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interacción de *Entamoeba histolytica* patógena y no patógena con el complemento humano

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Resumen

La resistencia a la lisis mediada por complemento puede ser un mecanismo importante de la virulencia de las cepas invasoras de *E. histolytica.* El suero humano normal (NHS) produjo lisis con rapidez de cada uno de 34 aislamientos de sujetos asintomáticos (66.8 ± 12.1 por ciento), en tanto que las cepas obtenidas de 23 pacientes que sufrían enfermedad invasora manifestaron resistencia al NHS (3.2 ± 5.5 por ciento de lisis) y al suero inmune (66.8 ± 11.3 por ciento). La conservación de la resistencia al complemento de las cepas patógenas pareció depender de las condiciones de crecimiento, ya que dos cepas resistentes se volvieron sensibles durante el proceso de axenización. La activación de la vía alternativa tuvo importancia para la lisis de las cepas no patógenas, a juzgar por la lisis ocurrida en NHS (59.3 ± 4.5 por ciento), NHS + EGTA 5 mM en C4 (71.0 ± 9.1 por ciento) lo mismo que suero humano deficiente en C2 (70.7 por ciento), pero no por NHS + EDTA 5 mM. En contraste, las cepas patógenas fueron resistentes a la lista por las vías tanto clásica como alternativa. Tanto las cepas resistentes al complemento como las sensibles al mismo agotaron el complemento de manera relativa, a juzgar por las actividades hemolíticas de CH50, C3, C7, y C5-9. Además, se agotó más de 98 por ciento de la actividad hemolítica de C4 por la acción de las cepas tanto patógenas como no patógenas, lo que indica que actúa la vía clásica aunque no sea necesaria para la lisis. Estos estudios demuestran que las cepas patógenas de *E. histolytica* activan al complemento pero son capaces de evadir la citotoxicidad mediada por el mismo.

Abstract

Resistance to complement-mediated lysis may be an important virulence mechanism for invasive strains of *E. histolytica.* Each of thirty-four isolates from asymptomatic patients were rapidly lysed (66.8 ± 12.1 per cent) by normal human serum (NHS) while strains from 23 patients with invasive disease were resistant to NHS (3.2 ± 5.5 per cent lysis) and immune sera (9.8 ± 11.3 per cent). Maintenance of complement resistance of pathogenic strains appeared to be dependent on growth conditions as two complement resistant strains became sensitive during the process of axenization. Activation of the alternative pathway was important in the lysis of nonpathogenic strains as demonstrated by lysis in NHS (59.3 ± 4.5 per cent), NHS + 5 mM EGTA (60.9 ± 15.6 per cent) as well as C4-deficient guinea pig serum (71.0 ± 9.1 per cent) and C2-deficient human serum (70.7 per cent) but not by NHS + 5 mM EDTA. In contrast, pathogenic strains were resistant to lysis by both classical and alternative pathways. Both complement-sensitive and complement-resistant strains depleted complement comparably as assessed by CH_{50}, C3, C7, and C5-9 hemolytic activities. In addition, more than 98 per cent of the C4 hemolytic activity was depleted by both pathogenic and nonpathogenic strains, indicating that classical pathway activation occurs even though it is not necessary for lysis. These studies demonstrate that pathogenic strains of *E. histolytica* do activate complement but are able to evade complement-mediated killing.

The mechanisms of virulence of *Entamoeba histolytica* are poorly understood. For many years, no consistent morphological or biochemical differences had been found betwen strains from patients who were completely asymptomatic and those with invasive disease. The classification of clinical strains into zymodemes by Sargeaunt was a major breakthrough since there was a strong correlation between isoenzyme patterns and invasive disease.[1,2] This suggested that other biological properties might differentiate pathogenic and nonpathogenic amebae. One such biological property, resistance to complement-mediated lysis has been reported to be an important virulence factor in both bacterial and parasitic diseases.[3,4,5] We speculated that strains of *E. histolytica* which disseminate from the bowel through the blood stream to cause liver abscesses should be resistant to complement lysis. An apparent paradox existed as all previous work with axenic *E. histolytica* demonstrated rapid lysis of amebae by nomimmune serum.[6,7,8] In contrast, our earlier studies showed that recent clinical isolates from patients with colitis or liver abscesses were resistant to complement-mediated killing while nonpathogenic isolates were sensitive.[9] We therefore undertook these studies to examine the interactions of pathogenic and nonpathogenic strains of *E. histolytica* with human complement.

Materials and methods

Serum. Sera from at least three healthy volunteers without antibodies to *E. hisltolytica* (by agar gel diffusion[10] or counterimmunoelectrophoresis[11] were pooled and allowed to clot for 30 minutes at room temperature, centrifuged at 2000g for 10 minutes, and stored at —70°C.

C4-deficient guinea pig serum was obtained from congenitally deficient guinea pigs (NIH strain) by cardiac puncture. Congenitally C2— deficient human serum was a gift from Susan Koethe, Ph.D. (University of Wisconsin).

Immune sera were obtained from four patients with amebic liver abscesses. All had titers above 1:128 by counterimmunoelectrophoresis. Complement was inactivated by heating the sera at 56°C for thirty minutes. Nonimmune human serum was added as a complement source.

E. histolytica cultures. Twelve pathogenic and five nonpathogenic strains were obtained from P. Sargeaunt. The other forty strains were obtained from cultures of stools or liver abscess aspirates submitted to the Microbiology Laboratory at UCSD Medical Center. Recent clinical isolates were cultured directly from stools or liver abscesses into Robinson's medium[12] and maintained in either Robinson's medium or Diamond's TYSGM[13] containaing *E. coli* 0111. Subcultures were made every two to three days. Axenic strains included HM-1:IMSS (HM-1), American Type Culture Collection, múm. 30458, UCSD:0283:1 (SD-1), and NIH 200 (ATCC núm. 30458). They were grown in TYI-S-33 medium containing 12 per cent bovine serum[14] with subculture twice weekly. To control bacterial overgrowth when cultures were transfered from Robinson's medium or TYSGM to TYI-S-33 media, Piperacillin 500 ug/ml and Amikacin 125 ug/ml or other antibiotics were added with each subculture as needed.

Isoenzyme electrophoresis. Lysates were made as described by Sargeaunt[1] and zymodemes assigned according to starch gel isoenzyme patterns of maleic enzyme, phosphoglucomutase, glucophosphoisomerase, and hexokinase.[1] All pathogenic strains in this study were isolated from patients with either symptomatic colitis or liver abscesses and were zymodemes II, XI, XIV. In contrast, strains isolated from asymptomatic patients were in nonpathogenic zymodemes (I, III, VIII).

Serum killing assay. Cultures were chilled on ice for ten minutes to promote detachment, centrifuged at 1000g for ten minutes, and resuspended in a holding medium, MEM-CH (HEPES buffered MEM with cysteine).[15] Equal volumes of the amebic suspension and either normal human serum (NHS) or C2-deficient serum (50 per cent v/v) were mixed and aliquots removed immediately for the zero time sample. The C4-deficient guinea pig serum was diluted 1:10 in phosphate buffered saline, pH 7.4 with 0.15 mM $CaCl_2$ and 0.5 mM $MgCl_2$ (PBS^{++}). Mixtures were incubated on a rocker at 37°C to insure complete mixing. At timed intervals, aliquots were removed for direct counts in a hemacytometer. Percentage lysis was calculated as $N_o - N_x/N_o \times 100$ per cent where N_o was the initial viable count and N_x, the count at time x. Complement sensitivity was defined as greater than 59 per cent lysis and resistance as less than 20 per cent lysis after sixty minutes incubation.

Purification of amebae. Amebic cultures were chilled on ice for ten minutes then centrifuged at 1000g for ten minutes. Pellets were resuspended in MEM-CH and layered on top of a discontinuous Percoll gradient[16] containing equal layers of 100 per cent and 50 per cent Percoll. After centrifugation at 3000g for 15 minutes, the starch was pelleted on the bottom, while the amebae were concentrated in the 50 per cent layer. After the 50 per cent layer was removed and washed three times with PBS at 500g for the minutes, the associated bacterial count was decreased by at least 99 per cent to 1×10^4 to 5×10^5/ml. Amebae were resuspended in MEM-CH and maintained at room temperature or 37°C until used. 1×10^5 to 1×10^6 purified ameabae/ml were used in all experiments unless otherwise indicated.

Complement Assays. Suspensions of 1×10^5 to 1×10^6 amebae in MEM-CH were mixed with an equal volume of NHS. A zero time sample was immediately removed and the remainder incubated on a rocker at 37°C. At timed intervals, aliquots were removed. Amebae were pelleted in a microfuge at 15,000g for five minutes, the supernatant removed, and the pellet resuspended in 1.0 ml MEM-CH. Both pellet and supernatant were frozen at −70°C until use.

Sheep erythrocytes were sensitized with rabbit anti-sheep hemolysin (EA). EACl cells were made by incubating EA cells 30 minutes at 30°C with human Cl.[17] EACl 4b cells were generated by incubating EACl cells with an optimal dilution of purified human C4[17] for 30 minutes at 4°C.

The total hemolytic activity (CH50) was determined by incubating EA cells with dilutions of the experimental supernatants and controls. The samples were incubated sixty minutes at 37°C, the unlysed erythrocytes removed by centrifugation and the hemoglobin in the supernatant measured by A_{414}.[18]

All component assays were performed using specific depleted sera — human serum with potassium bromide-inactivated C3,[19] C4-deficient guinea pig serum[20] or human sera depleted of C7 by affinity chromatography.[4] An excess of oxidized human C2,[21] Clq,[22] C3 (Cordis Corp., Miami, Fla.) and or C5 (Cordis Corp, Miami, Fla) was added as required so only the measured component was limiting. Following incubation, the hemoglobin in the supernatant was measured as above and the unites of functional complement in each sample were calculated.

Controls for media components. Amebic samples were purified over Percoll gradients with multiple washes as above. The number of bacteria remaining in the media was determined by plating serial dilutions of the purified amebic samples on rabbit blood agar or trypticase soy agar plates. The number of bacteria ingested by the amebae was also assessed by lysing the amebae in distilled water before making the dilutions. To assess the amount of complement depleted by various concentrations of *E. coli* 0111, bacteria were inoculated into sterile TYSGM and incubated overnight. Dilutions were made for quantitative bacterial counts in trypticase soy broth and plated on trypticase soy agar. In order to determine complemente consumption, dilutions of the TYSGM with *E. coli* were made in PBS and incubated with 50 por cent v/v NHS for 60 minutes at 37°C. After centrifugation at 15,000g for ten minutes, the supernatant was assayed for complement activity. As shown in figure 1, less than 5 per cent of the initial hemolytic activity was depleted by bacteria at concentrations of 10^7/ml while bacterial counts were less than 5×10^6/ml in all samples of amebae.

Results

Effect of NHS on pathogenic and nonpathogenic E. histolytica. Thirty-four nonpathogenic strains of *E. histolytica* isolated from asymptomatic patients and three axenic strains were rapidly lysed by 50 per cent v/v NHS (figure 2). In contrast, twenty-three stains from patients with symptomatic colitis or liver abscesses were resistant with less than 5 per cent lysis. The four pathogenic strains tested were also resistant to lysis by immune sera.

Media dependence of complement resistance. In order to understand the differences in complement-mediated lysis between axenic and recently isolated pathogenic strains of the same zymodeme, we followed the complement sensitivity of two strains during the process of axenization. Figure 3 A demonstrates an amebic strain initially isolated from an amebic liver abscess into Robinson's medium. Following axenization, the strain became serum sensitive but reverted to resistant within two weeks following transfer back into Robinson's medium. Figure 3B illustrates the course of a strain isolated from a patient with amebic colitis. After four weeks in TYI-S-33, the
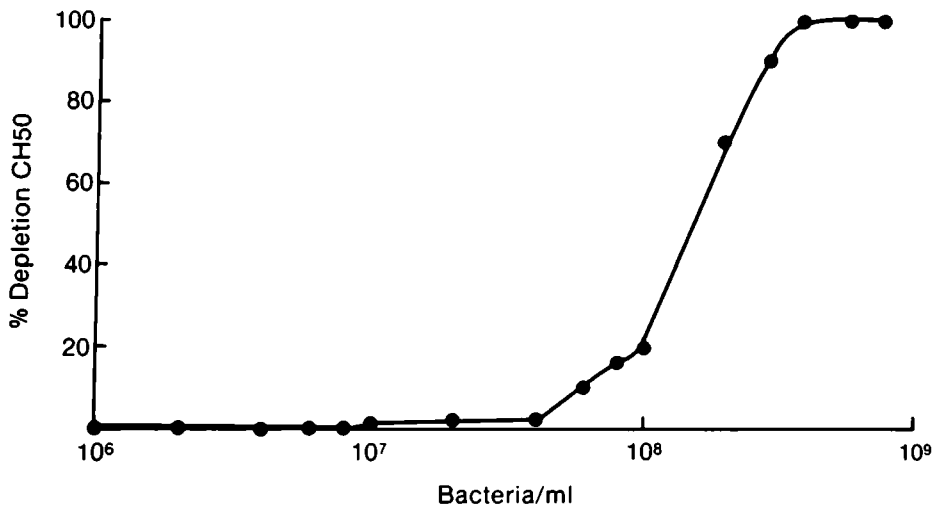
Figure 1: Control experiment showing the per cent depletion of complement by *E. coli* 0111. Values represent the mean per cent depletion of hemolytic complement activity (CH50) by dilutions of *E. coli* 0111 in PBS and 50 per cent v/v NHS in two experiments compared with controls containing PBS and 50 per cent v/v NHS. (Reprinted with permission from J. of Immunology.)
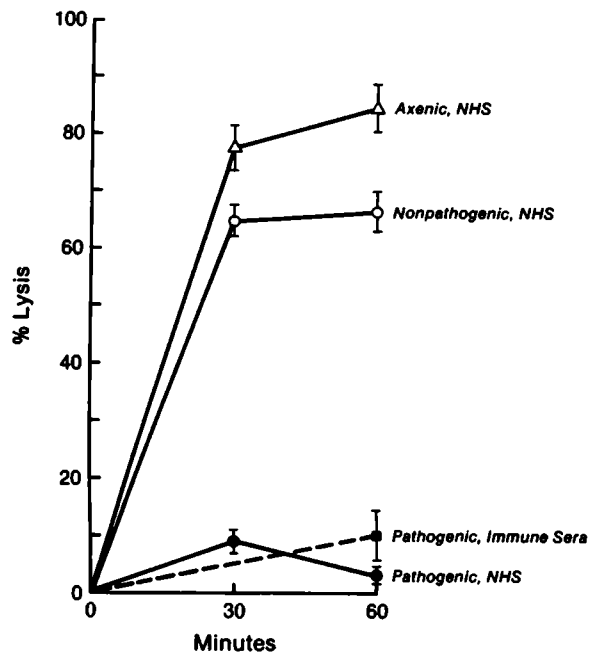

Figure 2. Per cent lysis of three axenic (△) thirteen nonpathogenic (O), twenty-one pathogenic (O) strains of *E. histolytica* by 50 per cent v/v NHS and four pathogenic strains (O) by 30 per cent heat-inactivated immune human sera and 20 per cent NHS.
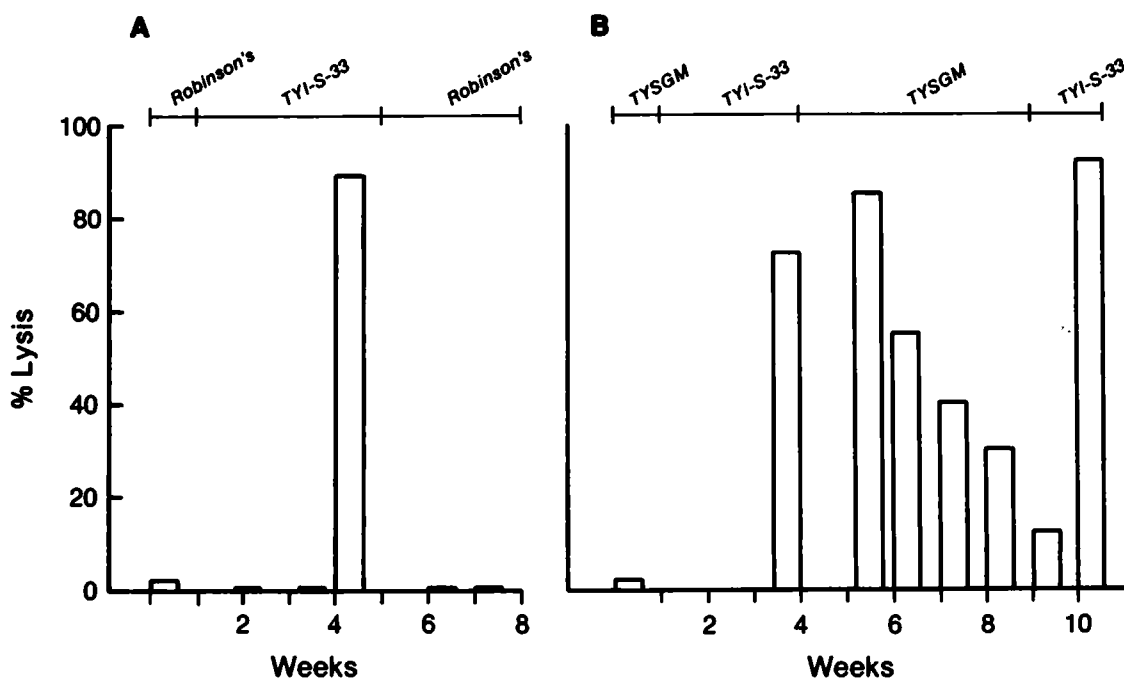
Figure 3. Time course of complement sensitivity of pathogenic strains in different culture media. A. Continuous sequence of tests of SD-1 during the course of its axenization. B. Composite of multiple transfers of a pathogenic strain which was never completely axenized but became complement sensitive.

strain became serum sensitive despite the continued presence of bacteria. Complement resistance was regained within four weeks following transfer back into TYSGM. Simultaneous controls tested at the same time points included the original culture kept in Robinson's media or TYSGM (<5 per cent lysis) or an axenic strain (> 80 per cent lysis). No change in zymodeme of any strain was observed. To date, we have not been successful in making HM-1 or NIH-200 complement resistant by bacterial reassociation.

Both Diamond's TYSGM and Robinson's media contain potential activators of complement. We deleted selected media components to assess the effect on the viability and complement resistance of the pathogenic amebae. A small amount of particulate starch was necessary for continued growth in TYSGM but less than 25 per cent of the amounts initially described[13] were necessary. Soluble starch was not sufficient alone. Similarly, growth could not be maintained without gastric mucin even with additional iron in the form of ferric nitrate. Because the bacteria added to the cultures, *E. coli* 0111 activate

complement, we attempted to grow axenic strains (HM-1 and SD-1) with bacteria which did not activate complement. The following strains were tested: *Staphylococcus aureus, Streptococcus viridans,* Group B *Streptococcus, Lactobacillus, Peptococcus asaccharolyticus, Clostridium difficile, Clostridium ramnosum* and *Staphylococcus epidermidis.* 10^8 bacteria of each strain depleted less than 10 per cent of the complement from 50 per cent NHS controls as measured by CH50. Cultures could not be maintained beyond two weeks with any of these bacterial strains. At this point, all *E. histolytica* tested were still complement sensitive.

The role fo the alternative and classical pathways in killing. We assessed the roles of the alternative and classical pathways in killing by comparing lysis in the presence of EGTA which blocks the classical pathway and with EDTA which blocks both alternative and classical pathways. Table 1 summarizes the results of three experiments with five amebic strains.[23] Nonpathogenic and axenic strains were lysed by NHS and + 5 mM EGTA but not by NHS + 5 mM EDTA.

TABLE 1. LYSIS OF *E. HISTOLYTICA* STRAINS BY CHELATED AND
COMPONENT-DEFICIENT SERA

Serum	Num. Anexic Strains	% Lysis	Num. Nonpathogenic Strains	% Lysis
45% NHS	2	81.6 ± 9.4%	3	59.3 ± 4.5
Heat-inactivated	2	0%	2	0%
45% NHS + 5mM EGTA	2	82.1 ± 8.6%	3	60.9 ± 15.6%
45% NHS + 5mM EDTA	2	14.3 ± 24.8%	3	0%
50% C2-deficient Human Serum	1	70.9%	1	70.7%
10% C4-deficient Guinea Pig Serum	1	76.4%	2	71.0 ± 9.1%

Axenic and nonpathogenic strains were also lysed by C4-deficient guinea pig serum and C2-deficient human serum (table 1). Less than 10 per cent of the amebae of pathogenic strains were lysed under identical conditions. These studies demonstrate that activation of the alternative pathway is sufficient for killing of sensitive amebae.

Complement activation. One possible explanacion for the complement resistance of pathogenic strains is that they do not activate complement. We assessed the amount of complement depleted from nonimmune serum incubated with different amebic strains by measuring CH50 in the supernatants. because the recent clinical isolates must be maintained in complex media containing potential activators of complement, we first evaluated the possibility that bacteria in the media might be depleting complement. Following purification, 1×10^4 to 5×10^6 bacteria/ml were present in the amebic samples, a level which caused essentially no complement activation (Figure 1).

The depletion of complement components by two nonpathogenic and three pathogenic amebic strains incubated in 50% NHS is shown in Table 2. CH50,

C3, C7, and C5-9 were depleted comparably by both pathogenic and nonpathogenic strains.[23] In addition, more than 98 per cent of the C4 hemolytic activity was depleted by both pathogenic and nonpathogenic strains, indicating that classical pathway activation occurs although it is not necessary for lysis.

Discussion

This study extends our earlier observation that recent clinical isolates from patients with invasive amebiasis are resistant to the lytic action of complement in normal human serum in contrast to nonpathogenic and axenic strains which are readily lysed (figure 2). The serum resistance of pathogenic clinical isolates is in marked contrast to earlier observations of lysis of axenic strains (HK-9, NIH 200[7,8] and HM-2[6]) by normal human serum. A decrease in the ability of some axenic strains to cause liver abscesses in animal models[24,25] or to destroy tissue culture monolayers[26] has been well documented. We have been able to demonstrate the gradual loss of serum resistance in two pathogenic strains during the process of axenization

TABLE 2. DEPLETION OF COMPLEMENT COMPONENTS BY PATHOGENIC AND NONPATHOGENIC AMEBAE

% Initial Titer ± S.E.

% Initial Titer ± S.E.

Sample	CH50	C3	C4	C7	C5-9
Control	96.2±2.9	93.3±6.7	90.6±9.4	94.7±5.4	93.8±6.0
Nonpathogenic Amebae	12.9±7.7	34.0±17.0	1.3±0.1	51.5±0.9	22.7±10.7
Pathogenic Amebae	4.0±4.0	12.5±7.1	1.3±0.4	21.2±9.7	4.0±4.0

Values represent per cent of the initial (0 time) hemolytic titer of control samples (50% v/v NHS in MEN-CH), nonpathogenic, and pathogenic amebae incubated in 50% NHS for 60 minutes at 37°C. Component titrations were performed as described in Materials and Methods.

as well (figure 3). In addition, complement resitance was restored upon readapting to growth in Robinson's or TYSGM media with bacteria.

The interactions of *E. histolytica* with the complex ingredients of *in vitro* culture systems has been a source of much interest. The virulence of axenically grown strains of *E. histolytica* has been shown to increase following reculture from hepatic abscesses in animals,[24,27] longtern growth with additional cholesterol in the medium,[28,29] and reassociation with bacteria.[16,30,31,33] The interactions of axenically grown *E. histolytica* with different bacterial strains has been intensively studied by Mirelman et al. who found that damage to tissue culture monolayers and the ability to cause hepatic abscesses in hamsters was increased after only fifteen minutes of incubation with bacterial strains possessing mannose-binding components.[16,32] Bacterial species without mannose-binding capacity would only attach to the amebae if coated with concanavalin A or oposonized with immune sera.[16] In our studies much longer periods of bacterial reassociation (weeks to months) were required to lose or regain complement resistance. Our inability to affect complement resistance with a number of bacterial strains which do not activate complement is probably due to lack of attachment and inability to support growth. The bacteria are clearly an important component of Diamond's TYSGM and Robinson's media but are not sufficient

alone to maintain the complement resistance of pathogenic strains as complement resistance was lost before bacteria were completely eliminated from the culture. Elucidation of other critical media components was limited by decreased viability of the amebae. The loss and restoration of complement resistance appears to be a gradual process, and the exact mechanism is unknown. It is important to stress, however, that complement resistance is not just a media-evoked phenomenon. Nonpathogenic strains cultured under identical conditions never became complement resistant. To our knowledge, no strain from a nonpathogenic zymodeme has ever been completely axenized and remained avirulent. Mirelman et al.,[33] however, reported that an uncloned strain from an asymptomatic carrier changed zymodemes and acquired the ability to produce hepatic abscesses in hamsters during the process of axenization. We have never detected any zymodeme changes during axenization or reassociation with bacteria.

We have not been successful to date in making HM-1 or NIH 200 complement resistant by reassociation with bacteria. Presumably, the amebae are genetically capable of expressing complement resistance as demonstrated by Calderon's selection of complement

resistance through continued exposure of axenic trophozoites to increasing concentrations of NHS.[34]

Activation of the alternative and classical complement pathways by axenic amebae has been demonstrated previously[7,8,35,36] as well as the importance of complement in animal models of liver abscesses.[37] We are the first to demonstrate that the alternative pathway plays a critical role in the lysis of nonpathogenic clinical isolates as established by killing in the presence of EGTA and by C2- and C4-deficient serum (table 1). Classical pathway activation also occurs but is not necessary for lysis. Pathogenic clinical isolates were not lysed by complement alone or in the presence of specific antibodies.

One possible explanation for complement resistance of pathogenic strains is that they fail to activate complement. Instead, we found that both pathogenic and nonpathogenic strains rapidly depleted both early and late components of complement (table 2). Further studies are underway to explore the interactions of individual complement components at the cell surface. Initial studies have demonstrated that equivalent amounts of C3 are bound by pathogenic and nonpathogenic amebae and that the same C3 degradation products (C3bi, C3dg) are deposited on the amebic cell surface.[38] Although the mechanism is not presently known, these studies demonstrate that pathogenic strains of *E. histolytica* do activate complement but are able to evade an important host defense, complement-mediated killing.

Referencias
References

1. SARGEAUNT, P.G.; WILLIAMS, J.E.; GREENE, J.D.: *The differentiation of invasive and noninvasive E. histolytica by isoenzyme electrophoresis.* Trans. Roy. Soc. Trop. Med., 1978; 72:519.

2. Sargeaunt, P.G.; JACKSON, T.F.H.G.; SIMJEE, A.: *Biochemical heterogeneity of Entamoeba histolytica especially those from liver abscesses.* Lancet, 1982; 1:1386.

3. JOINER, K.A.; HAMMER, C.H.; BROWN, E.J.; et al: *Studies on the mechanism of bacterial resistance to complement-mediated killing. I. Terminal components are deposited and released from Salmonella minnesota s218 without causing bacterial death.* J. Exp. Med., 1982; 155:797.

4. HARRIMAN, G.R.; PODACK, E.R.; BRAUDE, A.I.; et al: *Activation of complement by serum-resistant Neisseria gonorrhoeae. Assembly of the membrane attack complex without subsequent cell death.* J. Exp. Med., 1982; 156:1235.

5. SAMUELSON, J.C.; SHER, A.; CAUFIELD, J.P.: *Newly transformed schistosomula spontaneously lose surface antigens and C3 acceptor sites during culture.* J. Immunol., 1980; 124:2055.

6. ORTIZ-ORTIZ, L.; SEPULVEDA, B.; CHEVEZ, A.: *Nuevos estudios acerca de la accion de sueros humanos normales e immunes sobre el trofozoito de E. histolytica.* Arch. Invest. Med. (Mex.), 1974; 5:337.

7. ORTIZ-ORTIZ, L.; CAPIN, R.; CAPIN, N.R.; et al: *Activation of the alternative pathway of complement by Entamoeba histolytica.* Clin. Exp. Immunol., 1978; 34:10.

8. HULDT, G.; DAVIES, P.; ALLISON, A.C.; et al: *Interactions between Entamoeba histolytica and complement.* Nature, 1979; 277:214.

9. REED, S.L.; SARGEAUNT, P.G.; BRAUDE, A.I.: *Resistance to lysis by human serum of pathogenic Entamoeba histolytica.* Trans. Roy. Soc. Med. Hyg., 1983; 77:248.

10. POWELL, S.J.; MADDISON, S.E.; WILMOT, A.J.: *Amoebic gel-diffusion precipitin test. Clinical evaluation in amoebic liver abscess.* Lancet, 1965; 2:602.

11. KRUPP, I.M.: *Comparison of counterimmunoelectrophoresis with other serologic tests in the diagnosis of ameabiasis.* Am. J. Trop. Med. Hyg., 1974; 23:27.

12. ROBINSON, G.: *The laboratoy diagnosis of human parasitic amoebae.* Trans. R. Soc. Trop. Med., 1968; 62:285.

13. DIAMOND, L. S.: *A new liquid medium for xenic cultivation of Entamoeba histolytica and other lumen-dwelling protozoa.* J. Parasit., 1982; 68:958.

14. DIAMOND, L.S.; HARLOW, D.R.; CUNNICK, C.C.: *A new medium for the axenic cultivation of Entamoeba histolytica and other Entamoeba.* Trans. Roy. Soc. Trop. Med. Hyg., 1978; 72:431.

15. GILLIN, F.D; SHER, A.: *Activation of the alternative complement pathway by* Trichomonas vaginalis. *Inf. and Immun.*, 1981; *34:*268.

16. BRACHA, R.; KOBILER, D.; MIRELMAN, D.: *Attachment and ingestion of bacteria by trophozoites of* Entamoeba histolytica. Inf. Immun., 1982; *36*(1):396.

17. RAPP, H.J.; BOROS, T.: *Molecular basis of complement action, Appleton-Century Crofts, New York, 1970. Pag. 75.*

18. KABAT, E.A.; MAYER, M.M.: *Experimental immunochemistry,* Charles C. Thomas, Springfield, 1961. Pag. 133.

19. COOPER, N.R.; MULLER-EBERHARD, H.J.: *The reaction mechanism of human C5 in immune hemolysis.* J. Exp. Med., 1970; *132:*775.

20. GAITHER, T. A.; ALLING, D.W.; FRANK, M.M.: *A new one-step method for the functional assay of the fourth component (C4) of human and guinea pig complement.* J. Immunol., 1974; *113:*574.

21. POLLEY. M.J.; MULLER-EBERHARD, H.J.: *Enhancement of the hemolytic activity of the second component of human complement by oxidation.* J. Exp. Med., 1967; *126:*1013.

22. REID, K.B.M.: *Preparation of human Clg, a subcomponent of the first component of the classical pathway of complement.* En Lorand, L. (ed), *Methods in Enzymology,* Academic Press, New York, 1981; *80:*16.

23. REED, S.L.; CURD, J.G.; GIGLI, I.; et al.: *Activation of complement by pathogenic and nonpathogenic* Entamoeba histolytica. J. Immunol., 1986; *136:*2265.

24. DIAMOND, L.S.; PHILLIPS, B.P.; BARTGIS, L.L.; *A comparison of the virulence of nine strains of axenically cultivated* E. histolytica *in hamster liver.* Arch. Invest. Med. (Mex), 1974; *5*(Suppl 2):423.

25. PHILLIPS, B.P.; DIAMOND, L.S.; BARTGIS, L.L.; et al: *Results of intracecal innoculation of germ-free and conventional guinea pigs and germ-free rats with axenically cultivated* Entamoeba histolytica. J. Protozol., 1972; *19:*498.

26. MATTERN, C.F.T.; KEISTER, D.B.; CASPAR, P.A.: *Experimental amebiasis. III. A rapid in vitro assay for virulence of* Entamoeba histolytica. Am. J. Trop. Med. Hyg., 1978; *27:*882.

27. LUSHBAUGH, W.B.; KAIRALLA, A.; LOADHOLT, C.B.; et al: *Effect of hamster liver passage on the virulence of axenically cultivated* Entamoeba histolytica. Am. J. Trop. Med. Hyg., 1978; *27:*248.

28. DAS, S.R.; GHOSHAL, S.: *Restoration of virulence to rat of axenically grown* Entamoeba histolytica *by cholesterol and hamster liver passage.* Ann. Trop. Med. Parasit., 1976; *70:*439.

29. MEEROVITCH, E.; GHADIRIAN, E.: *Restoration of virulence of axenically-cultivated* Entamoeba histolytica *by cholesterol.* Arch. Invest. Med. (Mex), 1978; *9*(Suppl 1): 253.

30. PHILLIPS, B.P.; BARTGIS, I.L.: *Effects of growth in vitro with selected microbial associates and of encystation on the virulence of* Entamoeba histolytica *for guinea pigs.* Am. J. Trop. Med. Hyg., 1954; *3:* 621.

31. WITTNER, M.; ROSENBAUM, R.M.: *Role of bacteria in modifying virulence of* E. histolytica. *Studies of amebae from axenic cultures.* Amer. J. Trop. Med. Hyg., 1970; *19:*755.

32. BRACHA, R.; MIRELMAN, D.: *Virulence of* Entamoeba histolytica *trophozoites. Effect of bacteria, microaerobic conditions and metronidazole.* J. Exp. Med., 1984, *160:*353.

33. MIRELMAN, D.; BRACHA, R.; CHAYEN, A.: Entamoeba histolytica: *Effect of growth conditions and bacterial associates on isoenzyme patterns and virulence. Exp. Parasit., 1986; 62:*142.

34. CALDERON, J.; TOVAR, R.: *Resistance to serum lysis in* Entamoeba histolytica *after several treatments with human complement.* Fed. Proc., 1985; *44:*3341.

35. MERI, S.; RICHARD, G.; LINDER, E.: *Complement activation by antigenic fractions of* Entamoeba histolytica. Parasit. Immunol., 1985; *7:*153.

36. CALDERON, J.; SCHREIBER, R.B.: *Activation of the alternative and classical complement pathways by* Entamoeba histolytica. Inf. Immun., 1985, *50:*560.

37. CAPIN, R.; CAPIN, N.R.; CARMONA, M.; et al: *Effect of complement depletion on the induction of amebic liver abscess in the hamster.* Arc. Invest. Med. (Mex). 1980, *11*(Suppl 1): 173.

38. REED, S.L.; GIGLI, I.: *C3 binding of complement-resistant and sensitive strains of* Entamoeba histolytica. 26th Interscience Conference on Antimicrobial Agents and Chemotheraputics, 1986.