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Entamoeba histolytica: ANTIGENIC CHARACTERIZATION OF AXENIC STRAINS FROM BRAZIL

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SUMMARY

Trophozoites from cultures of **Entamoeba histolytica** strains isolated and grown axenically in Brazil (ICB-CSP, ICB-462 and ICB-32) were used for immune sera production and for characterization of their antigens by using electrophoretic and glycoproteic profiles, in parallel with a standard strain isolated and kept under axenic conditions in USA (HK-9). Hyperimmune sera, presenting high antibody titers with homologous and heterologous antigens, were obtained. The four strains in study revealed similar and complex electrophoretic and glycoproteic profiles showing polypeptides with molecular weights ranging from 200 to less than 29 kDa. No significant differences were detected between the pathogenic and non-pathogenic strains.

KEY WORDS: Entamoeba histolytica; Axenic strains; Antigens.

INTRODUCTION

Studies on characterization of **Entamoeba histolytica** antigenic components have been widely performed and are relevant for the differentiation of strains as well as for the improvement of the immunodiagnostic methods.

Early attempts to characterize the antigenic composition of **E. histolytica** gave poor results because amebae were grown with bacteria from intestinal tract of man, or in association with other protozoa[17]. Only after development of the axenic culture method[5] it was possible to prepare **E. histolytica** antigens without such contaminants.

The knowledge of the antigenic composition of axenically cultivated amebae is still limited. Many investigators have pointed out the complexity of antigenic preparations used in the immunodiagnostic tests[3, 9, 12].

Different strains of **E. histolytica** have common antigens[2, 4, 12]. The identification of specific antigens could increase the specificity of the immunologic reactions for the amebiasis diagnosis. Besides, as there are differences in the pathogenicity of the strains, the antigenic characterization could be used as another parameter for differentiation of strains.

Several **E. histolytica** strains were isolated and are kept under axenic conditions in Brazil[18]. Very few immunological studies have been done with these strains.

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The aim of the present study was to characterize antigenically pathogenic and non-pathogenic strains of **E. histolytica**, isolated and kept under axenic conditions in Brazil.

MATERIAL AND METHODS

Entamoeba histolytica strains — Trophozoites from four strains of **E. histolytica** grown axenically in TPS-1 medium according to DIAMOND[5] were used. Three of them were isolated and maintained under axenic conditions in Brazil, at the Amebiasis Laboratory (ICB/UFMG) in Belo Horizonte, Minas Gerais, by SILVA et al[18]: ICB-CSP from a symptomatic patient and a high virulence strain; ICB-32 from a patient with non-enteric colitis and a low virulence strain; ICB-462 from an asymptomatic patient and a low virulence strain too. The fourth strain HK-9, isolated in USA by Frye (cited by GEIMAN & BECKER)[7] from a symptomatic patient and kept in axenic conditions by DIAMOND[5], was used as a standard strain.

Antigens — Mass culture of amebae was performed at 37°C in 250 ml culture plastic flasks. Briefly, 72 to 96 hour cultures of amebae were washed five times in Phosphate Buffered Saline (PBS). The trophozoites masses from each **E. histolytica** strain were treated with protease inhibitors[14] and individually disrupted by ultrasonication. Whole extract, soluble extract and lipid free extract were prepared from homogenates of each strain. The soluble lipid free extract was obtained according to TANIMOTO-WEKI et al[21], while soluble extract consisted of the supernatant obtained after centrifugation of whole extract at 20,000 x g at 4°C during 2 hours. The protein content was determined by the method of LOWRY et al[11].

Immune sera — Immune sera to **E. histolytica** were raised by immunizing New Zealand rabbits with four doses of antigen from each amebae strain (two animals/strain). The interval between each dose was fifteen days. In the first dose, animals were given 5 x 10^6 whole trophozoites by intraperitoneal route; in the second dose, whole extract (5 mg protein) emulsified in Freund's Complete Adjuvant (v/v) on the rabbit's back; in he third dose, 5 mg of protein with Freund's Incomplete Adjuvant (v/v) on the haunch. The booster dose of 1.5 mg of protein with isotonic saline was given subcutaneously in the last third of the pads. The animals were bled to death 10 days after the booster dose, and the sera were titred by immunoenzimatic assay (ELISA).

ELISA — 2 μg of whole extract from each strain (20 μg protein/ml carbonate buffer pH 9.6) were adsorbed to microplates wells and incubated overnight at 4°C. The next day, the plates were washed with PBS containing 0.05% Tween 20 and incubated with several dilutions of rabbit's immune sera as well as with negative and positive (infected rabbit) control sera at room temperature for 1 h. All sera were diluted up to 1:20480 in PBS containing 0.3% casein. The plates were washed as before and 100 μl of peroxidase conjugate goat anti-rabbit IgG (diluted in PBS-casein) was added to each well of the plates and incubated as above for 1 h. After the wells had been washed in PBS-Tween 20, 100 μl of 0.002% 0-phenylenediamine (Sigma) in buffer solution (pH 5.0) containing 46 mM Na_2HPO_4, 100 mM citric acid and 0.012% H_2O_2 was added to each well. The reaction was interrupted after 30 min at room temperature by the addition of 20 μl of 4N H_2SO_4. Intensity of colour developed in each well was recorded as optical density (OD) at 492 nm wavelengh.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) — Samples of soluble extract and soluble lipid free extract (60 μg protein) from each strain were solubilized in sample buffer (v/v) containing 10% SDS and 5% 2-mercaptoethanol and heated for three minutes. Electrophoresis was performed, as described by LAEMMLI[10], in a 5 to 12% gradient for separating gels. Molecular weight markers (Sigma) were always used. After electrophoresis, gels were stained with Comassie blue or transferred to nitrocellulose sheets.

Concanavalin-A (Con-A) blot — Glycoproteins binding Con-A were detected on nitrocellulose sheets by a modification of the method of HAWKES[8]. Proteins of soluble extracts and soluble lipid free extracts were previously separated by SDS-PAGE as described above and transferred to nitrocellulose sheet (0.45 μm). After transference, the sheets were washed with PBS containing 0.3% Tween 20 and imersed in lectin buffer (20 mM Tris, 500 mM NaCl, 1 mM MnCL and

0.03% Tween 20) containing 50 mg/ml Con-A (Sigma) for 2 hours at room temperature with gentle rotation. After incubation the supernatant was removed and the sheets were washed three times (15 minutes each) in lectin buffer. The wash solution was removed and replaced with a solution containing 50 μg/ml horseradish peroxidase (Sigma) in lectin buffer. After 1 h at room temperature with gentle rotation, the sheets were washed twice, 10 min each with lectin buffer, followed by two 10 min washes in PBS alone. Glycoprotein binding Con-A were detected using the following substrate solution: 120 mg of 3,3'-diaminobenzidine (Sigma) in 120 ml of PBS and 60 mg of 4-chloro, 1-α-naphthol (Sigma) dissolved in 20 ml of methanol and 100 ml of PBS, 100 μl of 30% H_2O_2.

RESULTS

Immune sera production

Rabbit hyperimmune sera to **E. histolytica** were raised by immunizing the animals with the antigens from HK-9, ICB-CSP, ICB-42 and ICB-462 strains. When tested in ELISA with antigens from homologous and heterologous strains they showed titers ranging from 2560 to 10240 (Table 1).

Electrophoretic characterization of soluble amebae extracts

Samples of soluble extracts and soluble lipid free extracts from each axenic strain were characterized by polyacrylamide electrophoresis (Figures 1 and 2). Better results were seen when soluble lipid free preparations were used presenting more distinct polypeptide bands in SDS-PAGE (Figure 2). The strains revealed a complex profile of polypeptide bands with molecular weights (M.W.) ranging from 200 kDa to less than 20 kDa (Figures 1 and 2). The Brazilian strains ICB-CSP, ICB-462 and ICB-32 showed similar profiles and did not differ significantly from the

TABLE 1
Sera titers in ELISA of rabbits immunized with antigens from axenic strains of **E. histolytica**.

Antigens	Anti-**E. histolytica** sera titers			
	HK-9	ICB-CSP	ICB-462	ICB-32
HK-9	5120	2560	2560	10240
ICB-CSP	10240	5120	5120	5120
ICB-462	5120	5120	5120	10240
ICB-32	5120	5120	2560	5120

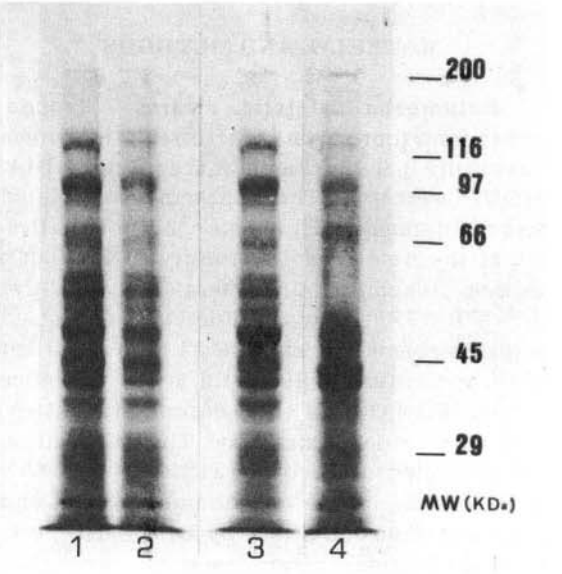

Fig. 1 — Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble extracts from axenic strains of **E. histolytica**. (1) ICB-CSP, (2) ICB-32, (3) ICB-462, (4) HK-9. The protein samples (60 μg/well) were analysed by using gradient gel 5-12% acrylamide and Comassie blue stainning.

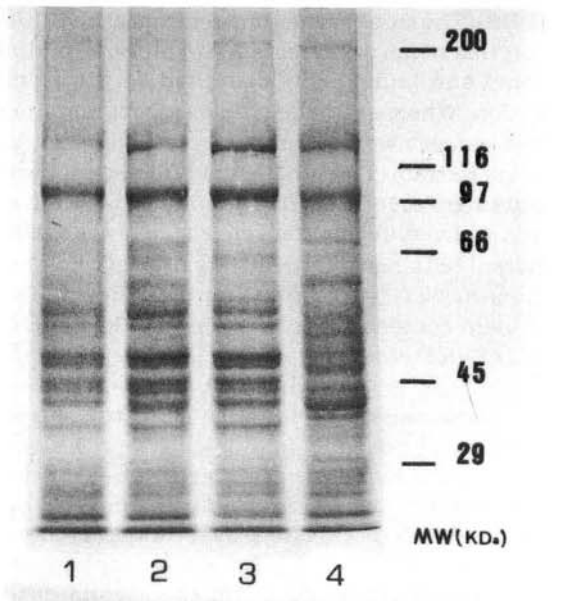

Fig. 2 — Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of soluble lipid free extracts from axenic strains of **E. histolytica**. (1) ICB-32, (2) ICB-CSP, (3) ICB-462, (4) HK-9. The protein samples (60 μg/well) were analysed by using gradient gel 5-12% acrylamide and Comassie blue stainning.

standard strain HK-9. Most of the polypeptides detected in SDS-PAGE were observed in all the four strains analysed.

Glycoproteic characterization of soluble amebae extracts

Glycoproteins binding Con-A present in soluble extracts and soluble lipid free extracts were detected in nitrocellulose membrane (Figure 3). The four strains showed a complex and apparently homogenous glycoproteic profile. The glycoproteic profiles of the strains were very similar without significant differences, showing glycoproteins with molecular weight ranging from 200 kDa to 29 kDa. When soluble lipid free extracts were used, the bands observed were more clear (Figure 3B).

DICUSSION

The characterization of the antigenic components of **E. histolytica** has been one of the objectives in studying this parasite. Their definition is relevant for the improvement of diagnostic methods and for differentiation of axenic strains.

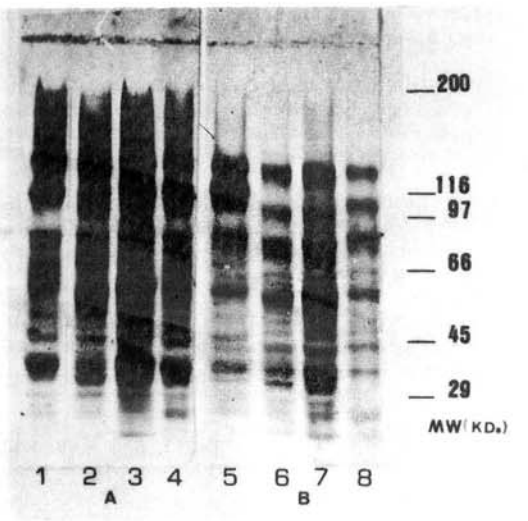

Fig. 3 — Glycoproteic profile of soluble lipid free extracts (A) and soluble extracts (B) from axenic strains of **E. histolytica**. (1) HK-9, (2) ICB-462, (3) ICB-CSP, (4) ICB-32, (5) HK-9, (6) ICB-462, (7) ICB-CSP, (8) ICB-32. Glycoproteins were detected in nitrocellulose sheets by using Con-A. The protein samples (60 µg/well) were previously fractionated by SDS-PAGE.

Antigens from Brazilian axenic strains (ICS-CSP, ICB-32 and ICB-462) and from the standard strain HK-9 showed to be similarly immunogenic, as well as were able to induce production of antibodies showing high titres with homologous and heterologous antigens. This cross-reactivity suggests the occurrence of common antigens between **E. histolytica** strains and have been demonstrated by other investigators[2,4,9].

According to SDS-PAGE analyses, soluble extracts of **E. histolytica** strains revealed complex and similar electrophoretic and glycoproteic profiles and did not differ significantly one from another. Soluble extracts showed more distinct electrophoretic and glycoproteic profiles than whole extracts. Better resolution was observed when soluble lipid free extract was used. Many polypeptides detected in SDS-PAGE showed molecular weight identical or nearly to those detected by AUST-KETTIS et al[2] and MATHEWS et al[12].

Several glycoproteins detected in nitrocellulose membrane showed the same molecular weight to those polypeptides observed in polyacrylamide gels, which demonstrated the glycoproteic composition of many antigens of **E. histolytica**. Furthermore, in the soluble preparations we detected glycoproteins with molecular weights similar to those detected on the surface of the parasite by others investigators[1,2,13,14]. According to AUST-KETTIS et al[2], this coincidence could be explained considering the pronounced phagocytic power and the rapid and continuous cell membrane turnover allowing the presence of antigenic components both on and within the amebae.

We showed that axenic **E. histolytica** strains from Brazil, present a pattern as complex as others strains described in the literature. None of polypeptides identified could differentiate between pathogenic and non-pathogenic strains.

Recent studies by SARGEAUNT and co-workers have demonstrated that **E. histolytica** strains isolated from patients with dysentery show isoenzymatic patterns that differ from those isolated from asymptomatic carriers[15,16]. More recently, it has been reported that monoclonal antibodies (MAbs) and DNA probes also could distinguish between pathogenic and non-patho-

genic strains[6, 19, 20, 22]. However, in spite of the several studies on amebae antigenicity, the immunology of the components responsible for pathogenicity or nonpathogenicity is not well understood.

RESUMO

Entamoeba histolytica: **caracterização antigênica de cepas axênicas do Brasil.**

Trofozoítos obtidos de cultura de **Entamoeba histolytica** isoladas e axenizadas no Brasil (ICB-CSP, ICB-462 e ICB-32) foram utilizados para a produção de soros imunes em coelhos e para a caracterização de antígenos através dos seus perfis eletroforético e glicoproteico, em paralelo com uma cepa padrão isolada e axenizada nos Estados Unidos (HK-9). Obtiveram-se soros hiperimunes, reativos frente aos antígenos homólogos e heterólogos. As quatro cepas em estudo apresentaram perfis eletroforético e glicoproteico complexos e semelhantes, compostos por polipeptídeos com pesos moleculares variando de 200 kDa a menos de 29 kDa. Não se detectaram diferenças significativas entre as cepas patogênicas e não patogênicas.

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