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A Multicomponent Hemolytic System in the Pathogenic Amoeba *Naegleria fowleri*

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A hemolytic activity associated with postnuclear supernatant fractions of *Naegleria fowleri* has been partially characterized in an attempt to isolate cytolytic molecules that may participate in naeglerial cytopathogenicity. Hemolysis by naeglerial postnuclear supernatant fractions was sensitive to heat and trypsin hydrolysis, and was inhibited by divalent cations. The majority of the hemolytic activity was nonlatent and associated with a particle fraction sedimenting at 48,000 × *g* (maximum) for 1 h. This particle-associated hemolytic activity appears to be membrane associated, as high salt concentration, chelating agents, and pH extremes were ineffective in solubilizing the hemolytic activity, whereas treatment with 0.15% Zwittergent 3-12, a dipolar ionic detergent, results in 98% release of the sedimentable hemolysin. The sigmoidal nature of the progress curve of postnuclear supernatant hemolysis, as well as synergistic interactions between fractions of amoebal whole cell extracts, suggests that the hemolytic activity has a multicomponent nature, with at least two and possibly three components participating in the hemolytic event. The significance of these findings in the context of naeglerial cytopathogenicity is discussed.

The trophozoites of *Naegleria fowleri*, the causative agent of primary amoebic meningoencephalitis, possess a potent in vitro cytolytic capability (3, 19, 20). Although conclusive evidence is lacking, the mechanisms operating during the in vitro lysis of mammalian cell lines by intact trophozoites are thought to resemble those which result in the massive tissue destruction evident in human infection (15). Despite numerous investigations (see reference 15 for a review), the cytolytic mechanism remains poorly understood. Chang (7) has suggested that cytolytic factors are lipolytic enzymes released into the cell-free medium of growing amoebae. In possible support of this proposal, Hysmith and Franson (13, 14) found that lipolytic enzymes, released into the axenic media of growing amoebae, were able to degrade human myelin phospholipids. In contrast, a number of reports have maintained that naeglerial cytopathogenicity is purely a contact-dependent phenomenon (3, 19, 20), perhaps mediated by a spontaneously forming "sucker" apparatus (19). Surprisingly little data are available concerning the cytolytic capability of cell-free lysates of *N. fowleri* amoebae. Dunnebacke and Schuster (10) have reported a self-replicating cytopathogenic agent present in cell-free lysates of both *N. fowleri* and the nonpathogenic *Naegleria gruberi*.

As part of a study aimed at gaining a clearer understanding of the molecular events which operate during the lysis of target cells by amoebae, we have partially characterized a hemolytic activity associated with cell-free lysates of trophozoites. The simplicity and quantitative nature of hemolytic assays have provided a rapid and reliable method in other systems for the identification of those components which may be involved in cytolytic phenomena in vivo (30). In this report, we describe the existence of three distinct hemolytic activities in *N. fowleri* trophozoites and present evidence that at least two of them operate in a synergistic fashion in vitro. In addition, one of the components in this synergistic response is a basic protein, in keeping with other findings for a number of cytolytic molecules (6, 11, 12). Although hemolytic activities have been described for other pathogenic protozoa (16, 24), this is the first instance in which evidence of a multicomponent hemolytic system has been obtained.

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MATERIALS AND METHODS

Cultivation and maintenance of organisms. *N. fowleri*, Lee strain, (ATCC 30894) was maintained in Nelson medium containing 2% calf serum as previously described (26). Stock cultures were grown in tissue culture flasks (BD Labware, Oxnard, Calif.) at 30°C and were subcultured at weekly intervals. At monthly intervals, amoebae from stock cultures were used to infect mice intranasally (28), with new stock cultures being established at necropsy from infected brain tissue. In this manner, the virulence of the isolate was maintained and any variations in pathogenicity were precluded (9, 28). For mass cultivation, 1-liter volumes of Nelson medium, supplemented with 2% calf serum, were used according to Weik and John (26), with the exceptions that the pH was adjusted to 5.0 before autoclaving and siliconized glassware was not used. Amoebae used in experiments were in the late log phase of growth, being routinely harvested 72 h after inoculation.

Whole-cell homogenate and subcellular fractions. After the flask cultures were placed on ice for 10 min, the amoebae were recovered by centrifugation at 3,500 rpm (1,480 × *g* maximum) for 4 min (Sorvall GSA rotor) and subsequently washed twice with 0.25 M sucrose. The washed amoebae were then disrupted by grinding with glass beads (Sigma W, acid washed, 75 μm diameter) for 1.5 min in a chilled glass mortar. Treatments with a motor-driven Potter-Elvehjem tissue grinder and a hand-held Dounce homogenizer were found to be ineffective in disrupting amoebae, in keeping with an earlier observation on the difficulty in breaking *Naegleria* spp. amoebae (27). The use of glass beads, as described, was found to disrupt 85% of the amoebae as judged by phase-contrast microscopy.

The glass beads were sedimented by centrifugation at 800 rpm (77 × *g* maximum) for 1 min (SS34 rotor) and washed twice with 0.25 M sucrose, and the washings and original supernatant were pooled to yield a whole-cell homogenate.

The homogenate was centrifuged at 3,500 rpm (1,480 × *g* maximum) for 4.0 min (SS34 rotor with 145 adaptor) and after removal of the supernatant fluid, the loose pellet was washed with 0.25 M sucrose. The original and resulting supernatant fractions were pooled and constituted the post-nuclear supernatant (PNS) fraction. In some experiments, a high-speed pellet (P48) was obtained by centrifuging the PNS at 20,000 rpm (48,200 × *g* maximum) for 1 h (Sorvall SS-34 rotor with 145 adaptors).

Hemolytic assay. A quantitative hemolytic assay was adapted from a previously described method (24). For each experiment, 2 ml of fresh blood in Alsever solution (1) was washed three times in hemolysis buffer (0.12 M NaCl, 25 mM HEPES [*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid] [pH 7.4], 5 mM KCl, 0.7 mM $CaCl_2$, 1.0 mM $MgSO_4$) and suspended in an appropriate volume of hemolysis buffer so that upon lysis of 0.9 ml with ca. 5 mg of saponin, an absorbance at 415 nm of 0.800 was obtained. The assay was then initiated by the addition of 0.1 ml of amoebal fractions or smaller volumes diluted with hemolysis buffer. After 1 h at 37°C, the assay tubes were removed and placed in Sorvall HS-4 rotor blocks that were prechilled to 4°C and centrifuged immediately. The absorbance at 415 nm of the resulting supernatant fluids was then determined, together with controls, to account for the absorbance of amoebal fractions, the spontaneous lysis of erythrocytes, and 100% lysis. One unit of hemolytic activity was defined as the amount of hemoglobin released into the supernatant fluid of centrifuged assays in 1 h that resulted in an absorbance of 0.05 at 415 nm. The absorbance spectrum produced by the supernatant fluids of *N. fowleri*-lysed erythrocytes showed the characteristic absorption peaks at 415 and 541 nm of hemoglobin, with no other significant peaks.

Sensitivity to trypsin digestion. To 300 μg of PNS protein, 100 U of trypsin (TPCK [tolylsulfonyl phenylalanyl chloromethyl ketone]-treated, Worthington Diagnostics, Freehold, N.J.) was added to a solution containing 1.0 mM $CaCl_2$ and 50 mM HEPES (pH 7.5) in a final volume of 1 ml. After incubation for 1 h at 37°C, 500 μl of trypsin lima bean inhibitor (9 mg/ml) was added to terminate the reaction. The controls consisted of the digestion mixture, with water substituted for trypsin, and a solution containing only PNS diluted to 1.0 ml with water. Digested PNS and the controls were assayed for hemolytic activity as described above.

Sedimentation of hemolytic activity and dissociation of particle-associated activity. Whole, washed amoebae were disrupted, and a high-speed pellet was obtained as described above. The whole-cell homogenate, the nuclear fraction, and the high-speed supernatant (S48) and pellet (P48) fractions were compared for hemolytic activity as usual. In an attempt to dissociate hemolytic activity, a portion of fraction P48 was mixed 1:1 (vol/vol) with one of the compounds listed in Table 1 and stirred overnight at 4°C. The mixture was then centrifuged at 20,000 rpm (48,200 × *g*) for 1 h (Sorvall SS-34 rotor), with the resulting supernatant and pellet fractions being dialyzed against repeated changes of hemolysis buffer for 24 h. After dialysis, the supernatant and pellet fractions were either concentrated by ultrafiltration under nitrogen (Diaflo PM-10 filter, Amicon Corp.) or suspended in hemolysis buffer to the original volume of the P48 sample and assayed for hemolytic activity.

Column chromatography. A column (30 by 1.5 cm) of Polybuffer Exchanger 9-4 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) was equilibrated with either 0.025 M imidazole-acetate (pH 7.4) for pI 7 to 4 chromatofocusing, or 0.025 M ethanolamine-acetate (pH 9.4) for pI 9-6 chromatofocusing. A whole-cell extract prepared from the overnight treatment of washed amoebae with 0.15% Zwittergent 3-12 (Calbiochem, La Jolla, Calif.) was applied to the column and eluted with the appropriate Polybuffer solution (Polybuffer 9-6 [pH 6.0] or Polybuffer 7-4 [pH 4.0]; Pharmacia) at a flow rate of 20 ml/h, 3-ml fractions being collected. All buffers used in chromatofocusing experiments contained 0.15% Zwittergent 3-12. The pH and optical density at 280 nm of the resulting fractions were then determined. Protein peaks were pooled and dialyzed extensively for 24 h against hemolysis buffer before being assayed for hemolytic activity.

Attempts to demonstrate secretion of hemolytic activity. Late log phase cultures were harvested and washed as described above and suspended into a volume of Page saline (23) equivalent to that from which they were harvested. The amoebae were allowed to incubate at 37°C with agitation for 2.5 h, after which they were sedimented by centrifugation and the cell-free supernatant fluid was assayed for hemolytic activity. The same procedure was employed with Page saline containing 2.0 and 6.6 μM polystyrene beads at a ratio of two beads per amoeba and with the saline containing 0.1% glucose. During these assays, the amoebae remained 99% viable as determined by trypan blue exclusion, and only 4% of the amoebae transformed into flagellates. Secretion of hemolytic factors was also examined by using an assay developed for the detection of secreted substances by activated macrophages (25). Amoebae were seeded into 240-well microtiter plates and allowed to grow at 37°C for 24 h in Nelson medium with 2% calf serum. At the end of this time, the medium was aspirated and the amoebae monolayers were carefully washed twice with Page saline. Suspensions of autoclaved *Escherichia coli*, autoclaved rat erythrocytes,

TABLE 1. Release of hemolytic activity from a P48 particle fraction

Compound[a]	Hemolytic units			% Soluble	Final recovery (%)
	P48[b]	Non-sedimentable[c]	Sedimentable		
5 mM EDTA	3,072.0	0.0	2,712.0	0	88.3
5 mM EGTA	1,139.0	12.0	400.0	3.0	36.2
1.0 M KCl	1,139.0	23.0	881.0	3.0	79.4
2% Na_2CO_3 (pH 11.5)	1,139.0	5.6	40.0	14.0	4.0
100 mM acetate (pH 4.0)	8,406.0	788.0	4,662.0	17.0	64.8
Sonication[d]	3,596.0	525.0	3,828.0	14.0	121.0
0.15% Zwittergent 3-12	3,233.0	1,200.0	26.0	98.0	37.9

[a] Each compound tested was added to the P48 fraction at the final concentration indicated. After 12 h at 14°C, the extract was centrifuged at 20,000 rpm (48,000 × *g* maximum, for 1 h (Sorvall SS-34 rotor), and the supernatant and pellet fractions were separated and dialyzed against repeated changes of distilled water.
[b] Refers to activity present in P48 particle fractions before exposure to the treatments.
[c] Non-sedimentable refers to hemolytic activity released into the supernatant fluid after extraction and centrifugation.
[d] Sonication refers to a 10-min exposure to 300 W with a Braunsonic microprobe.

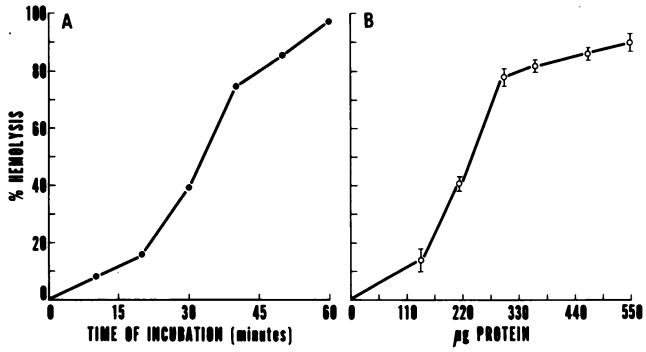

FIG. 1. Progress curves of the hemolytic reaction of an *N. fowleri* PNS fraction, with rat erythrocytes used as target cells. (A) Percent hemolysis as a function of increasing incubation time. The results from two experiments are shown, with each point representing the mean of these measurements. (B) Percent hemolysis as a function of increasing PNS protein concentration. Each point represents the mean of three experiments. Vertical bars indicate the standard deviation.

or Page saline alone were added. After incubation at 37°C for 2.5 h, the supernatant fluid was removed from each well, examined by phase-contrast microscopy to confirm the absence of amoebae, and assayed for hemolytic activity and protein.

Protein determination. Protein was routinely determined by the method of Lowry et al. (17); insoluble cell fractions were allowed to solubilize for 2 h at room temperature in 0.5 N NaOH. For pooled column fractions, protein content was calculated from the absorbance at 260 and 280 nm.

RESULTS

Hemolytic activity of PNS fractions. In preliminary experiments, PNS fractions of axenically grown amoebae were observed to possess hemolytic activity towards sheep, rat, and human erythrocytes. The specific hemolytic activity (in units per hour per milligram of protein) of PNS was greater for rat erythrocytes (55.7 ± 6.9, five experiments) than for human erythrocytes (1.2 ± 0.07, three experiments). Variation in erythrocyte sensitivity to other hemolysins has been noted previously (2). Due to the greater sensitivity of rat erythrocytes to hemolysis and the ready availability of fresh blood from an established colony of animals, rat erythrocytes were chosen as target cells in all subsequent assays.

The progress curve of the hemolytic reaction was sigmoidal with respect to both increasing time of incubation and increasing PNS protein concentration (Fig. 1). A standard curve of erythrocyte lysis generated by the addition of saponin was, as expected, linear over the range represented. The sigmoidal nature of the progress curve is a constant feature that has also been obtained with not only rat erythrocytes but also sheep and human erythrocytes (data not shown).

For the compounds listed in Table 2, a consistent finding was the ability of divalent cations to inhibit hemolysis. Calcium, cobalt, manganese, and magnesium were all found to exert an inhibitory effect at a final concentration of 5 mM, with inhibition ranging from 56% for manganese to 23% for cobalt. In view of this, it was not unexpected to find that 5 mM EDTA significantly stimulated hemolysis. In the 1.0 to 5.0 mM range, a linear inhibition of hemolytic activity was noted for $CaCl_2$. A complicating factor was the need to include 0.7 mM $CaCl_2$ and 1.0 mM $MgSO_4$ in the hemolysis buffer to avoid unacceptable spontaneous lysis; thus, a true assessment of the inhibitory effect of divalent metals was prevented.

TABLE 2. Effect of potential modulators on the hemolytic activity of a PNS fraction

Compound[a] (5 mM)	Hemolytic units	% of control
$CoCl_2$	5.7	77.0
$CaCl_2$	3.9	53.6
$MnCl_2$	3.2	43.4
$MgCl_2$	4.0	54.8
EDTA	12.1	164.0
Dithiothreitol	6.5	88.0
Cysteine	5.3	71.6
None	7.4	100.0

[a] All compounds were added at a final concentration of 5 mM, and for each a blank containing no PNS fraction was subtracted. All blanks were less than 15% of the values shown. Results shown are the means of two experiments.

Heat inactivation kinetics and trypsin digestion. The proteinaceous nature of the hemolysin(s) was suggested by the ability of heat to inactivate the hemolytic activity (Fig. 2). Treatment of PNS at 92°C reduced hemolytic activity by 88% within 5 min, although treatments at 56 and 75°C for 30 min had no significant effect. The involvement of a protein component(s) in the hemolytic process was reinforced by the effect of limited trypsin digestion of PNS, which resulted in a 54% inhibition of hemolytic activity compared with control values.

Particle association of hemolytic activity. A prominent feature of the naeglerial hemolytic activity was its sedimentability. After fractionation of the PNS fraction by centrifugation at 20,000 rpm (48,200 × *g* maximum) for 1 h (Sorvall SS-34 rotor), most of the hemolytic activity was recovered in the P48 fraction (85.6 ± 14.4%, three experiments). Although strongly particle associated, PNS hemolytic activity was nonlatent, as neither eight cycles of freeze-thawing (dry

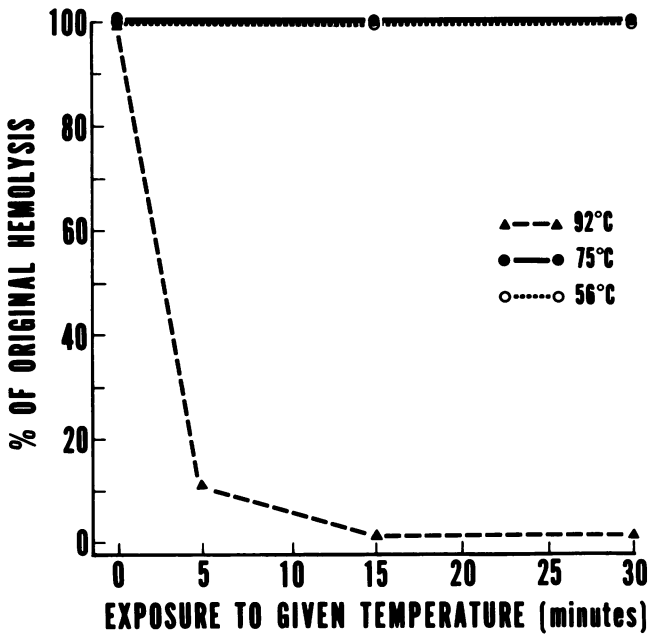

FIG. 2. Heat inactivation kinetics of *N. fowleri* hemolytic activity, using a PNS fraction. Plot shows hemolytic activity after exposure to 92, 75, and 56°C as described in the text.

TABLE 3. Synergistic interaction between P48 and S48 fractions obtained from a PNS fraction

P48 dilution[a]	% Hemolysis[b]		
	P48	S48 (1:2)	P48 + S48
1:35	9.2	26.4	85.0
1:30	15.0	26.4	87.0
1:25	22.1	26.4	92.1
1:20	77.1	26.4	>100.0

[a] Dilutions of the P48 and S48 shown were made in hemolysis buffer (see the text).

[b] Percent hemolysis refers to the hemolysis given by each fraction divided by the hemolysis obtained after the addition of saponin to the same concentration of erythrocytes.

TABLE 4. Demonstration of synergistic interaction between hemolytic components recovered after chromatofocusing

Pooled fraction(s)	Hemolytic units[a]	Degree of[b] synergism
4	2.4 ± 0.8	
6	2.8 ± 0.5	
9	1.6 ± 0.7	
4 + 6	12.4 ± 0.5	2.38
4 + 9	11.4 ± 0.5	2.85

[a] Values are expressed in hemolytic units and represent the mean of three experiments plus or minus the standard deviation.

[b] The degree of synergism is expressed as the hemolytic activity obtained for two given fractions assayed together compared with the sum of the hemolytic activities obtained after the same two fractions were assayed individually.

ice-ethanol bath to 37°C waterbath) nor a 1-min sonication (Braunsonic, 300-W output microprobe) was found to increase the activity of fractions osmotically protected in 0.25 M sucrose.

Release of the sedimentable component of the hemolytic activity was attempted with a variety of compounds. KCl (1.0 M), EDTA (5 mM), and EGTA (ethyleneglycol-bis(β-aminoethyl ether)-*N*-*N*-tetraacetic acid) (5 mM) were ineffective in releasing any additional activity (Table 1). Sonication, 2% (wt/vol) sodium carbonate (pH 11.5), and 100 mM acetate (pH 4.0) released small amounts of hemolytic activity; however, the high and low pH treatments drastically reduced recovery. In contrast, after treatment of the P48 fraction with 0.15% Zwittergent 3-12, 98% of the recovered hemolytic activity was soluble, this being 37.9% of the original activity present in the particle fraction.

Synergistic interactions between amoebal fractions. Mere separation of the PNS into S48 and P48 fractions resulted in a 25 to 45% loss in hemolytic activity. Upon addition of the S48 fraction to the P48 fraction in a hemolytic assay, a synergistic release of hemoglobin resulted as compared with the hemolysis of equivalent amounts of either fraction alone. Table 3 shows a representative experiment in which increasing dilutions of the P48 fraction were added to a constant amount of the S48 fraction. Similar results are obtained when the S48 fraction is diluted and assayed in the presence of a constant amount of the P48 fraction. The apparently synergistic nature of this phenomenon was confirmed by the chromatofocusing experiment shown in Fig. 3. Peaks 4, 6, and 9 were found to possess hemolytic activity, each having isoelectric point ranges of 8.33 to 7.91, 7.60 to 7.51, and 6.93 to 6.64, respectively. The most conspicuous hemolytic activity of *N. fowleri* elutes with the greatest protein peak (peak 4) and accounts for 24.0% of the hemolytic activity contained in the whole-cell extract. Peaks 6 and 9 also displayed hemolytic activity, with all three hemolytic components accounting for 28.0% of the original whole-cell extract hemolytic activity. The three peaks in which hemolytic activity eluted comprised 16.3% of the total cell protein of *N. fowleri*.

When components 4, 6, and 9 were appropriately diluted and assayed alone and in the presence of each other, synergistic hemolysis was noted between peaks 4 and 6 and between peaks 4 and 9 (Table 4). No synergy was observed between peaks 6 and 9 or between peak 4 and the nonhemolytic peak 2 (data not shown). Hemolytic activity was not increased when fractions 4, 6, and 9 were mixed and assayed together, as compared with values obtained for 4 and 6 or 4 and 9 assayed separately. When chromatofocusing was performed in the pI 7 to 4 range, all of the activity eluted at a pI of 6.95, with no subsequent activity peaks (data not shown).

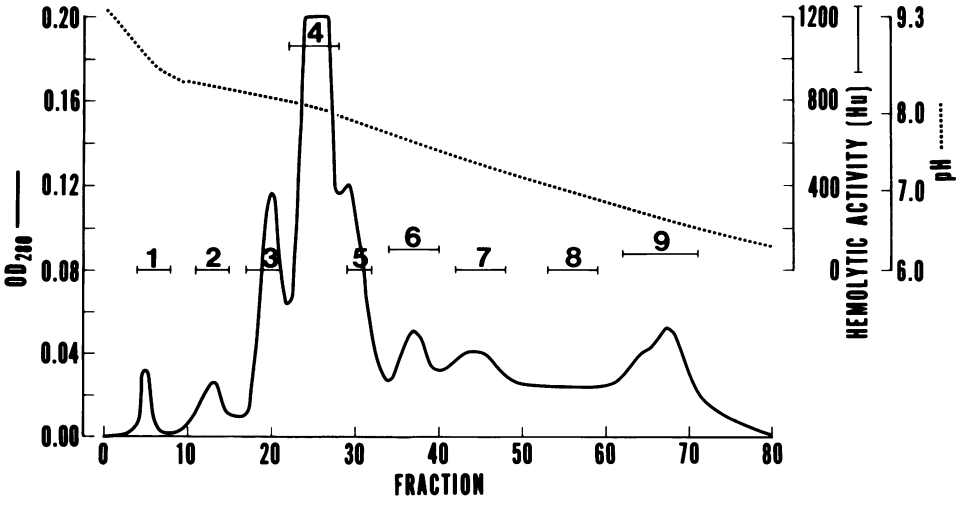

FIG. 3. Separation by means of chromatofocusing of hemolytic components present in a Zwittergent extract of *N. fowleri* trophozoites. The optical density at 280 nm, the pH for each tube, and the total hemolytic activity of each pooled fraction are shown. Experimental conditions are described in the text. Protein peaks were pooled and dialyzed against hemolysis buffer to remove detergent before the assay of hemolytic activity.

Attempts to demonstrate secretion of hemolytic activity. The possibility that hemolytic activity is released by intact *N. fowleri* trophozoites was examined by using assays previously used to determine secretion phenomena in *Tetrahymena pyriformis* (21) and in activated macrophages (25). No evidence was obtained to indicate any release of hemolytic factor(s) into the media of amoebae, nor was it possible to effect release upon stimulation by 2.0 or 6.6-μm polystyrene beads, autoclaved *E. coli*, autoclaved rat erythrocytes, or 0.1% glucose in Page saline.

DISCUSSION

From the results described above, there is considerable evidence that the hemolytic activity of naeglerial PNS fractions involves more than one component. The progress curve of hemolysis with respect to either time or protein is sigmoidal, similar to that observed for snake venoms (8) in which hemolysis clearly involves two components. Separation of a whole-cell freeze-thaw lysate into pellet and supernatant fractions consistently produces a small, non-sedimentable hemolytic activity which, when combined with the sedimentable activity, produces a synergistic response. The concept that the activity present in the supernatant fluid is distinct from that of the pellet is suggested by the fact that the sedimentable activity cannot be released from its particle association except by relatively drastic treatments, i.e., extensive sonication and treatment with detergent. Finally, chromatofocusing of an amoebal whole-cell extract yielded three hemolytic peaks displaying synergistic hemolysis. Synergistic action between components of other hemolytic and cytolytic model systems has also been described. The direct lytic factor of cobra venom (8), mellitin of bee venom (12), and the bactericidal–permeability-increasing protein of rabbit polymorphonuclear leukocytes (11) all act synergistically with associated phospholipases to facilitate lipid perturbation of membranes in target cells. In view of the presence of active lipolytic enzymes in *N. fowleri* homogenates (13), the presence of a synergistic cofactor becomes an attractive possibility. Moreover, the direct lytic factor, mellitin, and the bactericidal–permeability-increasing protein are all basic proteins, a property shared by one of the three hemolytic components described in this report. The sensitivity of the hemolytic reaction to inhibition by divalent cations agrees with data obtained for other basic cytolytic proteins (11, 22).

The observed sedimentability of the hemolytic activity, when considered with the fact that 0.15% Zwittergent 3-12, a dipolar ionic detergent, releases virtually all hemolytic activity from its sedimentable form, implicates a component(s) of the reaction as being membrane associated. This finding is not unusual, as both a hemolytic activity (24) and a pore-forming activity (18) in *Entamoeba histolytica* are largely particle associated and a membrane association has been suggested for each. Our results do not support the concept that the hemolytic activity is latent within a limiting, subcellular membrane, e.g. lysosome, as neither freeze-thawing nor sonication of osmotically protected PNS fractions resulted in an increase in hemolysis.

As accumulating evidence suggests that *N. fowleri* lyses target cells solely by a contact-dependent mechanism (3–5, 19, 20), the demonstration of a membrane-associated cytolysin may offer an explanation for the molecular events surrounding the destruction of target cells. We propose that *N. fowleri* trophozoites may possess potent cytolytic molecules on either the outer membrane or some subcellular membrane or particle which interacts with the target cell membrane with subsequent lysis. A likely role for an outer membrane cytolytic component might be to increase target cell membrane fluidity. Such a process would seem necessary to account for the claims that intact portions of cultured mammalian cell line target cells are "pinched" off, leaving an intact yet damaged cell (5, 20).

Our failure to demonstrate secretion of hemolytic factors by *N. fowleri*, and experiments in which chicken erythrocytes were found to be internalized in an intact form by trophozoites (4), suggest that the described membrane-associated, multicomponent hemolytic system operates at a subcellular level. It is possible that the hemolytic activity has a bimodal subcellular distribution, with one component on the outer membrane of the amoeba and another associated with a subcellular particle. Upon phagocytosis, these components could interact synergistically at the level of the food vacuole to facilitate digestion of portions of phagocytized cell. As this is the first report of a multicomponent, cytolytic system in a pathogenic protozoan, the phenomenon may prove to be quite unique from any process described so far. Experiments to further purify and determine the subcellular localization of the hemolytic components are currently in progress.

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