possibilidade de conversão de amebas avirulentas em virulentas, como também a possibilidade de aumento de virulência de cepas virulentas seguido da associação com bactérias. Para tal utilizamos 5 cepas de *E. histolytica*, 2 avirulentas e 3 virulentas. As amebas foram associadas com as bactérias *Escherichia coli* 055 e 0115, demonstradas previamente como hábeis para modificar o comportamento patogênico da *E. histolytica*. As modificações na virulência das amebas foram avaliadas através do efeito citopático sobre células de mamífero em cultivo e eritrofagocitose.

A virulência das cepas patogênicas foi significativamente aumentada depois da associação com bactérias em oposto ao observado para as cepas não patogênicas, as quais não foram influenciadas pela associação com bactérias.

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