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Entamoeba histolytica: surface proteolytic activity and its relationship with *in vitro* virulence

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Abstract. We determined the surface-associated proteolytic activity in three *Entamoeba histolytica* Schaudinn, 1903 strains (monoxenic HM1, axenic HM1, and HK9) of known virulence and its relationship with collagenase activity. Both activities were also determined in axenic HM1 amoebae trophozoites which were sensitive and resistant to complement-mediated lysis. Surface proteolytic activity was determined in glutaraldehyde-fixed *E. histolytica* trophozoites, which degraded the insoluble substrate, hide powder azure, and cleaved the human immunoglobulin G heavy chain in a time-dependent fashion, at neutral pH, in presence of 2-mercaptoethanol as cysteine protease activator. Surface proteolytic activity was strain dependent: monoxenic HM1 > axenic HM1 > axenic HK9. This activity correlated with collagenolytic activity ($p < 0.05$). Acquisition of resistance to complement-mediated lysis by axenic HM1 strain did not modify either surface proteases or collagenase expression. Our results suggest that this surface proteolytic activity could be used as an *in vitro* virulence marker for *E. histolytica*.

Entamoeba histolytica Schaudinn, 1903 (cited by Martínez-Palomo 1982) is a protozoan parasite that infects humans, causing amoebiasis. This enteric protozoan infects more than 500 million people. *E. histolytica* trophozoites can invade the intestine causing mucosal ulceration and colitis; in addition, amoebae can enter the bloodstream and cause liver abscesses (Walsh 1986). The pathogenesis of *E. histolytica* is a multifactorial process dependent on expression of amoebapore (Leippe 1997), collagenase (Muñoz et al. 1982), and proteases (McKerrow et al. 1993) as well as the ability to evade the host's immune response. Immune evasion by amoebae includes mechanisms such as downregulation of macrophage function, suppression of T-cell function (Campbell and Chadee 1997), capping of membrane-bound antibodies (Calderón et al. 1980), resistance to complement-mediated lysis (Calderón and Tovar 1986) and degradation of C3a and C5a complement-derived proteins (Reed et al. 1995), as well as immunoglobulins (Tran et al. 1998). Likewise, membrane and secreted cysteine proteases have been implicated in amoeba-host interplay (Que and Reed 1997). Proteases on the amoeba's surface could also damage cell surface proteins on target cells, besides other cytotoxic events dependent on cell-contact (Arias-Negrete and Chadee 1996). Several amoebae proteolytic activities associated with membranes have been described (Ostoa-Saloma et al. 1989, Jacobs et al. 1998); a 70 kDa major constituent has been identified in amoebic membrane preparations which can be re-incorporated into artificial membranes (Avila and Calderón 1993); this activity has been defined as surface proteolytic activity (SPA). Virulent isolates of *E. histolytica* were resistant to complement lysis (Reed et al. 1983), and axenic HM1 trophozoites resistant to complement-mediated lysis showed increased levels of N-acetylglucosaminidase (Arias-Negrete et al. 1992). The purpose of this study was to evaluate whether SPA could be utilised as a virulence marker of *E. histolytica* by contrasting it with collagenase activity in amoebae strains of known *in vivo* virulence, as well as in amoebae which are susceptible and resistant to complement-mediated lysis.

MATERIALS AND METHODS

Cultures and strains of *Entamoeba histolytica.* Trophozoites of *E. histolytica* HM1:IMSS and HK9 axenic strains were cultured in TYI-S-33 medium (Diamond et al. 1978) at 37°C. Monoxenic HM1 strain of *E. histolytica* was cultured with *Clostridium symbiosum* (ATCC No. 14940) (Anaya-Velázquez et al. 1985) under similar conditions. Trophozoites were harvested in logarithmic phase of growth for all the experiments.

Surface proteolytic activity. *Entamoeba histolytica* rapidly turns over its plasma membrane every 20 minutes (Gitler and Mirelman 1986) and secretes proteases (Que and Reed 1997). To ensure that proteolytic activity was due to proteases present on the amoebae surface and not to endocytosis of substrate or protease secretion, trophozoites were fixed with glutaraldehyde as described by Etges et al.

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(1986) in studies of surface proteolytic activity in *Leishmania. E. histolytica* trophozoites (viability ≥ 98%) were harvested, washed once with phosphate-buffered saline (PBS-A, 175 mM NaCl, 0.15 mM phosphate, pH 7.2), fixed with 2.5% glutaraldehyde (Grade II, Sigma) in PBS-A for 30 min at 4°C, treated with 250 mM glycine (pH 7) for 30 min (hereafter glutaraldehyde fixed-*E. histolytica*, GF-Eh), and washed twice again with PBS-A (Avila and Calderón, 1993). Proteolytic activity was determined as follows: 1×10^6 GF-Eh in a 0.5 ml final volume were incubated with 5% 2-mercaptoethanol (2-ME) and 1 mg Azure (Sigma). After 40 min of incubation, the absorbance (600 nm) of the supernatant was measured (Rinderknecht et al. 1968). Human immunoglobulin G (IgG; 100 µg) was also used as substrate; after centrifugation, the cell-free supernatant was analysed by 10% SDS-PAGE (Laemmli 1970). SPA was determined in presence of trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) and *p*-hydroxymercuribenzoate (*p*-HMB), which are cysteine protease inhibitors.

Collagenolytic activity. Collagenolytic activity was evaluated by incubating amoebae (20,000; viability ≥ 98%) for 16 h with human type I collagen films (Muñoz et al. 1984). Following incubation, the remaining films were stained with Syrian Red, washed with 0.1 N HCl, and the collagen-bound dye was eluted by treatment with 0.01 N NaOH in methanol, and the OD (540 nm) was measured. One unit of collagenase corresponds to 1 µg of digested collagen per minute at 37°C.

Induction of resistance to complement-mediated lysis in amoebae. *Entamoeba histolytica* HM1 axenic strain trophozoites were repeatedly exposed to human lytic serum as previously described (Calderón and Tovar 1986). As controls, other group of amoebae were also exposed to heat-inactivated human serum and to TYI-S-33 culture medium. Resistance to complement lysis was assessed by exposure to 10 and 25% v/v lytic serum. Heat-inactivated human serum was utilised as a control.

RESULTS

Micromethod for SPA detection. One million GF-Eh HM1 trophozoites were sufficient to detect surface proteolytic activity (SPA) using the insoluble substrate, Azure. After fixation to stop endocytosis and exocytosis, amoebae were incubated for 1 hour at 37°C, the supernatants and pellets were separated, and proteolytic activity was measured in both fractions. Pelleted GF-Eh had most of the proteolytic activity (90%), whereas the cell-free supernatant had the 10% of the proteolytic activity (Fig. 1). This indicated that little shedding of membrane-bound proteases was occurring. For the purpose of this work, SPA was determined in GF-Eh without separation of released protease.

Effect of reducing agents on SPA. This surface proteolytic activity is a cysteine protease, since its activity was inhibited by cysteine proteinase inhibitors: irreversibly by 50 µM E-64 (96% inhibition), and reversibly by 0.5 mM *p*-HMB (91% inhibition); 5% v/v 2-ME reversed *p*-HMB inhibition (by 80%). Basal SPA

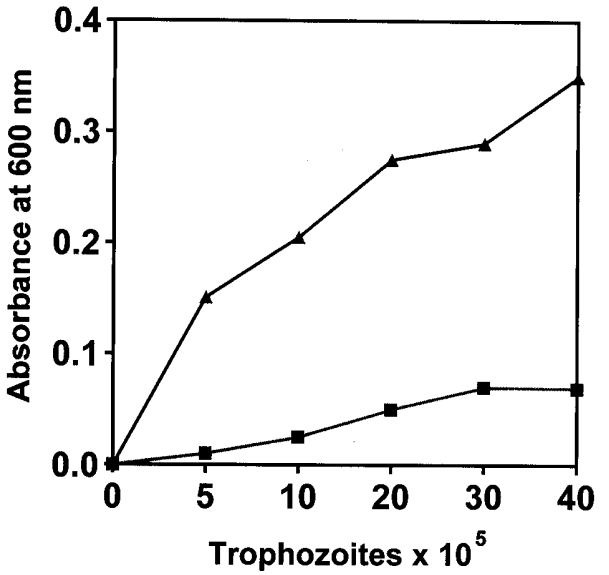

Fig. 1. Surface proteolytic activity of *Entamoeba histolytica.* Increasing amounts of trophozoites from 72 hours of culture were fixed with glutaraldehyde (GF-Eh) and after 30 min of incubation were centrifuged; pellet (1×10^6 GF-Eh) (▲) and its supernatant (■) were obtained and incubated with Azure (1 mg) for 40 min. Absorbance (600 nm) was determined. This experiment was repeated twice by duplicate.

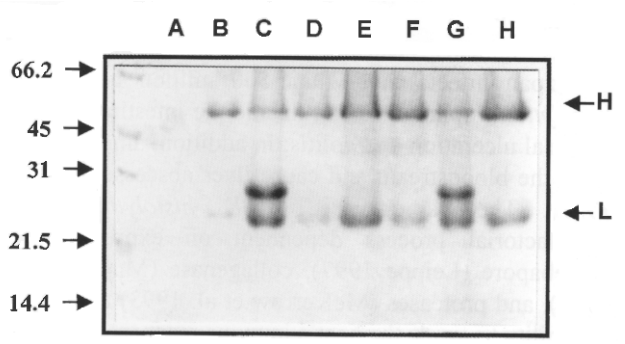

Fig. 2. Effect of cysteine protease inhibitors on SPA. Trophozoites were fixed with glutaraldehyde; then 5×10^5 GF-Eh were incubated with 100 µg of human IgG at 37°C for 90 min under the following conditions: (A) supernatant from GF-Eh incubated without human IgG, (B) no 2-ME was added, (C) 5% v/v 2-ME, (D) 50 µM E-64, (E) 50 µM E-64 plus 5% v/v 2-ME, (F) 0.5 mM *p*-HMB, (G) 0.5 mM *p*-HMB plus 5% v/v 2-ME, (H) native human IgG. After incubation time, the cell-free supernatant was electrophoresed. H and L, at right, correspond to IgG light and heavy chains. Numbers at left correspond to the molecular weight (kDa) of markers. This is a representative result of two independent experiments.

(0.1 O.D.) was activated (up to 2.6 times) by 2-ME in a dose-dependent fashion (data not shown). To further support that SPA is a cysteine protease, degradation of native human IgG was analysed in the presence of

cysteine protease inhibitors and/or reducing agents. PAGE analysis indicated that SPA from GF-Eh did not degrade human IgG in the absence of 2-ME (Fig. 2, lane B), whereas 2-ME activated SPA and a degradation product (PD) was generated, possibly from the IgG heavy chain (Fig. 2, lane C); E-64 inhibited irreversibly SPA from GF-Eh, since human IgG was not degraded even after 2-ME addition (Fig 2, lanes D and E). SPA Inhibition by *p*-HMB was reversed by 2-ME (Fig. 2, lanes F and G).

Dependence of SPA activity on pH. The effect of pH on SPA was also evaluated. This proteolytic activity was detected in a wide pH range (4-8.5). Maximum activity was found between pH 6.5 and 7.5 (Fig. 3) suggesting that SPA is a neutral protease.

SPA degraded human immunoglobulin G. Native human IgG was incubated with GF-Eh in the presence of 2-ME for 5, 30, 90 and 120 min and the proteolytic products were evaluated in the supernatant. No IgG degradation was detected in the control without amoebae. A time-dependent degradation of the heavy chain of IgG was found following incubation with GF-Eh (Fig. 4). Supernatant from GF-Eh incubated without IgG did not show any material detectable in Coomassie blue stained gels.

Temperature fixation affects SPA. Amoebae trophozoites were fixed with glutaraldehyde at 4°C, 20°C and 37°C, and the SPA was determined at 37°C. The proteolytic activity was higher in trophozoites fixed at 4°C, whereas only 40% and 10% of this activity was observed when amoebae were fixed at 20°C and 37°C respectively (Fig. 5). Therefore, the temperature selected for the amoebae fixation was 4°C.

Surface proteolytic activity and collagenase in amoebae strains of known virulence. Three strains of *E. histolytica* of known *in vivo* virulence (Anaya-Velázquez et al. 1985): monoxenic HM1 > axenic HM1 > HK9 were used to determine SPA (Fig. 6A). The monoxenic HM1 strain showed a 25% higher SPA compared with the axenic HM1 strain ($p < 0.05$), considered as the reference strain (100%). Moreover, differences between axenic HM1 and HK9 strains were observed: *E. histolytica* HK9 strain only showed 9% of the SPA of the reference strain (HM1 > HK9; $p < 0.05$). Collagenolytic activity showed a similar pattern as SPA (Fig. 6B).

Collagenolytic and surface proteolytic activities in amoebae resistant to complement-mediated lysis. After repeated *in vitro* exposure to lytic human serum, amoebae from axenic HM1 strain became resistant to complement lysis, whereas those exposed to heat-inactivated human serum or modified TYI-S-33 remained susceptible as described by Calderón and Tovar (1986). When SPA and collagenolytic activity were assayed in these trophozoites, it was observed that both susceptible and resistant amoebae had similar levels of SPA and collagenase (Fig. 7).

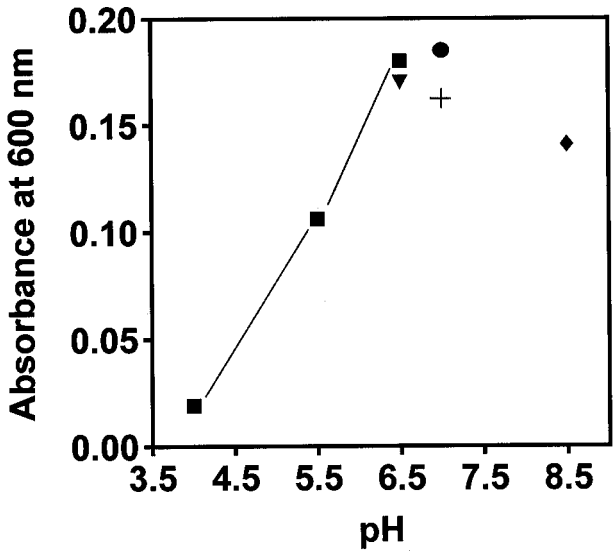

Fig. 3. Dependence of SPA on pH. Trophozoites were fixed with glutaraldehyde at pH 7.5. Afterwards, fixed amoebae were resuspended in buffers at different pH's. The following buffers were employed: (■) citrate-phosphate for pH 4, 5.5 and 6.5; (▼) PIPES for pH 6.5; (●) PBS-A or (+) Tris-HCl for pH 7.5; (◆) Veronal for pH 8.5. This is a representative result of two independent experiments.

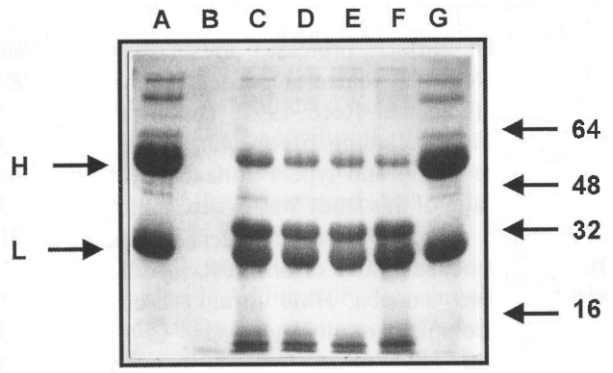

Fig. 4. Amoebae SPA degrade human immunoglobulin G. One million GF-Eh were incubated with human IgG for 5, 30, 90 and 120 min, afterwards the cell-free supernatants were electrophoresed in PAGE gels (lanes C-F respectively). (A) corresponds to undegraded human IgG (arrows indicate heavy and light chains). (B) is the supernatant of GF-Eh incubated for 120 min in absence of immunoglobulin. (G) corresponds to immunoglobulin incubated for 120 min in PBS-A. Numbers at right correspond to the position of molecular weight markers (kDa). Gel was stained with Coomassie blue. Results from a representative experiment repeated twice.

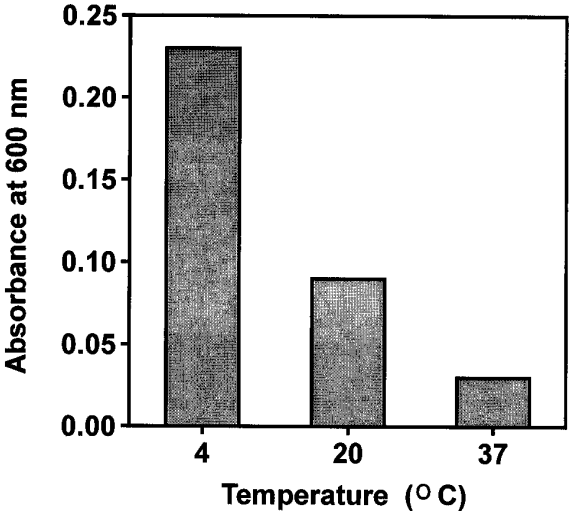

Fig. 5. Temperature of fixation affects surface proteolytic activity. *Entamoeba histolytica* HM1 strain trophozoites were fixed with glutaraldehyde at the indicated temperatures; afterwards SPA was determined at 37°C. Absorbance (600 nm) was determined in the supernatant. Results from a representative experiment performed by duplicate.

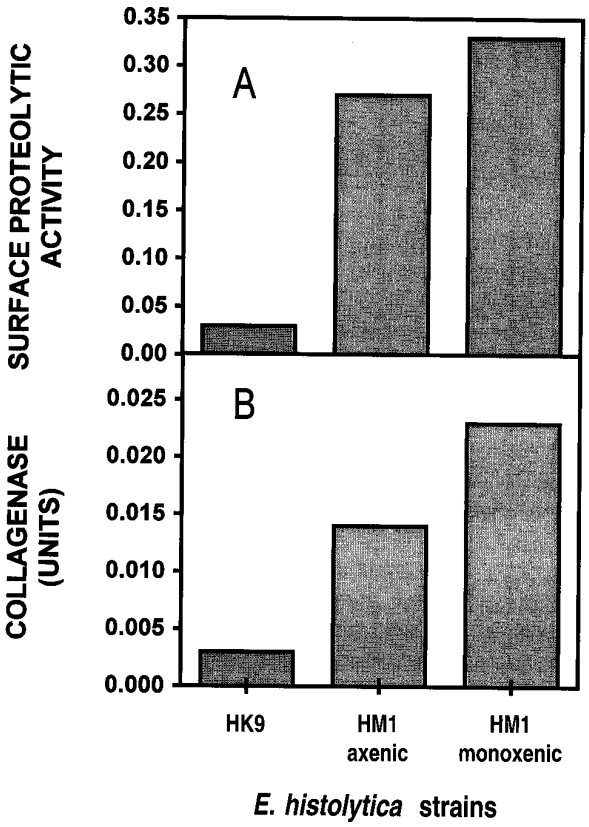

Fig. 6. Comparison between collagenolytic activity and SPA in amoebae strains. Surface proteolytic activity as well as collagenolytic activity was tested in three glutaraldehyde-fixed *Entamoeba histolytica* amoebic strains by using Azure as substrate; absorbance (600 nm) was measured in the supernatant **(A)**. Collagenolytic activity by live trophozoites was measured on human type I films. One unit of collagenase corresponds to 1μg of digested collagen per minute at 37°C **(B)**. These experiments are representative of three independent experiments performed by duplicate.

DISCUSSION

Proteases play a crucial role in host-parasite interplay in protozoan infections (McKerrow et al. 1993). Accordingly, surface proteases must likely play an important role in contact-dependent damage by *E. histolytica* (Que and Reed 1997) and in cleavage of matrix proteins including fibronectin, laminin (Schulte and Scholze 1989) and type I collagen (Muñoz et al. 1989). The aim of this study was to determine the role of SPA and to compare it with collagenase (Muñoz et al. 1984) in amoebae strains of different virulence, as well as in axenic amoeba HM1 strain susceptible and resistant to complement-mediated lysis.

In several *Leishmania* species, a major surface protease (p63) was detected in glutaraldehyde-fixed promastigotes (Etges et al. 1986). A similar approach was utilised to study amoeba surface proteases, because of the active exocytosis-endocytosis by *E. histolytica* trophozoites (Gitler and Mirelman 1986). Glutaraldehyde fixation conserved and stabilised proteases present on the amoeba surface, in addition to preventing exocytosis/endocytosis of substrates. SPA corresponds to the 50% of the proteolytic activity detected in live trophozoites activated with 2.85 mM cysteine and was inhibited (90%) by 100 μM E-64 (data not shown). Amoebae membrane integrity was previously assessed by ruthenium red exclusion (Avila and Calderón 1993). Therefore, Azure and human IgG were mainly degraded by SPA from *E. histolytica,* although 10% of the SPA was shed from GF-Eh during the assay period. The main released proteolytic activity has been shown to be a 70 kDa protein by SDS substrate gels under non-reducing conditions; this suggests a weak association of proteases with plasma membrane. Therefore, SPA is possibly the result of several proteases present on amoeba surface; some of them were being secreted or weakly bound to plasma membrane, as previously suggested Scholze et al. (1992).

At present, six genes coding for 27 to 30 kDa mature cysteine proteinases have been reported (Reed et al. 1993, Bruchhaus et al. 1996, Mirelman et al. 1996), but the location of each genetic product is not known, with the exception of the protease EhCP5 which is proposed to be surface-associated (Jacobs et al. 1998). The main difficulty in assessing the location of each protease is the similarity of SH-proteases (43% to 87% amino acid identity with many conservative changes) (Jacobs et al.

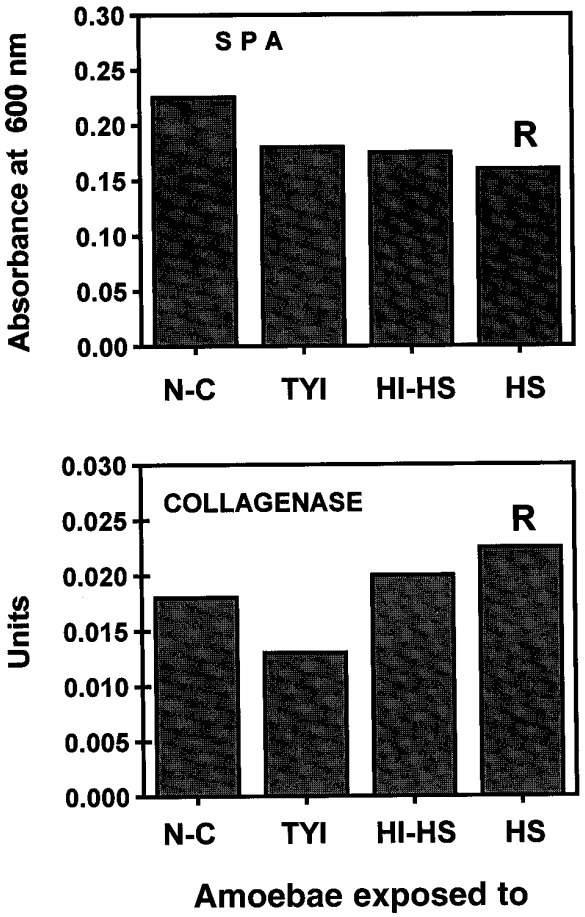

Fig. 7. Collagenolytic activity and SPA in amoebae resistant to lysis by complement. Both activities were determined in amoebae treated as described in Fig. 6. N-C corresponds to amoeba normal cultures taken as control sensitive to complement lysis. "R" indicates that amoebae exhibited resistance to complement-mediated lysis, the other groups remained susceptible. These results are representative of three independent experiments performed by duplicate.

1998); furthermore, differential inhibitors or substrates to distinguish among them have not been described. At present, knowledge of proteases gene sequences does not fully explain the heterogeneity of the different molecular weight amoebae proteases described.

Proteases ranging from 73 to 23 kDa have been detected in substrate gels (Ostoa-Saloma et al. 1989, Avila and Calderón 1993). Proteases with molecular weights as high as 56 and 60 kDa have been purified (Reed et al. 1995, Spinella et al. 1997). Some kind of aggregation of low molecular weight proteases could explain these differences; however, definitive evidence of the relationship among proteases encoded by different genes is lacking.

In order to understand amoeba-host interplay, it must also be considered that host inhibitors might circumvent amoeba protease activity. Accordingly, human colostrum antibodies containing anti-amoeba IgA antibodies partially inhibited proteolytic activity by *E. histolytica* (Rico-Mata and Avila 1997). In this study we described the cleavage of human IgG by SPA. Degradation of human IgA by *E. histolytica* proteases was already documented (Kelsall and Ravdin 1993). The purified extracellular cysteine proteinase and the live *E. histolytica* degraded IgG, products of the proteolysis of the heavy chain of IgG were found in amoebic pellets (Tran et al. 1998), but this is the first report indicating human IgG degradation by SPA, which suggests another possible mechanism to evade the host immune response.

The SPA we described corresponds with a neutral cysteine protease since its activity was dependent on sulfhydryl groups active at a neutral pH. SPA was also inhibited by E-64 and *p*-HMB, which are specific cysteine-protease inhibitors. Moreover, SPA activity is well preserved when amoebae are fixed with glutaraldehyde at a low temperature, suggesting that prevention of SPA degradation or possibly conformational changes could modulate its activity.

Collagenase is a virulence marker (Muñoz et al. 1984) which is secreted in electron-dense granules (EDG) after 16 h of incubation with collagen films (Muñoz et al. 1990). From our results, comparison of SPA and collagenase in three amoebae strains (axenic HM1 and HK9, and monoxenic HM1 strains) showed a good correlation (r = 0.98). As mentioned above, collagenase activity is time- and substrate-inducible, whereas SPA is constitutive; suggesting those are independent proteolytic activities. Furthermore, cysteine and bovine serum present in normal amoebae culture interfere with collagenase expression (Muñoz et al. 1982).

Therefore, SPA could also be utilised as an amoeba virulence marker. Additionally, erythrophagocytosis (Orozco et al. 1983) and cytotoxicity (Arias-Negrete et al. 1991) also correlate with amoebic virulence, and with our results. However, other studies are controversial, since Montfort et al. (1992) described variations in the total proteolytic activity and a lack of correlation with phagocytosis in several HM1 pathogenic strains of *E. histolytica*. In our study, no significant variations in SPA and collagenase were observed in independent experiments by utilising three amoeba strains with different virulence.

The complement system is a natural defence mechanism against microorganisms (Joiner 1988, Tomlinson 1993). *E. histolytica* trophozoites resist complement-mediated lysis (Calderón and Tovar, 1986) by binding CD59 complement regulatory protein from human erythrocytes (Flores-Romo et al. 1994, Gutiérrez-Kobeh et al. 1997). In addition, the galactose-specific adhesin also regulates complement deposition

(Braga et al. 1992). We were interested in determining if, along with development of resistance to complement lysis by *E. histolytica* HM1 strain, a modification in SPA and collagenase could be induced. Previous studies have indicated that amoeba resistant to complement lysis has increased its N-acetylglucosaminidase levels (Arias-Negrete et al. 1992). Co-culture of amoebae with *Clostridium symbiosum* also increased *E. histolytica* virulence (Anaya-Velázquez et al. 1985). Amoebae resistant to complement-mediated lysis as well as the susceptible controls showed similar SPA and collagenase expression. These results indicate that acquisition of resistance to complement-mediated lysis and a modification in SPA and collagenase by amoebae are probably independent phenomena. It is possible that different or additional stimuli are needed to modify virulence factors.

In conclusion, our results confirmed that SPA is a sulfhydryl activity corresponding with a neutral protease that degrades the human immunoglobulin G heavy chain. Importantly, a correlation of SPA with collagenase type I activity was demonstrated in several amoeba strains. No differences between susceptible and resistant amoebae were found. Based on these results, we proposed SPA as a virulence marker in *E. histolytica* strains with different degree of virulence.

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