Lysis of Complement-sensitive *Entamoeba histolytica* by Activated Terminal Complement Components

Initiation of Complement Activation by an Extracellular Neutral Cysteine Proteinase

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Abstract

Activation of complement by *Entamoeba histolytica* may be initiated by the extracellular 56-kD neutral cysteine proteinase which cleaves the α chain of C3. To determine the relationship between the fluid-phase activation of complement and our observation that only strains isolated from patients with invasive disease are resistant to complement-mediated lysis, we investigated the fate of C3 with recent amebic isolates. When ^{125}I-C3 was incubated with trophozoites in serum, C3 in the fluid phase was cleaved to C3b or C3bi, but the α chain of the C3 molecules on the cell surface appeared intact. Since the lysis of nonpathogenic strains takes place in the absence of bound C3b, we demonstrated that this reaction occurs by reactive lysis initiated in the fluid phase: (*a*) the killing of nonpathogenic strains was enhanced when alternative pathway activation was accelerated by the addition of cobra venom factor; (*b*) nonpathogenic strains were lysed by purified terminal components; and (*c*) sera incubated with pathogenic *E. histolytica* produced passive lysis of chicken erythrocytes. These results demonstrate for the first time that complement-sensitive *E. histolytica* are lysed by activation of the terminal complement components in the fluid phase where the 56-kD neutral cysteine proteinase cleaves C3, and not by the surface deposition of activated C3. (*J. Clin. Invest.* 1990. 86:1815–1822.) Key words: amebas • complement • passive lysis • protease • reactive lysis

Introduction

Amebiasis is a major cause of morbidity and mortality worldwide, but the factors controlling the severity of infection are poorly understood. An estimated 90% of patients infected with *Entamoeba histolytica* are asymptomatic, while the remaining 10% develop dysentery or metastatic abscesses, particularly of the liver. Although isolates from symptomatic and asymptomatic patients are morphologically indistinguishable, those strains capable of causing invasive amebic disease appear to have unique virulence properties. Pathogenic isolates are characterized by distinct isoenzyme patterns (1), production of a 56-kD extracellular cysteine proteinase which may play a role in tissue invasion by degrading collagen and laminin (2, 3), the presence of unique surface antigens (4), and distinct DNA sequences (5, 6). In addition, we have shown that invasive isolates from patients with colitis or liver abscess are able to evade an important host defense mechanism, complement-mediated lysis (7). To demonstrate these differences in complement sensitivity, it was necessary to use recent clinical isolates which are grown in culture conditions mimicking those in the human bowel with bacteria and starch. Axenic strains, which have been adapted to grow without bacteria, were initially isolated from patients with invasive disease, but have lost their virulence to variable degrees (8) and have become sensitive to complement-mediated lysis (9). Complement-sensitive and resistant isolates, as well as axenic strains, have been shown to activate complement in the fluid phase as demonstrated by complement consumption and the generation of complement fragments (10, 11).

The third component of complement is central to the subsequent deposition of the membrane attack complex (C5b-9). C3 is a glycoprotein composed of a 115-kD α chain linked to a 70-kD β chain by disulfide bonds (12). After complement activation, the α chain is cleaved by classical and alternative pathway C3 convertases, releasing C3a, a 9-kD peptide with anaphylatoxin activity. A reactive thioester is exposed in the remaining 105-kD α' chain of C3b which is transiently capable of binding to surfaces (13, 14). C3b may be inactivated through cleavage of the α' chain into 68- and 46-kD peptides by the serum enzyme, factor I, after binding to a serum cofactor, H (15, 16).

We have shown previously that activation of complement may be initiated in the fluid phase by the major extracellular proteinase of *E. histolytica*, a 56-kD neutral cysteine proteinase that cleaves the α chain of C3 one amino acid residue distal to the cleavage site of the serum C3 convertases (17). The resulting molecule was structurally and functionally equivalent to C3b as demonstrated by its participation in the activation of the alternative pathway of complement and its susceptibility to the action of factor I in the presence of factor H (17). These experiments did not address whether this C3b-like molecule bound to the membrane of *E. histolytica*. Calderon and Schreiber (11) detected specific binding of radiolabeled C3 to axenic amebic trophozoites, however the molecular form of C3 on the cell surface was not evaluated. To determine the relationship between the fluid-phase activation of complement by *E. histolytica* and the observed differences in complement sensitivity, we investigated the fate of C3 and the late-acting complement components incubated with recent pathogenic and nonpathogenic amebic isolates.

Methods

Complement components and sera. C3 and C5b6 were prepared by published techniques (18, 19). C7, C8, and C9 were purchased from Quidel Laboratories (San Diego, CA). The thiol ester of C3 was inacti-

This work was presented in abstract form at the 13th International Complement Workshop, San Diego, CA, 1989.

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Received for publication 10 April 1990 and in revised form 10 July 1990.

J. Clin. Invest.

0021-9738/90/1815–1822 $2.00
Volume 86, December 1990,

vated by incubating C3 (1 mg/ml) in a final concentration of 0.1 M methylamine in 0.1 M Tris, 10 mM EDTA, pH 8.0, for 4 h at 37°C, followed by dialysis overnight in 0.1 M Tris, pH 7.5, at 4°C (20). C3 and methylamine-treated C3 were radiolabeled with ^{125}I by the iodogen method (21) to a specific activity of 1–2 × 10^6 cpm/μg of C3 protein. In several experiments, the radiolabeled C3 was subjected to gel filtration on a 1.5 × 56-cm Sephacryl S-300 column (Pharmacia, Inc., Piscataway, NJ) to insure that aggregrates were not formed during labeling. ^{125}I-C3b was generated by incubating ^{125}I-C3 with tosylamide-phenylethylchloromethyl ketone-trypsin (Sigma Chemical Co., St. Louis, MO) (1:100 dilution wt/wt) in PBS for 8 min at room temperature, followed by a 10-fold excess of soybean trypsin inhibitor.

To demonstrate that ^{125}I-C3 was functionally active, the labeled protein (2 μg) was added to 50% normal human sera (NHS)[1] and incubated for 60 min at 37°C with zymosan at a concentration of 4 mg/ml (14). The zymosan was removed by centrifugation at 10,000 *g* for 10 min, and the C3 cleavage products in the supernatant were evaluated by SDS-PAGE (22) under reducing conditions on a 5–15% gel. The gels were dried and subjected to autoradiography at −70°C using Kodak XAR-5 film (Eastman Kodak Co., Rochester, NY). The C3 cleavage fragments generated were quantified by densitometric scanning of the autoradiographs using a model 2400 Gel Scan XL (LKB Instruments, Inc., Gaithersburg, MD).

The susceptibility of ^{125}I-C3 and methylamine-treated ^{125}I-C3 to cleavage by EAC14b2a cells was evaluated by incubating 0.2 μg of ^{125}I-C3 or methylamine-treated ^{125}I-C3 with 5 × 10^9 EAC14b2a cells at 30°C for 2 h. The erythrocytes were removed by centrifugation at 10,000 *g* for 2 min and the C3 cleavage fragments assessed by SDS-PAGE (22) under reducing conditions on a 5–15% gel.

The autolytic fragmentation pattern of the radiolabeled C3 was evaluated by incubating 2 μg of ^{125}I-C3 and 10 μg of unlabeled C3 in a final concentration of 0.1 M Tris, pH 9.0, 1% SDS, and 4 M urea. After heating to 100°C for 15 min, the sample was chilled, made 20 mM with dithiothreitol (DTT) and incubated for an additional 30 min at 37°C. The samples were then alkylated by adding 10% vol/vol of 0.5 M iodoacetamide in 1 M Tris, pH 7.5 (23, 24), and evaluated by SDS-PAGE under reducing conditions.

To measure the hemolytic activity of the complement components and sera, sheep erythrocytes were sensitized with rabbit anti-sheep hemolysin (EA). EAC14b cells were prepared by incubating EA cells in 10% NHS with 10 mg/ml of methylprednisolone (25). EAC14b2a cells were prepared by incubating EAC14b cells with C2oxy (26). The total hemolytic activity (CH50) was determined by incubating EA cells with dilutions of NHS for 60 min at 37°C, removing the unlysed erythrocytes by centrifugation, and measuring the hemoglobin in the supernatant at A414 (27). C3 hemolytic activity was assayed with EAC14b cells, 50 U/ml C2oxy (26), and potassium bromide-inactivated serum (28) supplemented with C5 (Diamedex Corp. Miami, FL). The C5b6 activity was assayed by measuring the hemolysis of unsensitized sheep erythrocytes in guinea pig sera with 10 mM EDTA as a source of terminal complement components. In experiments to measure the fluid-phase generation of C5b-9, chicken erythrocytes (Colorado Serum Co., Denver, CO) were used in sodium barbital buffer (3.3 mM) containing 0.1% (wt/vol) gelatin, 0.5 mM $MgCl_2$, and 150 mM NaCl (gelatin/veronal-buffered saline with Mg^+).

Normal human blood was obtained from at least five healthy volunteers and allowed to clot for 30 min at room temperature. Then the serum was separated by centrifugation, pooled, and stored at −70°C until use. All volunteers had negative serologies for *E. histolytica* as measured by agar gel diffusion (LMD Laboratories, San Diego, CA). Sera were depleted of C7 by passage over an affinity column with polyclonal goat anti-C7 (a gift of Dr. H. Müller-Eberhard, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany) linked to Affi-Gel (Biorad Laboratories, Richmond, CA).

1. *Abbreviations used in this paper:* EA, sheep erythrocytes sensitized with rabbit anti-sheep hemolysin; NHS, normal human sera.

E. histolytica strains. Two pathogenic and two nonpathogenic strains were obtained from P. Sargeaunt (London School of Hygiene and Tropical Medicine). The other five pathogenic and seven nonpathogenic strains were obtained from cultures of stools or liver abscess aspirates submitted to the Microbiology Laboratory at UCSD Medical Center. Clinical strains were first isolated in Robinson's medium (29) and maintained in either Robinson's medium or TYSGM (30) containing *E. coli* 0111. Subcultures were made every 2 d. Axenic strains included HM-1:IMSS (HM-1, No. 30459, American Type Culture Collection, Rockville, MD) and UCSD:0283:1 (SD-1), which were grown in TYI-S-33 media containing 12% bovine serum (31) and subcultured twice weekly.

Strains were classified as pathogenic or nonpathogenic based on the electrophoresis patterns on starch gels of maleic enzyme, phosphoglucomutase, glucophosphoisomerase, and hexokinase as defined by Sargeaunt and Williams (1) and were consistent with the clinical history.

Amebas were purified from the culture media by layering pelleted trophozoites (1,000 *g* for 10 min) over a discontinuous Percoll gradient to remove starch and by multiple low speed spins to remove > 99.9% of the bacteria. The levels of bacteria which remained did not affect complement activation (10).

C3 binding assays. Purified trophozoites (1 × 10^6/ml) were suspended in PBS with 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 20 mM cysteine (PBS-Cys^{++}) and mixed with 1–2 μg of ^{125}I-labeled C3 or methylamine-treated ^{125}I-C3 which had been added to 10% NHS. The presence of cysteine had no effect on the hemolytic activity of C3, but prolonged trophozoite survival. Samples were incubated on a rocker at 37°C to ensure complete mixing. At timed intervals, 100-μl duplicate samples were removed and added to 0.5 ml of cold PBS-Cys^{++} in a 1.5-ml microcentrifuge tube and centrifuged in the cold at 12,000 *g* for 10 min. After an additional wash in PBS-Cys^{++}, the supernatant and pellets were separated and the radioactivity of the samples determined. The total molecules of C3 bound per parasite were calculated based on the specific activity of the labeled C3 and the ratio of labeled to unlabeled C3 in the sample.

To evaluate inhibition of ^{125}I-C3 binding by C3a des Arg, aliquots of trophozoites (2 × 10^5) were incubated in triplicate in PBS-Cys^{++} alone or in the presence of C3a des Arg (0.5–7.5 μg) for 30 min at 37°C. 1 μg of ^{125}I-C3 was then added and the samples incubated an additional 30 min before separating the pellets and supernatants as above.

The susceptibility of surface-bound C3 to pronase cleavage was tested by incubating 2–4 × 10^5 amebas with 1 μg of ^{125}I-C3 added to 10% NHS in PBS (final volume of 0.5 ml). After 10 min of incubation at 37°C, the supernatants were aspirated, and the pellets were washed three times in PBS. Duplicate samples of the pellets were counted or resuspended in 200 μl of PBS alone or PBS containing 3.5 mg/ml of pronase (type XXV, Sigma Chemical Co.) After an additional 15-min incubation at 37°C, the pellets were washed three times, the washes were combined with the supernatants, and the samples were counted. The percentage of counts removed by pronase was calculated by comparing counts in the pronase- and buffer-treated pellets.

In experiments designed to evaluate the specificity of C3 binding in the absence of serum, 500 ng to 5 μg of ^{125}I-C3 were added to triplicate samples of 2 × 10^5 washed trophozoites of axenic strain HM-1 in PBS-Cys^{++}. Nonspecific binding was assessed by adding a 100-fold excess of cold C3. After incubation for 30 min at 4°C, the pellets and supernatants were separated by centrifugation at 10,000 *g* for 5 min. The pellets were washed one time in PBS-Cys^{++}, resuspended in 200 μl, and layered over a mixture of 50% (vol/vol) dibutyl phthalate and dioctyl phthalate (Eastman Kodak Co.) in 400-μl microfuge tubes and centrifuged for 3 min at 15,000 *g*. The pellets were cut from the tube and the supernatant and the oil interface were counted separately. The amount of ^{125}I-C3 bound was calculated as above.

Molecular forms of C3. The chain structure of the C3 molecules bound to the trophozoites and free in the fluid phase was determined by incubating 2 × 10^5 amebas with 1–2 μg of ^{125}I-C3 in the presence of either cold C3 (130 μg/ml) or 10% NHS for 30–60 min at 37°C. The

pellets were separated from the supernatants by centrifugation at 12,000 *g* for 5 min and washed twice with PBS-Cys^{++} containing 1 mM *N*-ethylmaleimide, 1 mM tosyl-lysyl-chloromethyl ketone, 1 mM EDTA, 1 mM PMSF, and 100 μM *trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64, Sigma Chemical Co.) (PBS-I). An aliquot of the supernatants and the pellets were then boiled in PBS-I, and reduced with 2.5% 2-mercapatoethanol and 2% SDS in 0.1 M Tris. To cleave the covalent ester linkage between C3 and the parasite membrane, duplicate samples of the pellets were solubilized in 2% SDS, heated to 100°C, and then incubated in 100 mM carbonate buffer containing 25 mM methylamine, pH 11.0, at 37°C for 60 min (32). The resulting supernatant was then processed as above. To evaluate the susceptibility of bound C3 to cleavage by the C4b2a convertase, 2×10^5 trophozoites were incubated with 1–2 μg of ^{125}I-C3 for 30 min at 37°C. The cells were washed with PBS and incubated an additional 30 min with 5×10^8 EAC14b2a cells or buffer alone. The pellets and supernatants were separated and the proteins were analysed on a 5–15% gradient SDS-PAGE. The gels were dried and subjected to autoradiography, and the C3 cleavage fragments were quantified by densitometric scanning as above.

The molecular form of C3 on the amebic pellets and in the supernants was also evaluated by immunoblotting with goat anti–C3 (from Dr. H. Müller-Eberhard). Trophozoites (1×10^6) were incubated in 10% NHS for 60 min at 37°C, and the pellets and supernatants processed as above. The gel was transfered to nitrocellulose (33), blocked for 1 h at room temperature in PBS containing 5% bovine serum albumin, then incubated in a 1:200 dilution of the polyclonal antisera in PBS-0.05% $Tween_{20}$ for 1 h. After six washes in PBS-Tween, the blot was incubated with alkaline phosphatase-labeled rabbit anti–goat IgG (1:2,000 dilution, Bio Rad Laboratories), washed an additional six times, and developed with nitroblue tetrazolium (0.3 mg/ml in 100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5) and 5-bromo-4-chloro-3-indolyl phosphate (0.15 mg/ml, Sigma Chemical Co.)

Fluid-phase activated components. The killing of *E. histolytica* by fluid-phase activated complement components was investigated by incubating amebas (5×10^5) in 20% NHS to which 20 U (100 U/ml) of cobra venom factor was added (Quidel Laboratories). Controls included amebas incubated in 20% NHS or cobra venom factor in buffer alone. The samples were incubated at 37°C on a rocker. Aliquots were removed at 0, 10, 20, 30, and 60 min for direct cell counts in a hemocytometer and calculation of the percentage of lysed trophozoites.

Lysis of amebas by purified complement components was determined by adding 2×10^5 amebas to 200 μl of either PBS alone, 10% NHS in PBS, or PBS to which the terminal complement components, C5b6 (20 U), C7 (5 μg), C8 (5 μg), and C9 (5 μg), were sequentially added at 5-min intervals. After 60 min of total incubation at 37°C, direct cell counts were determined in a hemocytometer (Fisher Scientific Co., Pittsburgh, PA), and the percent lysis was calculated.

The participation of the cysteine proteinase in the lytic process was investigated by measuring lysis in the presence and absence of a cysteine proteinase inhibitor, E-64. Lysis of complement-sensitive amebas by 10% NHS was determined by preincubating aliquots of 2×10^5 amebas in MEM-CH media (Eagle's minimal essential medium, HEPES buffer solution, ascorbic acid, and cysteine [34]) or MEM-CH containing 100 μM E-64 for 30 min at 37°C. Samples from each mixture were then incubated for an additional 60 min in MEM-CH alone, 10% NHS in MEM-CH, or 10% NHS in MEM-CH containing 100 μM E-64. The percent of trophozoites lysed in each mixture was determined as described above.

To further demonstrate that *E. histolytica* trophozoites generate a fluid-phase C5b-9 complex, the passive lysis of chicken erythrocytes by amebic supernatants was measured. Trophozoites (1×10^6) were incubated for 60 min at 37°C in 20% C7-deficient sera, and the supernatants were separated. The amebic supernatants were then incubated with chicken erythrocytes (5×10^7) in GVB+ with and without added C7 (5 μg) for 60 min at 37°C, and the percent lysis was calculated by measuring the hemolysis at A414. Control samples included C7-depleted sera incubated with zymosan at 4 mg/ml and erythrocytes incubated in C7-depleted sera alone.

Results

Binding of ^{125}I-C3 by pathogenic and nonpathogenic E. histolytica. The structure of the C3 molecule in the fluid phase and bound to the cell membranes was assessed by SDS-PAGE after the incubation of pathogenic and nonpathogenic trophozoites with ^{125}I-C3 in the presence of 10% NHS for 30 min at 37°C. In 22 experiments, 12 with pathogenic strains, 6 with nonpathogenic strains, and 4 with axenic strains, the α chain of C3 in the fluid phase (90% of total counts) was cleaved to yield fragments consistent with C3b and/or C3bi when NHS was present. In contrast, only C3 molecules with an intact α chain were detected on the cell surface (10% of total counts) (Fig. 1). No high molecular mass complexes were detected, making the presence of a covalent C3–acceptor complex unlikely. When the amebic pellets were solubilized in SDS followed by treatment with methylamine, which is capable of cleaving the covalent bond between C3 and acceptor molecules (13, 20), only the intact α chain was detected in nine experiments (three with pathogenic, three with nonpathogenic, and three with axenic strains). In three additional experiments testing the effect of methylamine on two pathogenic strains and one nonpathogenic strain, a mean of 20.5±11.5% of the counts of the

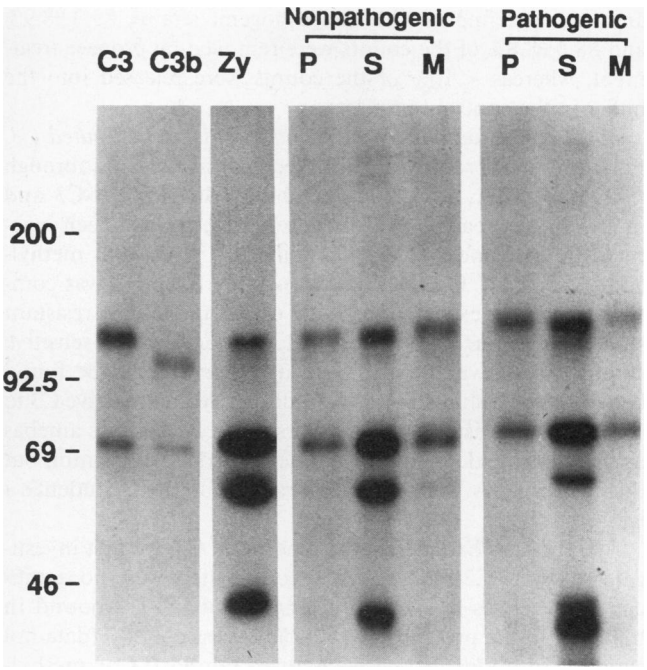

Figure 1. Molecular form of C3 in the fluid phase and bound to cell pellets of pathogenic and nonpathogenic amebas. Pathogenic and nonpathogenic trophozoites were incubated with 10% NHS to which ^{125}I-C3 was added, and the pellets and supernatants were separated. Identical pellets were subsequently incubated in the presence of 25 mM methylamine to break covalent ester bonds between C3 and the parasite membrane. All samples were analyzed by 5–15% SDS-PAGE under reducing conditions. C3b, trypsin-cleaved C3; Zy, cleavage of C3 in zymosan-activated sera; P, pellets; S, supernatants; M, supernatant after methylamine treatment of the pellet.

α chain was present in fragments equivalent in size to the α' of C3b or the α-68 of C3bi (data not shown). Similar results were found on immunoblots of amebic supernatants and pellets after incubation in 10% NHS and reaction with goat anti-C3 (data not shown).

The presence of C3 molecules with an intact α chain on the amebic surface was not caused by the binding of hemolytically inactive molecules in the ^{125}I-C3 preparation. This conclusion was reached because, first, the α chain of ^{125}I-C3 underwent autolytic fragmentation to peptides of 46 and 74 kD in the presence of denaturants, a property of hemolytically active C3 (23, 24). Secondly, the ^{125}I-C3 preparation was susceptible to proteolytic cleavage by EAC14b2a cells while methylamine-treated ^{125}I-C3 was not (data not shown). Moreover, when the radiolabeled C3 was incubated with zymosan in the presence of NHS, > 85% of the α chain of C3 was cleaved (Fig. 1, lane *Zy*).

The possibility that bound C3 might have been internalized and removed from interactions with fluid-phase activators and control proteins had to be considered as it has been estimated that *E. histolytica* can internalize their entire surface membrane in 20 min (35). To address this issue, we attempted to cleave the ^{125}I-C3 bound to the amebic surface with pronase. Pathogenic and nonpathogenic trophozoites were first incubated with ^{125}I-C3 in 10% NHS for 10 min at 37°C, washed three times, and then incubated for 15 min in either PBS alone or PBS containing 3.5 mg/ml pronase. The samples were centrifuged, and the counts released into the supernatant were determined. In three experiments with one pathogenic strain and two experiments with nonpathogenic strains, 89.3±8.9% and 88.5±8.8% of the counts were removed by pronase treatment, whereas < 10% of the counts were released into the buffer in the control samples.

Binding of radiolabeled C3 and methylamine-treated C3. For additional proof that C3 molecules did not bind through their thiol ester, we compared the binding of ^{125}I-C3 and methylamine-treated ^{125}I-C3 whose thiol ester had been inactivated. The time course of binding of ^{125}I-C3 and methylamine-treated ^{125}I-C3 in the presence of 10% NHS was compared in three experiments with one complement resistant strain and in four experiments with two complement-sensitive strains. As shown in Fig. 2, no differences could be found between the binding of hemolytically active and inactive C3 to complement-sensitive or resistant strains. Pathogenic amebas appeared to bind less methylamine-treated C3 at 20 min, but these differences were not significant ($P > 0.10$, Student's t test).

To further characterize the binding of C3, we first investigated whether C3 binding was specific. There were no significant differences between the amount of ^{125}I-C3 bound in buffer or in the presence of 100-fold excess cold C3 (data not shown). In addition, the α chain of bound C3 or methylamine-treated C3 was not cleaved by incubation with EAC14b2a cells (data not shown). Thus, the binding of C3 appears to be nonspecific, and once bound, C3 is not functionally active.

One explanation for the lack of binding of C3b might be a requirement for the amino-terminal portion of the C3 molecule containing C3a; however, the addition of a 1,500-fold excess (molecule/molecule) of C3a des Arg did not inhibit binding of ^{125}I-C3 (data not shown).

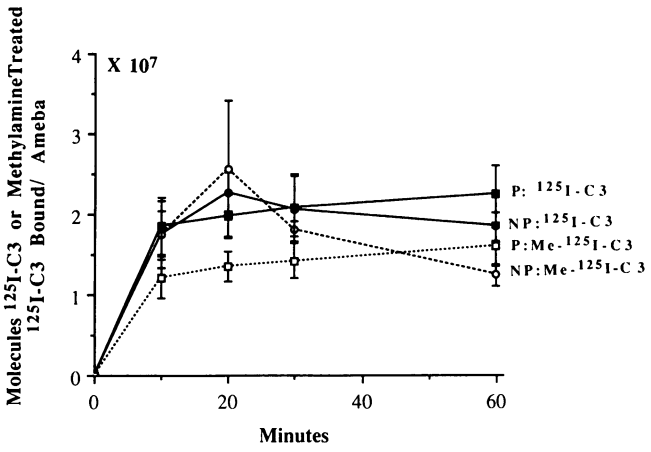

Figure 2. Binding of ^{125}I-C3 and methylamine-inactivated ^{125}I-C3 to pathogenic (*P*) and nonpathogenic (*NP*) strains. ^{125}I-C3 was added to trophozoites in 10% NHS. At timed intervals, aliquots were removed and the molecules of C3 bound were determined in the pellets. Values represent the mean±SEM of three experiments with one complement-resistant strain (+^{125}I-C3 [■], P; + methylamine-inactivated ^{125}I-C3 [□], P:Me) and four experiments with two complement-sensitive strains (+ ^{125}I-C3 [●], NP; + methylamine-inactivated ^{125}I-C3 [○], NP:Me).

Passive lysis by fluid-phase components. Since lysis of nonpathogenic strains takes place in the absence of bound C3b, we asked whether the lytic effect of complement occured by reactive lysis initiated in the fluid phase by cleaved C3. We first tested this hypothesis by comparing the lysis of pathogenic and nonpathogenic strains in the presence of 20% sera to which 100 U/ml of cobra venom factor was added to enhance activation of the alternative pathway (36). As shown in Fig. 3, nonpathogenic amebas were more efficiently killed by NHS in the presence of cobra venom factor (53.8±5.1%) than by 20% NHS alone (38.5±4.0%, $P < 0.05$ by Student's unpaired t test).

To confirm that the lysis of nonpathogenic *E. histolytica* was indeed caused exclusively by the terminal complement components, complement-sensitive and resistant strains were incubated with C5b6 followed by C7, C8, and C9 at concentrations equivalent to that of 20% NHS. Nonpathogenic amebas were killed by terminal complement components (44.7±1.6%) while pathogenic amebas were not (2.8±2.8%, $P = 0.005$, Student's t test, Fig. 4).

Effect of cysteine proteinase inhibitors. If the observed complement-mediated killing of nonpathogenic strains by NHS was initiated by the fluid-phase cleavage of C3 by the neutral cysteine proteinase, then inhibition of the proteinase should block killing. This was tested with axenic amebas which are the most sensitive to the action of complement. After preincubation of trophozoites for 30 min at 37°C with 100 μM E-64, an irreversible cysteine proteinase inhibitor, only 35.0±5.9% of amebae were lysed compared to 56.0±3.1% with 10% NHS alone ($P = 0.02$, Student's unpaired t test, Fig. 5). This concentration of inhibitor was not sufficient to fully inhibit proteolytic activity, however, attempts to inhibit killing with higher concentrations of E-64 were lethal to the amebas.

Formation of fluid-phase C5b-9. To demonstrate that the C5b6 complex was formed in the fluid phase, amebic trophozoites were incubated in 20% C7-depleted sera. The extent of

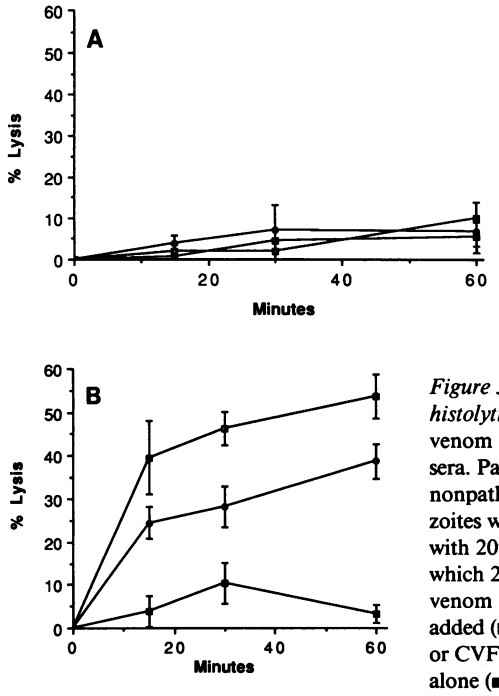

Figure 3. Lysis of *E. histolytica* by cobra venom factor-activated sera. Pathogenic and nonpathogenic trophozoites were incubated with 20% NHS to which 20 U of cobra venom factor had been added (□), 20% NHS (●) or CVF in PBS-Cys^{++} alone (■). At timed intervals, aliquots were removed and the percent lysis was calculated. Values represent the mean±SEM of six experiments with three pathogenic strains (*A*) and six experiments with four nonpathogenic strains (*B*).

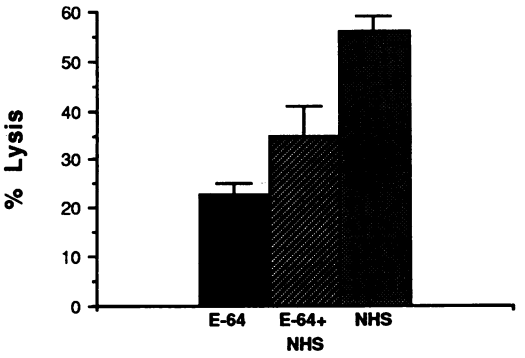

Figure 5. Inhibition of serum lysis by cysteine proteinase inhibitors. Aliquots of axenic *E. histolytica* were preincubated 30 min at 37°C in MEM-CH alone or with 100 μM E-64. Samples were subsequently incubated an additional 60 min in MEM-CH with 100 μM E-64, 10% NHS, or 10% NHS with 100 μM E-64. The percent lysis was determined from direct cell counts. Values represent the mean±SEM of four experiments.

the complement activation was then evaluated by measuring the lysis of chicken erythrocytes in the presence of the amebic supernatants and added C7. As shown in Table I, passive lysis of chicken erythrocytes was equivalent in the sera incubated with pathogenic *E. histolytica* and zymosan.

Discussion

We had previously shown that the purified major extracellular proteinase of *E. histolytica,* a 56-kD neutral cysteine proteinase, cleaves the α chain of C3 in the fluid phase yielding a hemolytically active molecule (17). Accordingly, we investigated the interactions of complement-sensitive (nonpathogenic) and resistant (pathogenic) strains with C3 to determine whether the fluid-phase activation of complement contributed to the observed differences in complement sensitivity. Trophozoites of *Entamoeba histolytica* have been shown to consume components of both the classical and alternative pathways of complement in the fluid phase (10, 11), a finding which was not unexpected in light of their carbohydrate-rich cell surface (37, 38). However, our results show that, although activation of both the classical and alternative pathways may result in cleavage of the third component of complement in the fluid phase, only C3 with an intact α chain could be detected on the surface of both complement-sensitive and resistant amebas in 31 experiments (Fig. 1). No higher molecular mass complexes were detected to suggest that C3b or C3bi might be covalently linked to parasite acceptor molecules, as demonstrated with *Leishmania major* (32) and *Toxoplasma gondii* (39). To detect even a small amount of covalently bound C3 activation fragments, pellets were treated with methylamine to disrupt the ester linkage. Minor cleavage of the α chain to fragments compatible in size with C3b (105 kD α′ chain) or C3bi (68, 46, or 43 kD) (20.5±11.5%) was detected in only 3 of 12 experiments (two in complement-resistant and one in a complement-sensitive strain). Therefore, differences

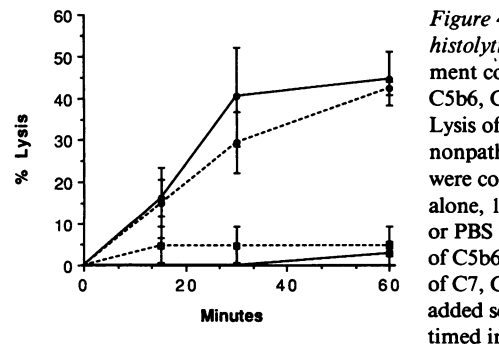

Figure 4. Lysis of *E. histolytica* with complement components C5b6, C7, C8, and C9. Lysis of pathogenic and nonpathogenic amebas were compared in PBS alone, 10% NHS in PBS or PBS to which 20 U of C5b6, and 5 μg each of C7, C8, and C9 were added sequentially. At timed intervals during incubation at 37°C, aliquots were removed for direct cell counts. Values represent the mean±SEM of three experiments with three pathogenic strains (NHS [□]; purified complement [■]) and three experiments with two nonpathogenic strains (NHS [○]; purified complement [●]).

Table I. Passive Lysis of Chicken Erythrocytes by Sera Activated by Pathogenic E. histolytica and Zymosan

	Lysis	
	Zymosan	Pathogenic *E. histolytica*
	%	
C7-depleted sera	10.7±3.0	8.0±0.3
C7-depleted sera + C7	81.7±1.4	91.1±2.2

C7-depleted sera (final concentration 20%) was incubated with 1 × 10^6 trophozoites from three pathogenic strains or zymosan at 4 mg/ml. The ability of the supernatants to cause lysis of chicken erythrocytes was then evaluated with or without added C7 (5 μg). Values are given as mean±SE.

in sensitivity to complement-mediated killing could not be attributed to the distinct C3 cleavage products on the surface of the parasite. We had previously reported that C3bi and C3d were detected on amebic cell surfaces after incubation in 20% sera depleted of C3 (40). In retrospect, these findings were probably caused by the incomplete inactivation of amebic proteinases during the reduction of samples, as the 56-kD neutral cysteine proteinase can rapidly and nonspecifically cleave denatured C3 (17).

Experiments to characterize the binding of C3 further demonstrated that C3 did not bind through the thiol ester since amebas bound equivalent amounts of native C3 and C3 with the thiol ester inactivated by methylamine (Fig. 2). Binding of C3 was not saturable, negating the presence of a specific receptor. Although major surface glycoproteins or lipophosphoglycans have been described as C3 acceptors in *Trypanosoma cruzi, Leishmania major,* and *Leishmania mexicana* (reviewed in reference 41), no receptors to intact C3 have been identified in protozoa. C3 did not bind through the amino-terminal portion of the molecule in that cold C3a des Arg did not inhibit binding of ^{125}I-C3. Finally, the thiol ester of bound C3 was inactive as further cleavage by C3 convertases could not be demonstrated.

We undertook multiple controls to insure that the finding of intact C3 bound to the cell surface was not an artifact produced by the addition of hemolytically inactive ^{125}I-C3. The radiolabeled C3 was present in a monomeric form and retained > 80% of the hemolytic activity of the unlabeled protein. The α chain of ^{125}I-C3, but not methylamine-treated ^{125}I-C3, was cleaved by the C3 convertase (EAC14b2a cells) in the fluid phase and underwent autolytic fragmentation. More than 85% of the radiolabeled C3 was cleaved in the presence of zymosan in NHS. In addition, we confirmed our results using immunoblotting of amebas incubated in sera with native C3. Finally, we demonstrated that all of the bound C3 was susceptible to pronase, indicating that it was not internalized, but, once bound, it was susceptible neither to the action of the convertases, nor fluid-phase control proteins.

The observation of fluid-phase C3 activation in NHS points out interesting differences between the interactions with C3 of intact amebas and the purified 56-kD proteinase (17). The purified proteinase may be completely inhibited by 5% bovine or human serum (2), while fluid-phase cleavage of C3 may be detected with intact amebae even in the presence of 50% NHS (data not shown). The fluid-phase cleavage was inhibited by preincubating the amebas with E-64, an irreversible cysteine proteinase inhibitor (42), making the participation of other proteinases unlikely. These findings may reflect the higher activity of the proteinase in the environment immediately adjacent to the amebic trophozoites. In addition, we could detect C3 cleavage by nonpathogenic strains, even though they release significantly less cysteine proteinase activity as assessed by enzymatic activity (3). Thus, it appears that C3 is extremely sensitive to cleavage by this cysteine proteinase (17), so that even the low level of proteinase released by nonpathogenic strains suffices to cleave C3 in the fluid phase.

Since complement-sensitive strains are lysed in the absence of bound C3b, we investigated whether lysis was the result of assembly of the membrane attack complex initiated by fluid-phase cleavage of C3. Cobra venom factor produces unrestricted complement activation because of the increased stability of the cobra venom factor-dependent C3 convertase (CVF,Bb) and its resistance to down regulation by the regulatory proteins, factors H and I (36). We found that complement-sensitive strains were more efficiently killed in the presence of cobra venom factor added to 20% NHS than by 20% NHS alone (Fig. 3). Moreover, nonpathogenic strains could be lysed by the addition of purified C5b6, C7, C8, and C9 (Fig. 4). Lysis of complement-sensitive amebas by 10% NHS could be partially prevented by preincubation with E-64, a specific, irreversible inhibitor of the 56-kD cysteine proteinase (Fig. 5). Finally, equivalent passive lysis of chicken erythrocytes was produced by C7-depleted sera incubated with pathogenic *E. histolytica* or zymosan (Table I).

The concept of reactive or passive lysis was first introduced by Thompson and Rowe (43) in 1968 to describe lysis of erythrocytes in the absence of antibody and the early components of complement and was subsequently shown to involve the attachment of C5b-9 to the erythrocyte membrane independently of C3b (44–46). Although lysis of a rough, serum-sensitive strain of *Escherichia coli* by C5b67, C8, and C9 was enhanced by pretreatment of the bacteria with EDTA and addition of an additional serum factor from the pseudoglobulin fraction (47), passive lysis initiated by release of a proteinase from a potential pathogen appears to be unique.

These results demonstrate for the first time that complement-sensitive *E. histolytica* are lysed by the formation of the membrane attack complex, C5b-9. Complement activation is initiated in the fluid phase by the cleavage of C3 by the 56-kD neutral cysteine proteinase (17), and not by the surface deposition of activated C3, as we have now shown. Clearly, the cleavage of C3 is not the only function of the proteinase; it appears to promote invasion by degrading extracellular matrixes, including fibronectin, laminin, and type I collagen (2). The role of complement activation by the cysteine proteinase in the pathogenesis of amebic disease may be significant and could include the participation of the anaphylotoxins, C3a and C5a.

The mechanism by which pathogenic amebas evade complement-mediated killing has not yet been delineated. It could involve the formation of a nonfunctional membrane attack complex similar to that seen with serum-resistant *Neisseria gonorrhoeae* (48, 49) or more rapid membrane repair as has been shown in a variety of nucleated cells (50–52). Alternatively, pathogenic strains might synthesize membrane proteins similar to the recently described inhibitor of reactive lysis (53) or homologous restriction factor (54). These molecules prevent assembly of the membrane attack complex at the level of C7 and C8 or the binding and polymerization of C9, thereby preventing the formation of a lytic channel.

Acknowledgments

We thank Dr. Tony Hugli for his generous gift of the C3a des Arg, Scott Herdman for technical assistance, and Drs. Jim McKerrow and Charles Davis for helpful discussions.

Dr. Reed is a Lucille P. Markey Scholar and this work is supported in part by grants from the Lucille P. Markey Charitable Trust and National Institutes of Health grants DK-35108, and AI-20067.

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