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Relationship between free radicals produced by *Entamoeba histolytica* and its proteases complex activity

M.P. Crisóstomo-Vázquez,* M.P. Cervantes-Cervantes,** E. Jiménez-Cardoso,* and J.L. Muñoz-Sánchez**

ABSTRACT. *Entamoeba histolytica* is a parasite which causes health problems and there has been many approaches to know what is the factor causing its pathogenicity. In the present work, we assayed if the production of free radicals by the amoeba, has a relationship with the proteases activity. When we test the DMSO action (free radicals quenching activity) the specific activity of the proteases complex of the parasite were affected also. At 33.3% (V/V) concentration of DMSO it was present a maximal decrease of the initial activity (about 46% decrease), for to a higher concentrations existing a trend to recuperate the original activity, suggesting that the free radicals are an important factor for the hydrolysis grade of the protein substrate. All the differences except those between 46.7 and 66.6%, were significantly different compared with the control without any addition. The effects of Probucol and Probucol plus DMSO, compared to those caused by Metronidazol (MZ). We can observe that the quenchers caused a decrease on proteases activity similar to that of MZ (which is an antiparasite drug) and it was of c.a. 58% of activity decrease. These data suggest that the action of both, free radicals and proteases complex of *Entamoeba histolytica*, can account for the pathogenicity of the parasite.

Key words: *Entamoeba histolytica*, free radicals, proteases activity.

RESUMEN. La *Entamoeba histolytica* es un parásito que ocasiona problemas de salud y ha habido muchas propuestas para entender qué es lo que causa su patogenicidad. En este trabajo ensayamos si la producción de radicales libres por la amiba tiene relación con la actividad de las proteasas complejas. Cuando probamos el efecto del dimetilsulfóxido (DMSO) la actividad específica de las proteasas del parásito también fue afectada, la concentración de 33.3% (V/V) de DMSO causó la máxima disminución de la actividad inicial (alrededor de 46% de disminución); para concentraciones más altas tendía a recuperarse la actividad original, esto sugiere que los radicales libres son un factor importante para el grado de hidrólisis del sustrato proteico. Todas las diferencias, excepto las comprendidas entre 46.7 y 66.6%, fueron significativamente diferentes comparadas con los testigos (a los que no se les adicionaba nada). Los efectos del probucol y probucol más DMSO se compararon con los producidos por metronidazol (MZ) y se observó que tales supresores disminuían la actividad de las proteasas en forma similar que el MZ (58% de reducción). Estos datos sugieren que la acción de ambos, radicales libres y proteasas complejas de la *Entamoeba histolytica* pueden explicar la patogenicidad del parásito.

Palabras clave: *Entamoeba histolytica*, radicales libres, actividad de proteasas.

INTRODUCTION

The powerful lytic activity of *Entamoeba histolytica* that gave origin to its name has lead to many experimental approaches in order to study what characteristic makes that this protozoan presents pathogenicity. Almost every study on this subject has focused to search for a unique factor, such as: activity from some organelle, toxin or enzyme. Nevertheless it must be remarked that the cytopathic effect that this parasite exerts upon mammalian tissues can not be attributable to a unique factor, but rather to a combination of various chemical as well as mechanical properties.[1] Besides, the destructive effect of amoebas upon its mammalian hosts could not be due only to parasite action but also to the defense mechanisms of the host itself, including the cell lysis of inflammatory cell.[2] A detailed chemical characterization of intracellular factors of parasite harmful to mammalian cells, have discovered the presence of proteinases in it, some of them presenting a cell's lytic activity.[1] The isolation of this kind of proteins has resulted in the identification of various proteolytic activities as for example, a sulphydryllic enzyme with a catepsin B-like activity. Or another with catepsin L-like activity, as well as the presence of other proteolytic or collagenolytic activities on the surface of *E. histolytica* trophozoites.[2,3] In the other hand, the findings made at our laboratories that *E. histolytica* can produce free radicals, as well as the evidence that such free radicals are able to cause biological damage,[4] have prompted us to search if it is any relationship between the proteases complex and the free radical production of the parasite. Since both factors could be of importance to the pathogenicity of the ameba, we try to find if it is any correlation between them.

* Laboratorio de Investigación en Parasitología, Hospital Infantil de México "Federico Gómez".
** Laboratorio de Regulación Celular, Departamento de Bioquímica, ENCB, IPN.

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MATERIAL AND METHODS

Microorganism and Culture Media

Entamoeba histolytica strain HM-1 was axenically cultured in TSY-33 medium5 for 24 or 48 h.

Determination of Proteolytic Activity of E. histolytica *Culture Supernatants*

Cultures of *E. histolytica* with c.a. 10^6 trophozoites/ml were washed with saline 0.15M and diluted 1:10 with 0.1 M phosphate buffer pH 7.4. Further, samples ranging from 0.1 to 0.5 ml were taken and pre-incubated at 37ºC during 10 min. Then 1.0 ml of either 1% casein (Hammerstein) or 1% ovoalbumin (egg white, mildly prepared to avoid denaturation), or different concentrations of either azocasein or azoalbumin (SIGMA Co. St. Louis Mo.) ranging from 0.5 to 6.0 mg/ml was added and incubated at 37ºC for 15 min more. After this incubation 0.5 ml of 20% Trichloroacetic acid (TCA) were added. To avoid turbidity in supernatants, the samples were incubated overnight at 0-4ºC. Next day the tubes were centrifuged at 2000 x g; then supernatants were placed in clean tubes and absorbance at 280 nm measured (for casein and ovoalbumin) and at 435 nm (azocasein or azoalbumin) in a Shimadzu-2000 spectrophotometer. Blanks were prepared just the same as samples, but substrates were added after 20% TCA addition. A tyrosine-working curve was constructed by use of increasing concentrations of the aminoacid from 0 to 500 µg/ml and absorbance readings made at 280 nm. Protein measurements were made using Lowry[6] standard method.

Absorbance values were intercepted on working curve, the proteolytic activity was obtained and expressed as freed tyrosine (µg/min). Specific activity (SA) for casein and albumin substrates was obtained relating the activity to the total protein amount. For azoalbumin and azocasein, activity is presented as Absorbance units at its corresponding wavelength.

In some cases, protease inhibitors (p-Hydroxy-mercuribenzoic acid or Phenylmethyl-sulphonyl fluride, SIGMA Co. St. Louis MO) or free radicals quenchers (Dimethyl sulfoxide or Probucol) were added as indicated latter. Also we tested the effect of adding to the reaction medium Metronidazol, in order to compare its action to those of either the free radical quenchers or the inhibitors.

RESULTS

In Fig. 1, Panel A, we can observe the effect of Dimethyl sulfoxide (DMSO) upon the specific activity of the proteases complex (means ± SD of five determinations). At 33.3% (V/V) concentration of DMSO it was present a maximal decrease of the initial activity (about 46% decrease), for to a higher concentrations existing a trend to recuperate the original activity, suggesting that the free radicals are an important factor for the hydrolysis grade of the protein substrate. All the differences except those between 46.7 and 66.6%, were statistically significatives ($p < 0.05$). In Panel B are represented the effects of Probucol and Probucol plus DMSO, compared to those caused by Metronidazol (MZ). We can observe that the quenchers caused a decrease on proteases activity similar to that of MZ (which is an antiparasite drug) and it was of c.a. 58% of activity decrease. In Panel C, is depicted the effect of lower DMSO concentrations (ranging this time from 0.3 t0 1%), since we wanted to know if at these concentrations there was an effect free of the interference caused by the lowering of medium dielectric constant by DMSO, which causes a better activity of the proteases. As can we see in this figure, there exists a decrease in activity of proteases complex, even greater than that caused by 6.67% DMSO concentration (46% loss of activity in Panel A against 90% from results of Panel C at 1% DMSO concentration). In Panel D we present the results of inhibiting either cysteinic or serinic proteases or both, by action of (in former case) either of p-Hydroxy-mercuribenzoic acid (p-HMB) or Phenylmethyl-Sulphonyl Fluoride (PSF) or a mixture of both. The inhibition caused by p-HMB accounts for a 26% of total proteases activity, while that caused by PSF is an 18% of the whole. When both inhibitors were used together, a 54% inhibition of total protease activity was obtained.

DISCUSSION

In the experiments to assess the DMSO effect, the fact of that there is a decrease in the proteases activity, suggest that oxygen free radicals produced by the amoeba are an important factor for the hydrolysis grade of protein substrate. Since it is unlikely that DMSO were an inhibitor of parasite proteases, then the results would indicate that free radicals produced by *E. histolytica* helps to the proteases action by denaturing the substrate and in presence of DMSO, such denaturation is in a lesser extent, providing so a resistance of protein substrate to be digested. The later rise at 47 and 66% DMSO (whose differences in specific activity were not significatives $p < 0.05$) , could be due to an improvement of the enzymatic activity caused by the decrease of medium dielectric constant, which causes a rearrangement of enzyme structure making it to be more efficient in its catalytic capacity.

In regard to the results obtained in the experiments were we assayed the effect of quenchers (Probucol and DMSO), comparing it with that of Metronidazol (an ati-

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parasite drug), they reinforce the former results obtained with DMSO, since Probucol as it was said in the case of DMSO, also is unlikely that it inhibits the proteases activity, rather it could act by impeding the substrate denaturation by amoeba free radicals action. Metronidazol could act in this context by an indirect way. When the drug acts

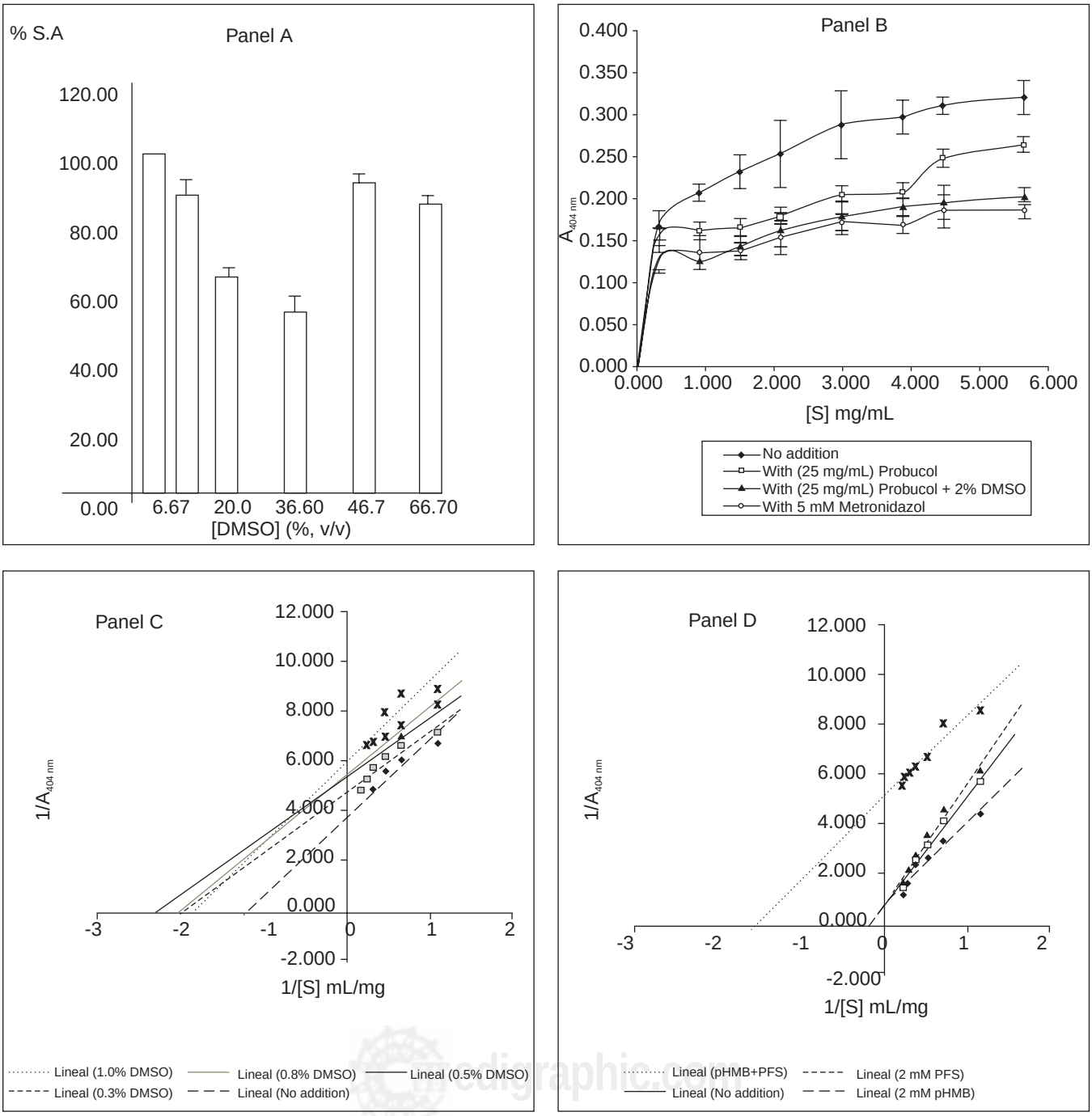

Figure 1. *Assessment of relationship between total proteolytic activity of* E. Histolytica *and free radical production by its trophozoites. Panel A. Effect of dimetyl sulfoxide (DMSO) on the specific activity of parasite proteases. Panel B. Comparision oh inhibitions caused by probucol, probucol plus DMSO and metronidazol to* E. Histolytica *proteolytic activity. Panel C. Lineweaver-Burk representation of inhibitory action of DMSO upon proteolytic activity of trophozoites. Panel D. Lineweaver-Burk representation of inhibitions caused by either pHMB, PFS or pHMB plus PFS.*

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upon the parasite, it could impair the free radical production as well as the protease synthesis of *E. histolytica*. The fact that Metronidazol rendered a similar inhibition of proteases activity than that of Probucol and DMSO together, could indicate that in a certain way, it possibly present too a quenching activity. The results depicted in panel C, for comparing the effect of DMSO at lower concentrations, confirmed our suspicion that what we observed in the results of Panel A, is a mixture of effects of DMSO (DC activating effect versus quenching activity of DMSO), and that indeed, there is a synergism between the free radicals action and proteases activity. Finally, from the results presented in Panel D, where the inhibition grade caused by p-HMB is a 26% while that of PSF is of 18% and the one when both acted together was of 54%, we interpreted this fact as a synergism between both inhibitors giving the mixture of both a better inhibition activity. The last figure of a 54% of inhibition, would mean that the activity of both, cysteinic and serinic proteases accounts for at least a 55% of the bulk *E. histolytica* proteases activity. Taking together the results of this work, we can say that the concerted action of both, free radicals produced by amoeba and the activity of the complex of proteases of it, could account for an important factor of pathogenicity of *Entamoeba histolytica*. It would be important since in nature no one factor alone could be responsible for a given characteristic1, as in the present case, the pathogenicity of this parasite.

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Correspondence to:

M.P. Crisóstomo-Vázquez
Dr. Márquez 162, México, D.F.
06720, México. Fax. 5761-0303
E-mail: enedina@servidor.unam.mx