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AN ATTEMPT AT REVERSIBILITY AND INCREASE OF THE VIRULENCE OF AXENIC STRAINS OF *Entamoeba histolytica*

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SUMMARY

In this study we have tried to verify whether the interaction "in vitro"with bacteria or small pieces of normal hamster liver would modify the pathogenic behavior of axenic strains of *E. histolytica:* avirulent ones (ICB-32 and ICB-RPS), of attenuated virulence (ICB-CSP and HM1) and of mean virulence (ICB-462). Every attempt to render virulent, recover or increase the virulence of axenic strains of E. histolytica has failed.

KEYWORDS: *Entamoeba histolytica*; Axenic cultures; Ameba-bacterium interaction; Virulence.

INTRODUCTION

Entamoeba histolytica is dispersed widely and about 10% of the world's population carry the parasite, although a much smaller percentage suffer disease. Clinical signs of infection vary in different parts of the world. These variations seem to be related to factors such as the parasites, the host and the environment. Several studies [2,15], have investigated the interplay between these factors, but conditions governing the change from an intestinal commensal organism to an aggressive, tissue invader have received little attention.

"In vitro" axenic cultivation of amebae[10,12] has enhanced knowledge about the virulence of parasites and has permitted studies on the pathogenicity of trophozoites grown in the absence of microorganisms with which they are usually associated within the human body. The degree of virulence of trophozoites of Entamoeba histolytica, isolated from different patients, can be assessed experimentally on the basis of three criteria: the ability to form hepatic abscesses in golden hamsters[11,16]; cytopathic and cytotoxic effects in cultured mammalian cells[3,19,23]; and erythrophagocytosis[20,27].

Assessment of the virulence of amebae is handicapped by the fact that prolonged culture in an axenic medium can diminish virulence[2,16,22], but virulence can be revived by "in vivo"passage of a strain in the liver of golden hamsters[8,11,14]. Virulence of a strain can also be enhanced by adding pieces of normal hamster liver to axenic cultures[1], or by incorporating bacteria[4,5,28].

In this study, we have tried to check whether the avirulent strains of *E. histolytica* (ICB-32 and ICB-RPS) would undergo any change regarding their pathogenicity, when associated with bacteria, since there are very few experimental data about avirulent axenic strains in literature. We have further tried to

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verify if the loss of virulence of the strains kept in axenic culture (HM1 and ICB-CSP) is a reversible phenomenon and investigate a possible modification in the pathogenic behavior of a mean virulent strain (ICB-462), following association with bacteria and incubation with small pieces of normal hamster liver.

MATERIAL AND METHODS

STRAINS OF E. histolytica USED:

- HM1-IMSS* : Isolated from a patient with symptoms of amebiasis by DE LA TORRE[9] in Mexico. DIAMOND[11] used it under axenic condition in 1971. It was considered virulent.
- ICB-CSP* : Isolated in 1969 from a patient with symptoms of acute amebic dysentery and regarded as highly virulent[8]. This strain has been cultured axenically since 1981.
- After prolonged axenic cultivation these strains are how considered to be attenuated, and do not produce liver abscesses when inoculated into hamsters.
- ICB-462: Isolated in 1980 directly from cysts in feces of a asymptomatic carrier. It was used under axenic conditions in 1981 and deemed to be of moderate virulence.
- ICB-32: Isolated in 1979 directly from cysts of a asymptomatic carrier, it was submitted to axenic conditions in 1981 and was considered avirulent.
- ICB-RPS: Isolated in 1989 directly from cysts of a asymptomatic carrier, is avirulent and has been cultivated axenically since 1990.

All the strains of *E. histolytica* that were utilized, excepting HM1, were isolated in Belo Horizonte, MG, Brazil and submitted to axenic conditions in TPS-1 or TYI-S-33 medium by SILVA et al.[24].

STRAINS OF BACTERIA:

- Escherichia coli O55 and O115, living were kindly provided by Dr. Mirelman, Weizmann Institute of Sciences, Israel. These bacteria have been described[5,17] as being able to increase the virulence of amebae.
- E. coli from the original flora of ICB-CSP strain.
- E. coli O55 and O115, killed by lethal radiation with 500.000 rads from a source of cobalt.

Interaction between ameba and bacteria

In each experiment, the amebae were exposed to a single strain of living bacteria, at intervals ranging from 1 to 72 hours in TPS-1 or TYI-S-33 medium at 37°C. After the time of association had elapsed, the amebae were washed in a 0.85% saline solution, to remove bacteria. The viability of trophozoites was checked through 0.125% of eosin and counted in Neubauer's chamber, in order to ascertain the size of the inoculum. Approximately 1 x 10^8 bacteria, lethaly irradiated by a cobalt source were also associated with amebae for 10 days, the medium containing the bacteria being changed every 48 hours.

Incubation with liver snips

Small pieces of approximately 1 mm^3 of normal hamster liver, were added to twenty-four hour cultures

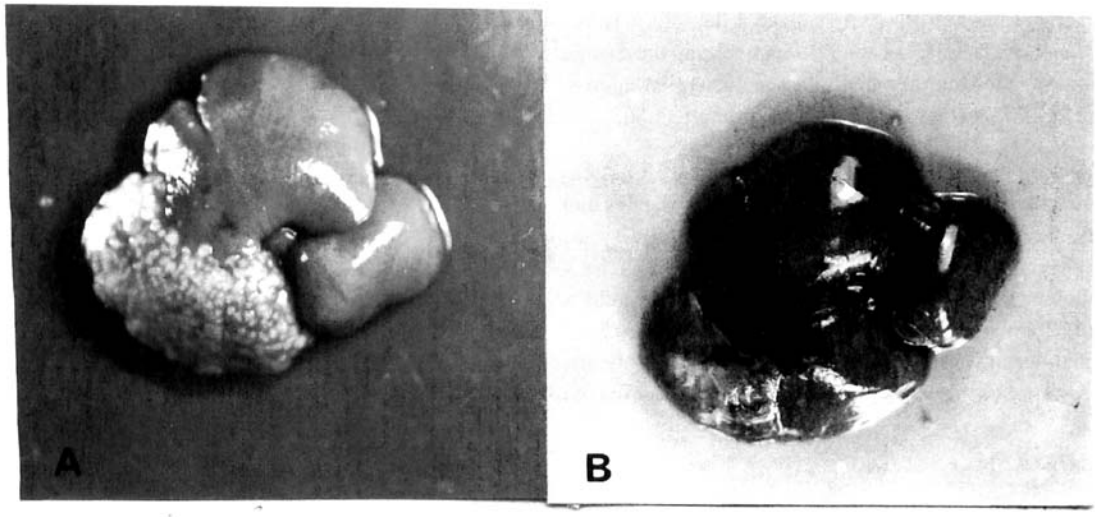

Fig. 1 - Liver of hamster inoculated with A) 1.0 x 10^6 trophozoites of the axenic strain ICB-462 of *E. histolytica* and B) associated 24 hours with *E. coli* 055

of amebae and incubated at 37°C for 48 hours. Following the period of incubation, the amebae were washed in a saline solution and the inoculum was determined by counting the viable trophozoites in Neubauer's chamber.

Inoculation in Hamsters

Both male and female hamsters, one month old, were intrahepatically inoculated under sterile conditions. Six days after the inoculation, the animals were sacrificed and their livers examined macroscopically and microscopically to detect parasites. Liver fragments were fixed in 10% buffered (pH 7.2) formalin, embedded in paraffin, sectioned (5 μm) and stained with hematoxylin-eosin (H.E.). Other bits of liver were inoculated in a culture medium containing *Crithidia fasciculata* as an attempt to reisolate the ameba.

RESULTS

The virulent though attenuated strains of *E. histolytica* (ICB-CSP and HM1) and the avirulent ones (ICB-32 and ICB-RPS), kept in axenic cultivation, produced no abscess when they were inoculated into the liver of hamsters. These strains were not reisolated in culture medium containing C. fasciculata and under histopathologic examination no amebae were found. ICB-462 strain (mean virulence) caused abscesses in 25% of the hamsters inoculated (Fig. 1A). The results were positive not only with regard to the isolated cultures starting from these animals, but also concerning the findings of the parasite in the slides stained with H.E. (Table 1).

The bacteria E. coli O55, O115 as well as *E. coli* from the original flora of the strain of E. histolytica ICB-CSP were inoculated into the liver of hamsters. They caused lesions in all the animals inoculated (100%).

The strain of *E. histolytica* ICB-CSP was associated with *E. coli* O55 for a period ranging from 1 to 48 hours. No experiment whatever showed the formation of amebic hepatic abscesses (Table 2). Only the typical bacterial lesion was found in the liver. All attempts at reisolation by using culture tecniques failed. From a histopathological viewpoint, it was observed a focal productive purulent hepatitis (formation of abscesses bearing a central area, almost exclusively constituted of both intact and degenerate granulocytes neutrophils, enveloped by a characteristic pyogenic membrane and the beginning of formation of a conjunctive capsule). A diffuse productive chronic inflammation with giant cell type, showing intense conjunctive neoformation together with inflitrates of mononuclear cells (granulocytes neutrophils, lymphocytes and macrophages) were evinced as well as fatty albuminous degeneration of hepatocytes. The research for amebae was negative. This strain was also associated with bacteria E. coli O115 and *E. coli* from its original flora, undergoing a treatment and showing results resembling those in Table 2, in order to be associated with *E. coli* O55.

Similar experiments concerning the strains of *E. histolytica* ICB-32, ICB-RPS and HM1 were carried out, varying only with reference to the size of the inoculum of the trophozoites. The same results as found for ICB-CSP strain were detected.

The results of the association of ICB-462 strain with *E. coli* O115 are shown in Table 3. Microscopically, we can observe several amebic abscesses at different stages: some with coliquative necrosis in the center, enveloped by several granulocytes neutrophilis and mononuclear cells among a large number of still intact amebae; others without central coliquative necrosis, but with many granulocytes already undergoing degenerative phenomena. Isolated nest of amebae intact or in

TABLE 1
Intrahepatic inoculation of Hamsters with different strains of *E. histolytica* from axenic culture.

STRAIN	INOCULUM	AMEBIC HEPATIC ABSCESSES	ISOLATION*	DEGREE OF INFECTION (I to V)
HM1-IMSS	9.5×10^6	0/16	0/16	nd
ICB-CSP	2.0×10^6	0/16	0/16	nd
ICB-462	3.5×10^6	4/16	4/16	II, III and V
ICB-RPS	5.0×10^6	0/16	0/16	nd
ICB-32	2.0×10^6	0/16	0/16	nd

* Fragments of liver were inoculated in culture medium containing *Crithidia fasciculata*.
nd = lesion was not detected.

TABLE 2

Intrahepatic inoculation of the strain of *E. histolytica* ICB-CSP after association with living *E. coli* 055 by various time intervals.

ASSOCIATION TIME (hours)	FINAL RATIO BACTERIA-TROPHOZOITES	INOCULUM	AMEBIC HEPATIC ABSCESSES*
1	1,500:1	3.0×10^6	0/8
6	1,000:1	2.0×10^6	0/8
12	2,000:1	1.5×10^6	0/12
16	2,500:1	1.7×10^6	0/9
24	6,000:1	1.0×10^6	0/10
48	10,000:1	4.5×10^5	0/6

* The hamsters were sacrificed 6 days after inoculation.

process of dissolution, with very little reaction around them, were also observed. Amebae were found only in the animals with amebic hepatic abscess. The experiments for reisolation only were positive when there were abscesses. ICB-462 strain was associated with bacteria E. coli O55 and *E. coli* from original flora of ICB-CSP strain. It showed no results different from the ones achieved when it associated with *E. coli* O115.

The strains of amebae associated with irradiated bacteria and the strains incubated with liver of normal hamsters behaved as the axenic strains showed in Table 1. They did not produce any lesions or abscesses in the hamsters inoculated, neither were they reisolated in cultures.

DISCUSSION

Several investigations have been carried out aiming at the understanding of the factors that determine the virulence of *E. histolytica*. Factors such as associated bacterial flora, cholesterol, food and passages through the liver of hamsters living and "in vitro"have shown to modify the virulence of *E. histolytica*. In this study, we have investigated two of these factors: bacterium associated with ameba and the treatment with small pieces of normal hamsters liver "in vitro".

It is known that the passage through the liver of hamsters increases the virulence of the amebae[8,14,18]. However, when its virulence is lost, following successive feedings to axenic cultures, one is no longer able to infect experimental animals with the attenuated strains. Therefore, it does not seem possible to restore the lost virulence, at least by this procedure. The incubation of *E. histolytica* with fragments of liver of normal hamster has been found to increase its virulence[1]. For the purpose of verifying whether this treatment would be able either to restore or to increase the virulence of strains of *E. histolytica* that we have used, the trophozoites were incubated with liver of normal hamsters. In no experiment did we manage either to change or to restore the virulence of the strains of *E. histolytica*. These results are in accordance with the observations of BOHL et al[1] concerning the reversibility of the virulence. Nevertheless, they differ in relation to the increase of the virulence, since ICB-462 strain has not changed its behavior following the incubation with liver of normal hamsters. The inoculation of this same strain into the liver of hamsters and its reisolation and subsequent inoculation, increase its virulence, as it has been proved by the greater number of animals with amebic hepatic abscess (unpublished data).

Irradiated bacteria were able to modify the zymodeme and the virulence of strains of *E. histolytica*[17]. In our experiments, however, the amebae associated with irradiated bacteria have not changed their pathogenic behavior.

Several investigators have shown that the virulence of *E. histolytica* increases after association with living bacteria[4,5,21,28]. Little do we know, however, about the ability of these bacteria to change a commensal ameba into a pathogenic one. This is due to the difficulty in submitting the avirulent strains to axenic conditions.

Axenic strains of *E. histolytica* that lost their pathogenic potential, because they had long been kept in a cultivation medium, were able to restore their virulence when associated with living bacteria[4,5,28]. Nevertheless, the same did not happen when we associated ICB-CSP and HM1 strains with bacteria, inclusively *E. coli* O55 and O115. These were associated with some strains of *E. histolytica*, leading to an increase of their virulence[4,5]. Later experiments[5] have established that the period of association ameba-bacterium is not a significant factor in so far as the increase of virulence is concerned. In spite of this, we

have carried out this association for periods ranging from 1 to 72 hours. So, we went beyond what was observed by WITTNER & ROSEMBAUN[28], who found it necessary a minimal period from 6 to 12 hours for association with living bacteria.

The approximate relation between bacterium and ameba suggested, as an important factor to alter the virulence of amebae, was 1,000/1[4]. In this work, we have utilized relations varying from 1,000/1 to 100,000/1. Nevertheless, even with such high relations, HM1 and ICB-CSP strains were not able to restore their virulence. This is in agreement with the findings of PHILLIPS[22], who tried to restore the virulence of NIH-200 strain by associating it with several types of bacteria, without any success. He concluded that the loss of virulence was an irreversible phenomenon.

Besides the bacteria *E. coli* O55 and O115, we have used *E. coli* from the original flora of ICB-CSP strain, which did not revert the virulence of HM1 and ICB-CSP strains either. The latter, when isolated, was submitted to monoaxenic conditions, with this same bacterium and it produced abscess in the liver of hamsters and in albino rats. Owing to these results, we are trying to submit the recently isolated strains of *E. histolytica* to axenic conditions, so that new associations with bacteria may tell if the reversibility of virulence depends on keeping the amebae in axenic cultivation for a long period or on keeping the bacteria in storage medium for long as well. At present, we are associating ICB-CSP strain with the flora from a strain of ameba recently isolated from a symptomatic patient and which showed to be pathogenic in the liver of hamsters. The association lasted 24 hours and the inoculum was of 4.5 x 10^6 trophozoites. No inoculated hamster presented amebic hepatic abscess.

With regard to the increase of virulence, no significant difference (Fisher's exact test) was observed before and after the association of ICB-462 strain with bacteria. This can be observed by comparison with Tables 3 and 1. As for the qualitative aspect of the lesions, the association with bacteria does not seem to have contributed to increase the degree of infecction of this strain (Fig. 1A and 1B).

The avirulent strains (ICB-32 and ICB-RPS) after association with bacteria, did not modify their original features, remaining avirulent. Considering that ICB-RPS strain was recently isolated as well as submitted to axenic conditions, and that this is one of the few studies carried out with avirulent strains in axenic cultivation, we can suppose that the conversion of a commensal ameba into a pathogenic one is not very likely to occur. In spite of this, it is necessary the inclusion of a larger number of avirulent axenic strains, so that we may agree with BRUMPT'S theory[6], reinforced by GRAHAN CLARK et al.[13]. They admit that *E. histolytica* comprises two distinct species: a pathogenic and a non pathogenic one. The fact is that the association ameba-bacterium has several obscure points which remain speculative, concerning how the bacteria interfere with the virulence of amebae.

RESUMO

Tentativa de reversibilidade e aumento de virulência de Cepas Axênicas de *Entamoeba histolytica*.

Neste trabalho procuramos verificar se a interação "in vitro" com bactérias e fragmentos de fígado de hamster normal, modificaria o comportamento patogênico de cepas axênicas de *E. histolytica* avirulentas (ICB-32 e ICB-RPS); virulentas, porém atenuadas (ICB-CSP e HM1) e de

TABLE 3

Intrahepatic inoculation of the strain of *E. hystolytica* ICB-462 after association with living *E. Coli* 0115 by various time intervals.

ASSOCIATION TIME (hours)	FINAL RATIO BACTERIA-TROPHOZOITES	AMEBIC HEPATIC ABSCESSES*	INFECTION DEGREE (I to V)
1	1,500:1	4/15	II-IV
6	100,000:1	5/16	II-IV
12	100,000:1	4/16	III-IV
16	5,000:1	5/15	I-IV
24	5,000:1	5/14	II-V
48	10,000:1	3/11	II-IV
72	20,000:1	3/10	II-IV

* Inoculation of 0.1 mL of amebae (1.0 to 2.5 x 10^6) in hamsters which were sacrificed 6 days afetr inoculation.

média virulência (ICB-462). Todas as tentativas de tornar virulentas, restabelecer ou aumentar a virulência das cepas axênicas de *E. histolytica* utilizadas fracassaram.

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