Free Fatty Acids Released from Phospholipids Are the Major Heat-Stable Hemolytic Factor of *Entamoeba histolytica* Trophozoites

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The major hemolytic activity of *Entamoeba histolytica* trophozoites is located in a vesicular fraction called P30 and known to be due to heat-labile and heat-stable hemolytic components whose effect increases up to 100 times during preincubation at 36°C. The heat-stable hemolytic activity (HSHA) was found in the chloroform-methanol extract of preincubated P30, whose partition with 2 M KCl yielded a lipid fraction, an interphase, and an aqueous phase. HSHA was detected only in the lipid fraction, where it amounted to 59% of the chloroform-methanol extract activity and increased 50% when supplemented with the interphase material; it was accounted for by the free fatty acids, whose potency increased 33% with the interphase material, and was blocked by delipidated bovine serum albumin. A parallel increase in free fatty acids and lysophospholipids and a corresponding decrease in phospholipids were observed during P30 preincubation. Most of the phospholipase activity of trophozoite homogenates was also found in P30. Therefore, most of the HSHA generated during preincubation was due to free fatty acids released from phospholipids by a P30 phospholipase that may contribute significantly to *E. histolytica* cytopathogenicity and virulence.

Cytolytic activity, considered a major determinant of *Entamoeba histolytica* cytopathogenicity and virulence (17, 24), has been studied in different experimental systems in which the effects of intact trophozoites (12, 17-19, 21, 25, 26) and their homogenates or subcellular fractions (15, 22, 26, 27, 35) were analyzed on animal cells in suspension (12, 15, 22, 26, 27) and on cell cultures (17-19, 21, 25, 26, 35).

Studying the interaction of *E. histolytica* trophozoite extracts with erythrocytes, we found that the cytolytic potency of amoebal extracts is strain specific and correlates with virulence (15). Hemolytic activity is located in a vesicular fraction called P30, whose effect is maximum in the presence of Ca²⁺ at pH 8 (27) and due to latent heat-labile and heat-stable components activatable by incubation at 36°C (28). From these findings, we suggested that the heat-labile hemolytic activity (HLHA) may be due to an enzyme (28). Two *E. histolytica* phospholipases that may contribute to HLHA have been described recently (13, 25).

In this paper we show that the increase in heat-stable hemolytic activity (HSHA) observed during preincubation of P30 at 36°C was accompanied by an increase in free fatty acids (FFA) and lysophospholipids and a concomitant decrease in phospholipids. FFA were the only heat-stable components with hemolytic activity, and their effect was increased by an amphipathic factor devoid of hemolytic activity per se. FFA thus determine the major HSHA of P30 and appear to be released from phospholipids by a phospholipase that could also be involved directly in the determination of *E. histolytica* cytopathogenicity and invasiveness.

MATERIALS AND METHODS

Parasites. Trophozoites of *E. histolytica* HK9 (14) were used. They were axenically cultured in TPS-1 medium (4), incubated at 36°C, and harvested at the end of the log phase.

Subcellular fractionation. Subcellular fractions and their protein content were obtained as described previously (27, 28). In a typical experiment, 8×10^7 trophozoites (containing 46 mg of protein) were washed twice in cold phosphate-buffered saline (4), suspended in 2 volumes of cold balanced salt solution (BSS: 1 mM CaCl₂, 5.5 mM glucose, 136.5 mM NaCl, 5.5 mM KCl, 1 mM MgSO₄, 1 mM Tris, 3 mM sodium azide [pH 8]; 290 mOsm/kg), and disrupted with a Potter-Elvehjem homogenizer. From total homogenates, the nuclear (N) fraction was obtained as the sediment formed after 5 min of centrifugation at $135 \times g$ and 4°C; the postnuclear supernatant was then centrifuged for 15 min at $30,000 \times g$ and 4°C, and the resulting pellet (vesicular fraction, P30) was separated from the corresponding supernatant (S30) and stored in liquid nitrogen.

Activation of hemolytic components. HSHA and HLHA were determined in P30 samples kept in liquid nitrogen and thawed by immersion in water at 36°C, suspended in 2 volumes of BSS, and then incubated for 24 or 36 h at 36°C. The heat-labile hemolytic component was inactivated by heating for 9 min at 90°C (28).

P30 partition. The procedure for P30 partition was based on the method of Folch et al. (5) as modified by Vorbek and Marinetti (37). P30 samples were precipitated with cold 5% trichloroacetic acid (TCA) and centrifuged for 10 min at $600 \times g$; the sediment was suspended with 8 volumes of methanol at 40°C and allowed to cool at room temperature, and to it 16 volumes of chloroform were then added to obtain the chloroform-methanol (CM) extract. This was centrifuged for 15 min at $600 \times g$, and its supernatant was subjected to

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partition with 1 volume of 2 M KCl and centrifuged for 15 min at $600 \times g$ to separate the lipid fraction, the interphase, and the aqueous phase. The hemolytic activity was assayed immediately in all the fractions obtained with this procedure, except the TCA-soluble one.

Preparation of lipids. A mixture containing ovoidlecithin, lysolecithin, and FFA was prepared by partial hydrolysis of ovoidlecithin with bee venom phospholipase A_2 ; the reaction was stopped with 5% TCA in butanol. The lipids of this reaction as well as total lipids from rat liver, egg yolk, and human serum were extracted and subjected to partition as for P30. FFA from rat liver and human serum lipids were purified and saponified by the methods described by Radin (23).

TLC and identification of lipids. Lipid chromatography was based on the method of Skipski and Barclay (29). Two solvent systems were used: chloroform-methanol-acetic acid-water (170:40:16:8) for polar lipids, and petroleum ether-ethyl ether-acetic acid (180:20:2) for nonpolar lipids (2). The relative migration (R_f) and specific color reaction (29) were determined and confirmed for each lipid used as a standard in thin-layer chromatography (TLC). Identification of polar and neutral lipids was performed by specific staining and by comparing the R_f and color of the amoebal lipid spots with those of standard lipids.

Identification of the HSHA. In each experiment, two types of chromatoplates (A and B) were prepared. Portions containing 360 μ g of total P30 lipids were applied at the origin of A plates; on B plates, identical samples of P30 lipids were applied next to samples containing 400 μ g of total rat liver and human serum lipids. On B plates, cholesterol and its esters were visualized as blue-violet spots after heating in the presence of concentrated sulfuric acid; other lipids were developed as brown spots after further heating of the plates (29). With the B plates as a reference, the areas corresponding to each of the separated lipid spots were scraped off from several A plates. The pooled silica gel particles were extracted three times with CM (4:1) at 25°C and then with ethyl ether at 40°C for 10 min; solvents were evaporated with an N_2 stream. The dry weight of the extracts was determined, and their hemolytic activity was tested in BSS immediately or after variable periods of storage in liquid nitrogen. The TLC-purified amoebal lipids having hemolytic activity were rechromatographed to confirm their purity; their chemical identity was verified by comparing their chromatographic migration with that of total rat liver lipids and with a mixture containing pure FFA and lysophosphatidylcholine.

Densitometry. Chromatograms of samples containing 2 to 16 μ g of ovoidlecithin, FFA, or lysophospholipids were developed by incineration with sulfuric acid. The optical density of the spots, determined with a plate densitometer (Schoeffel, Model SDC 300), increased linearly with the amount of lipids. The equations obtained for each lipid standard were used to calculate the content of amoebal lipids separated in the same plates.

Phospholipase activity in total extracts and subcellular fractions. The measurement of phospholipase activity was based on the method of Haberman and Hardt (8). Various amounts of crude amoebal extracts suspended in BSS containing 0.5% Triton X-100 were placed into 3-mm wells made in agar-egg yolk gels containing 0.02% sodium azide. The gels were then incubated in a humid chamber at 36°C for 20 h.

The logarithm of the area of hydrolysis (clear halo formed around each well) was a linear function ($r^2 = 0.982$) of the amount of bee venom phospholipase A_2 used as the enzyme standard. With the corresponding equation, the absolute phospholipase activity of the amoebal extracts and subcel-

lular fractions was calculated and expressed as milliunits at 20 h of incubation; 1 mU (amount of enzyme which hydrolyzed 1 nmol of L- α -phosphatidylcholine per min) produced a hydrolysis area of 130 mm². Although phospholipase A_2 was used to set up and standardize the assays, clear halos are also produced by other phospholipases (8).

Hemolytic assays. P30 or amoebal lipids were suspended in BSS, and their activity was measured after 1 h of incubation by colorimetric determination of the amount of hemoglobin released from Wistar rat erythrocytes (27). To assay amoebal lipids, variable volumes of either total extracts or TLC-separated lipids (dissolved at 10 mg/ml in CM [4:1]) were added to conical tubes; the solvent mixture was evaporated with an N_2 stream, the lipids were suspended in BSS by vigorous agitation, and 25 μ l of undiluted or diluted resuspended lipids was then mixed with the same volume of a 2% rat erythrocyte suspension (made up in BSS) and incubated at 36°C. Basal hemolysis was determined in control samples obtained from tubes treated with the solvent mixture devoid of lipids and processed like the experimental samples. Total (100%) hemolysis corresponded to the absorbance at 415 nm (A_{415}) of hemoglobin released from an equivalent amount of erythrocytes, lysed with distilled water as described (27). The activity was determined by interpolation in the linear function obtained by plotting the logarithm of hemolysis observed versus the amount of lipids tested (27). One hemolytic unit was defined as the amount of lipids present in the amoebal extract or subcellular fractions needed to release the hemoglobin contained in 3×10^6 erythrocytes. All hemolytic assays were performed with triplicate samples in at least three experiments.

Chemicals. The salts and organic solvents were from J. T. Baker (Mexico). Egg yolk phosphatidylcholine type V-E, egg lysophosphatidylcholine, bee venom phospholipase A_2 (1,300 U/ μ g), and bovine serum albumin (BSA) were from Sigma Chemical Co., St. Louis, Mo.

RESULTS

Location of HSHA in P30 lipids. When activated P30 (i.e., P30 incubated for 36 h at 36°C and then heated for 9 min at 90°C) was subjected to CM extraction, its heat-stable hemolytic components were twice as active as in the subcellular fraction from which they were obtained (Table 1).

TABLE 1. Location of hemolytic activity in FFA of activated P30

Fraction(s) tested ^a	Hemolytic activity ^b (mean $\pm$ SD)	
	Absolute (HU)	Specific (HU/mg per h)
Total activated P30	16,250 $\pm$ 1,829	42 $\pm$ 5
CME	33,500 $\pm$ 5,025	445 $\pm$ 19
Sediment 2	0	0
Lipid fraction	19,920 $\pm$ 1,395	268 $\pm$ 19
Interphase	0	0
Aqueous phase	0	0
Lipid fraction + interphase	29,570 $\pm$ 3,307	821 $\pm$ 65
FFA	19,970 $\pm$ 3,994	2,249 $\pm$ 449
FFA + interphase	26,569 $\pm$ 4,604	2,615 $\pm$ 453

^a Obtained as described in Materials and Methods, except for FFA, which were purified as described in the legend to Fig. 1. P30 was activated by 36 h of incubation at 36°C.

^b Hemolytic units (HU) in 387 mg (dry weight) of activated P30 or in samples of fractions obtained from the same amount of activated P30. Values are the mean of three experiments.

After partitioning the CM extract (CME) with 2 M KCl, its lipid fraction was only 59% as active, and both the interphase and the aqueous phase were inactive; however, nearly 90% of the CME activity was regained by mixing the interphase with the lipid fraction (Table 1).

Identification of the heat-stable hemolytic components. Since the heat-stable hemolytic components were located in the lipid fraction, we obtained the chromatographic pattern of total lipids before (Fig. 1, lane B) and after (lane C) P30 activation. Later on, we assayed chromatographically purified lipids to identify the hemolytic ones. Pure FFA obtained from activated P30 (Fig. 1, lane E) were the only lipids with HSHA, and their content increased during activation (Fig. 1, lanes B and C).

Addition of the interphase fraction to the assays increased 1.33 times the hemolytic activity of FFA, making the FFA-interphase mixture 90% as active as the total lipid fraction and 80% as active as the CME (Table 1).

Since the HSHA increased and FFA appeared to be the major hemolytic factor and to increase also with preincubation, we determined their relationship and found a linear increase in P30 FFA content during preincubation, coinciding with the exponential increase of the HSHA (Fig. 2).

If FFA were the HSHA, they could be neutralized by delipidated BSA (7, 30, 31). This prediction was fulfilled when we found that the hemolytic activity of activated P30 was inversely related to the amount of BSA added to assay mixtures above the threshold dose (Fig. 3).

FIG. 1. Lipid changes during P30 activation, and identification of FFA as the HSHA. The following samples were spotted at the origin of chromatographic plates that were developed with solvent for nonpolar lipids: (A) rat liver FFA (12 µg), (B) total lipids from fresh P30 (600 µg), (C) total lipids from P30 incubated for 24 h at 36°C (600 µg), (D) human serum total lipids, used as internal standards (600 µg), (E) purified lipid fraction containing the HSHA from preincubated P30 (5 µg), (F) human serum FFA (12 µg), and (G) FFA, lysophosphatidylcholine, and phosphatidylcholine obtained from the partial digestion of 30 µg of egg yolk phosphatidylcholine with bee venom phospholipase A₂. The numbers correspond to spots present in lanes C and D, whose identity was inferred from the chromatographic behavior of pure lipid standards: 1, minor unidentified component; 2, cholesterol esters; 3, fatty acid methyl esters; 4 and 5, minor unidentified components; 6, triglycerides; 7, FFA; 8, diglycerides; 9, cholesterol; 10, monoglycerides; 11 and 12, polar lipids.

FIG. 2. Correlation between HLHA and FFA content during P30 activation. At various times during incubation at 36°C, P30 samples (containing 5 mg of total lipids) were heated at 90°C and their hemolytic activity and FFA content were determined. Each point and standard deviation derive from three experiments.

Decrease in P30 phospholipids corresponding with increase in FFA plus lysophospholipids. To see whether the FFA increase originated from hydrolysis of phospholipids, we quantified them as well as FFA and lysophospholipids at different times during P30 preincubation. The amount of FFA plus lysophospholipids (putative hydrolysis products) increased linearly with time, while a corresponding linear decrease was observed in P30 phospholipid content (Fig. 4).

Hemolytic and phospholipase activities in P30. As FFA could have been released from P30 phospholipids by the action of a phospholipase, we investigated whether hemolytic and phospholipase activities occurred in the same subcellular fraction. In this way we confirmed that 85% of the hemolytic activity of amoebal homogenates resided in P30 and found that the same fraction also contained 90% of the trophozoite phospholipase activity (Fig. 5).

We also found that in fresh P30, the absolute hemolytic and phospholipase activities were higher (2.2 and 1.6 times, respectively), and their corresponding specific activities

FIG. 3. Inhibition of HSHA with BSA. Samples (50 µl) of activated and heated P30 (containing 67 µg of protein) were mixed with the same volume of a 2% rat erythrocyte suspension and variable amounts of delipidated BSA. The mixtures were incubated at 36°C for 1 h, and the amount of released hemoglobin was measured. Each point and standard deviation derive from three experiments.

FIG. 4. Correlation between FFA and lysophospholipid increase with P30 phospholipid decrease. Each point and standard deviation derive from three experiments.

were even higher (5.3 and 5.4 times, respectively), than in total amoebal homogenates (Fig. 5).

DISCUSSION

In previous papers we described the cytolytic effect of trophozoite homogenates obtained from several strains of *E. histolytica* and other *Entamoeba* species and proposed erythrocytes as model target cells to identify the cytolytic factors that might be involved in amoebal invasiveness (15, 26, 27). Using a similar approach, Prasad et al. (22) verified and extended our findings. *E. histolytica* extracts also lyse nucleated mammalian cells (15, 26), and such activity can be quantified in a cytolysis model developed recently (35).

The major hemolytic activity of *E. histolytica* trophozoites is maximal at pH 8 in the presence of 1 mM Ca^{2+} , resides in a vesicular fraction called P30 (27), and is mostly latent, since it increases greatly during incubation at 36°C (28). Expression of the latent activity is inhibited reversibly by incubation at 4°C and irreversibly by either heating at 90°C or repeated freeze-thawing (28). The hemolytic effect is due to heat-labile and heat-stable components: the first increases during the first 36 h of incubation at 36°C and then decreases rapidly, whereas the second increases during the first 24 h and then remains unaltered (28).

To identify the hemolytic components of P30, we took into account that most hemolysins are thiol activatable, hydrolases, or surface active (32). The last are usually heat stable and disrupt cells by intercalating in the lipid bilayer of the plasma membrane (32). The thiol-activatable ones are oxygen labile, and their effects are rapidly lost in impure preparations (6, 9). The hydrolases function in limited pH ranges and are usually heat labile, and some require Ca^{2+} (1). The characteristics of the P30 hemolytic activity found previously (27, 28) led us to assume that its labile component might be a hydrolase and that the stable one was amphipatic.

The idea that the amoebal HSHA is amphipatic was reinforced when we found that it resided in P30 lipids (Table 1) and was confirmed when we discovered that it corresponded to FFA (Fig. 1). The identity between FFA and the HSHA was supported by two additional pieces of evidence: (i) FFA content increased during P30 incubation at 36°C, while the HSHA activity also increased (Fig. 3), and (ii) the heat-stable hemolytic effect was blocked by delipidated BSA (Fig. 3), a protein with known carrier activity for FFA (7, 30, 31). The hemolytic effect of FFA was not entirely unexpected, because they are well-known cytolytic agents (3).

The hemolytic effect of FFA was potentiated by a component that remained at the interphase after partition of the lipid extract with KCl (Table 1). This amphipatic factor might be a peptide, because when we analyzed the interphase by TLC for polar lipids, we found three spots that stained with ninhydrin but not with rhodamine B (data not shown).

Accumulation of FFA during P30 activation was accompanied by a decrease in total phospholipids and a corresponding increase in lysophospholipids (Fig. 5), suggesting that FFA were released from P30 endogenous lipids and that a phospholipase A was involved in their generation (1). We did not detect hemolytic activity in assays performed with pure P30 lysophospholipids, probably because they are less potent than FFA (32) and did not reach a high enough concentration in our preparations. In accordance with these findings, Tizard et al. (33, 34) found that almost all of the nonenzymatic hemolytic activity of autolyzed *Trypanosoma congolense* is due to FFA, whereas the lysophospholipids generated by a phospholipase A during autolysis contribute only 12% to the overall hemolytic effect.

The P30 phospholipase that generates FFA could be the activatable HLHA (28). This possibility is supported by the following observations: (i) hemolytic and phospholipase activities were located mainly in P30 and their potency was higher in fresh P30 than in total homogenates (Fig. 5); (ii) when P30 activation is prevented (by incubation at 4°C, heating, or repeated freeze-thawing), production of the heat-stable component is also prevented (28), suggesting that FFA are not accumulated because the phospholipase activity is inhibited; (iii) P30 hemolytic activity is maximum

FIG. 5. Correlation between hemolytic and phospholipase activities in P30. Total extract (TE) was prepared by homogenization of trophozoite pellets. From this, the nuclear (N) fraction, the vesicular (P30) fraction, and the postvesicular supernatant (S30) were obtained. Absolute and specific hemolytic (hatched bars) and phospholipase (open bars) activities of each fraction were determined and normalized to the total extract activities. Hemolytic activity: absolute, 217 U; specific, 2.3 U/mg of protein. Phospholipase activity: absolute, 44 U; specific, 2.3 U/mg of protein.

around pH 8 and depends on calcium (27), as does one of the major *E. histolytica* phospholipases characterized by Long-Krug et al. (13); and (iv) both the calcium-dependent phospholipase A₂ (13) and the major hemolytic activity (27) are associated with trophozoite membranes. Furthermore, McCaul et al. (19) and Ravdin et al. (25) have shown that several phospholipase A antagonists prevent the cytolytic effect of intact *E. histolytica* trophozoites.

The enzyme found by us in P30 may be the alkaline and calcium-dependent phospholipase A₂ of Long-Krug et al. (13), because our experiments were performed at pH 8 in the presence of 1 mM calcium. If this phospholipase were a cytolysin, it could split not only the endogenous P30 phospholipids but the plasma membrane phospholipids of target cells as well.

This cytolytic model for *E. histolytica* is consistent with the pathogenic role that phospholipases appear to play in other protozoa (10, 11, 16, 20, 36). Virulent *Naegleria fowleri* trophozoites contain much larger amounts of phospholipases than attenuated *N. fowleri* and avirulent *Naegleria* sp. trophozoites (11). The potentiating role of an amphipathic factor in the phospholipase cytolytic mechanism has been proposed also for *N. fowleri* (16). Pathogenic *T. congolense* autolysates are highly hemolytic, whereas those of *T. lewisi* may be activated with bee venom phospholipase (33).

The major hemolytic effect of *E. histolytica* trophozoites thus appears to depend on at least three components. Two of them are heat-stable (FFA and the amphipathic factor), and one is a heat-labile cytolysin that might be the calcium-dependent alkaline phospholipase A₂ of Long-Krug et al. (13). We are currently investigating the relationship between these components and hemolysis by intact amoebae.

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