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Activation of the Alternative and Classical Complement Pathways by *Entamoeba histolytica*†

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Entamoeba histolytica **HM1 supported the activation of human alternative and classical complement pathways in the absence of ameba-reactive antibodies. Nonimmune serum depleted of C1q and factor D (NHS $\bar{s}$ C1q + D) and reconstituted with C1q was able to specifically deposit C3b onto trophozoites and produce lysis. This activity was not modified by the absorption of serum on *E. histolytica*. Serum depleted of factor B allowed C3b binding to amebae. Serum devoid of C4 effected only small amounts of C3 uptake. The kinetics of lysis of *E. histolytica* by serum in the presence of Mg-EGTA [ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] (lacking classical pathway function) or by NHS $\bar{s}$ C1q + D and reconstituted with factor D was slow and only produced one-half the amount of lysis produced by NHS $\bar{s}$ C1q + D supplemented with C1q. These results indicate that the surface of the ameba can promote complement activation by the classical pathway, without the participation of specific antibodies, and that the magnitude of this activation is greater than that induced by the alternative pathway.**

Entamoeba histolytica can live in the lumen of the large intestine of humans for long periods of time without producing clinical symptoms. In some cases, trophozoites invade the tissues and produce symptomatic infections observed with high frequency in endemic areas. However, little is known about the factors which influence the expression of the disease. To understand the immunological aspects of amebiasis, an analysis is required of the mechanisms employed by the host to resist parasite infection as well as the mechanisms employed by the parasite to circumvent destruction by the immune system.

It has been previously reported that trophozoites of *E. histolytica* activate the human complement system in the absence of antibodies (7, 12). These earlier studies indicated that the trophozoites selectively activated the alternative complement pathway, because only alternative pathway proteins were consumed when parasites were incubated with normal human serum (NHS), and cell lysis was effected in the absence of calcium ions. However, because the studies did not examine the direct binding of complement proteins to the cell surface, they could not conclusively rule out the participation of the classical complement pathway in host resistance to amebiasis.

In this communication, the specific deposition of C3b onto the ameba membrane was used as an indicator of direct complement-parasite interaction. By utilizing human serum depleted of its classical or alternative pathway function, the trophozoites were found to activate either complement pathway in nonimmune serum. Classical pathway activation was of greater magnitude than activation of the alternative pathway (both quantitatively and kinetically) and resulted in the lysis of amebae. These studies indicate that both complement pathways may participate in controlling the development of amebiasis in nonimmune hosts.

MATERIALS AND METHODS

Entamoeba **strains and culture conditions.** The *E. histolytica* strains HM1 and HK9 were obtained from M. de la Torre, Centro Medico Nacional, IMSS, in Mexico. Trophozoites were cultured axenically in TYI-S-33 medium (5) in borosilicate culture tubes by inoculating 5×10^4 exponentially growing amebae in 10 ml of culture medium containing 15% (vol/vol) heat-inactivated bovine serum. Trophozoites were harvested at the end of the logarithmic phase of growth and washed twice in cold Veronal-buffered saline for amebae (VBS-A) (Veronal buffer containing 18 mM diethylbarbiturate, 0.5 mM $MgCl_2$, 0.15 mM $CaCl_2$, and 193 mM NaCl [pH 7.2]).

Purified proteins. C3 (18), C1q (19), factor D (8), and factor B (6) were purified as previously described. C3 was radioiodinated to a specific activity of 0.5 to 1.0 μCi/μg by the iodogen method (9). C3 hemolytic activity was measured as described previously (4). Radioiodinated C3 retained full specific hemolytic activity.

Depleted human serum. Nonimmune NHS was immunochemically depleted of C4 or factor B as previously described (14) on columns containing monospecific antibodies to the appropriate proteins. By performing the absorption of factor B with buffers of high ionic strength, C1q was not removed and the classical pathway function of the serum was maintained (14). Serum lacking only C1q and factor D (NHS $\bar{s}$ C1q + D) was prepared by chromatography on BioRex 70 as described by Praz et al. (13). These methods have been used extensively in the past to prepare sera which are depleted in only one specific complement protein. All other complement components are present at their normal concentrations, and sera can be completely reconstituted by the addition of specific purified missing protein (14–16). Reconstitution of the sera was performed at physiological concentrations of the appropriate complement protein as

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TABLE 1. Deposition of C3b onto *E. histolytica*: correlation with cell lysis[a]

Serum	C3b Deposition		% Lysis
	%	No. of molecules/cell	
NHS	1.69	65.6×10^6	94
Absorbed NHS	1.25	48.5×10^6	86
NHS (56°C, 30 min)	0.35	13.5×10^6	0

[a] Trophozoites (10^6/ml) were incubated at 37°C for 10 min in VBS-A containing 40% (vol/vol) serum and ^{125}I-C3 (0.4 μg). Radioactivity in cell-free medium and in cell pellet was determined to calculate C3 deposition.

follows: C1q (70 μg/ml), factor B (200 μg/ml), and factor D (2 μg/ml).

Enzyme-linked immunosorbent assay (ELISA) for ameba-reactive antibodies. Sheep antibodies reactive with human immunoglobulin G were labeled with horseradish peroxidase by the method of Avrameas and Ternynck (1). The total extract of ameba antigen (*E. histolytica* HM1) at 5 μg/ml was absorbed on multiwell dishes (Costar) for 2 h at 37°C and then washed three times with 0.02% (vol/vol) Tween 20 in phosphate-buffered saline. NHS or serum from patients with amebiasis as positive controls were incubated in serial dilutions in the wells with bound antigen for 1 h. After being washed three times, the wells were exposed to horseradish peroxidase antibody for 1 h, washed again, and then incubated for 30 min with *o*-phenylenediamine (0.5 mg/ml) and H_2O_2 (0.012% [vol/vol]) in 17 mM citrate buffer (pH 5.0). The reaction was stopped with 4 N H_2SO_4. This assay detects all classes of human immunoglobulins, since the anti-human immunoglobulin G preparation also reacts with light chains.

Deposition of ^{125}I-C3 on trophozoites. Amebae (5×10^5/ml) were incubated at 37°C in VBS-A containing 40% (vol/vol) NHS, heat-inactivated serum, or NHS $\bar{s}$ C1q +D supplemented with a 0.4-μg/ml concentration of ^{125}I-C3. Reconstitution was accomplished with C1q (70 μg/ml), factor D (2 μg/ml), or where appropriate, both proteins. After incubation, samples of 80 μl, in duplicates, were layered onto 300-μl cushions of 20% sucrose in 0.4-ml microtubes and centrifuged for 4 min in a Beckman model 152 Microcentrifuge. Bottom tips containing cell pellets (5-mm height) were cut with a razor blade, and radioactivity present in cell pellets and cell-free medium was evaluated in a gamma counter. The percent specific ^{125}I-C3 binding was calculated by subtracting the percent nonspecific binding, obtained with heat-inactivated NHS, from experimental samples. In most assays with viable amebae, nonspecific binding of ^{125}I-C3 represented 20 to 30% of that bound in the presence of NHS. Further washes did not remove adsorbed C3 from trophozoites, which was consistent with the observed high binding of serum proteins by amebae.

Quantitation of C3 consumption. Reaction mixtures of 250 μl contained 100 μl of NHS $\bar{s}$ C1q + D, 1.25×10^5 *E. histolytica*, and the appropriate reconstituting protein. After 10 min at 37°C, the reaction was stopped by the addition of EDTA (final concentration, 10 mM), and the amebae were removed by centrifugation. C3 consumption was detected by effective molecule titration with EAC 142 cells as described elsewhere (4).

Quantitation of lysis of *E. histolytica*. Trophozoites at cell densities between 3.6×10^4 to 4.6×10^6/ml were mixed in VBS-A in the presence of 40% (vol/vol) human serum. The mixtures were incubated at 37°C for 1 h, diluted with cold VBS-A, and centrifuged for 3 s at 10,000 × *g* to separate trophozoites from the bulk of the serum proteins which would interfere with subsequent fixation. Residual trophozoites were suspended with VBS-A and fixed with 1% glutaraldehyde for 5 min, with continuous mixing of the suspension to avoid agglutination. The fixed amebae were washed, resuspended in 0.25 ml, and counted in a Fuchs-Rosenthal hematocytometer chamber. Percent lysis was defined as the decrease in viable trophozoites in NHS compared with the heat-inactivated serum control, calculated as follows: [(viable in control − viable in NHS)/viable in control] × 100. Thus, in a 20-min incubation at 37°C, only 30% of the amebae in NHS could be recovered due to the disintegration and solubilization by complement. Viable trophozoites showed refractile plasma membrane, while dead cells either appeared dim with various degrees of membrane disruption, observed by phase-contrast microscopy, or were lysed and were not observed in the microscopic field.

RESULTS

Deposition of ^{125}I-C3b onto *E. histolytica*. Table 1 demonstrates that when trophozoites were incubated with NHS, C3b was deposited onto the cells. The magnitude of the deposition (65.6×10^6 C3b molecules per cell) was nearly

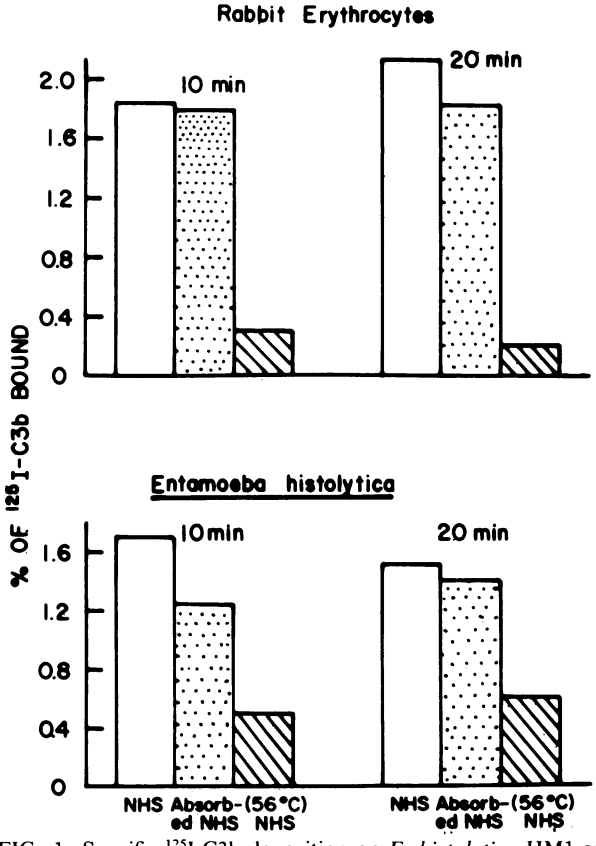

FIG. 1. Specific ^{125}I-C3b deposition on *E. histolytica* HM1 and rabbit erythrocytes. Trophozoites (1×10^6/ml) or rabbit erythrocytes (5×10^7/ml) were incubated with 40% NHS containing ^{125}I-C3 (0.4 μg/ml) in VBS-A. Serum was untreated, absorbed on 5×10^6 trophozoites, or heat inactivated (56°C, 45 min). ^{125}I present in the cell-free medium and cell pellet was determined to evaluate the percentage of C3 bound to cells.

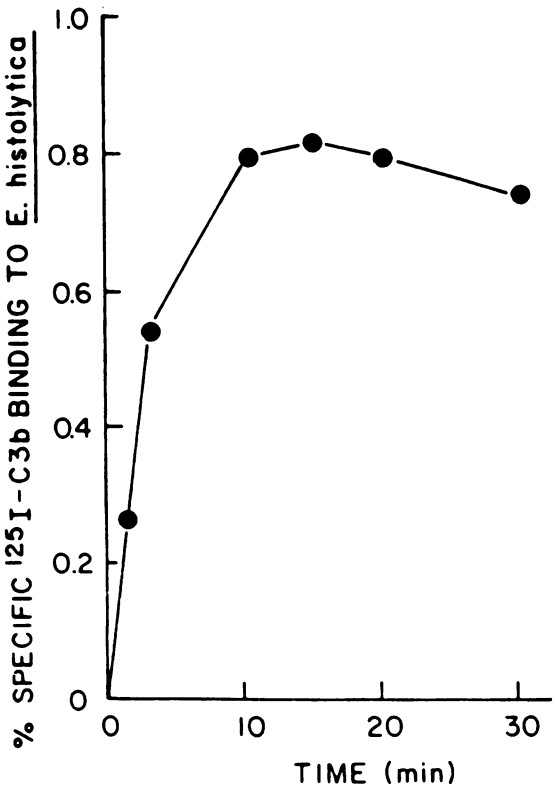

FIG. 2. Kinetics of specific C3 binding to *E. histolytica* HM1. Conditions were the same as described in the legend to Fig. 1.

five times greater than the nonspecific deposition from heat-inactivated serum (13.5 × 10^6 C3b molecules per cell). Antibody was apparently not required for complement activation, since serum devoid of ameba-reactive antibodies (as measured by ELISA) and absorbed with trophozoites (5 × 10^6/ml) could effect C3b deposition to an extent comparable with the untreated serum. The absence of ameba-specific antibodies in the sera was confirmed both by a negative ELISA reaction, with total ameba extract as immobilized antigen, and by a lack of reactivity of the sera on immunoblotting sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis with the same antigen source. In control experiments, the percentage of C3b deposited onto 10^6 amebae per ml was found to be equivalent to that deposited onto a concentration of rabbit erythrocytes of 5 × 10^7/ml (NHS, 1.8%; NHS absorbed with amebae, 1.78%; NHS at 56°C, 0.3% [Fig. 1]). The magnitude of the deposition correlated with the extent of complement-dependent killing of the ameba (Table 1). Untreated and absorbed serum displayed equivalent levels of killing, while heat-inactivated serum was devoid of lytic activity.

The kinetics of C3b deposition are shown in Fig. 2. Deposition increased in a time-dependent fashion during the first 5 to 10 min of incubation and reached a steady state after 10 min. Proportional C3b deposition resulted with increased serum concentrations and with increased cell densities (data not shown).

Demonstration that either complement pathway can deposit C3b onto *E. histolytica*. Two series of experiments were performed to identify the complement pathway responsible for depositing C3 onto the parasite. In the first experiment, serum was immunochemically depleted of either C4 or factor B and tested for its ability to support ^{125}I-C3b deposition onto trophozoites. With serum depleted of C4, no significant C3 deposition was observed (Fig. 3). This result correlated with that of serum treated with Mg-EGTA which effected only minimal specific C3 deposition. However, in serum lacking an intact alternative pathway (factor B-depleted serum), significant C3b deposition onto amebae occurred. The magnitude of the deposition was 60% that of NHS. The addition of factor B to the depleted serum totally reconstituted the complement activation.

In the second series of experiments, a single nonimmune serum was simultaneously depleted of both complement

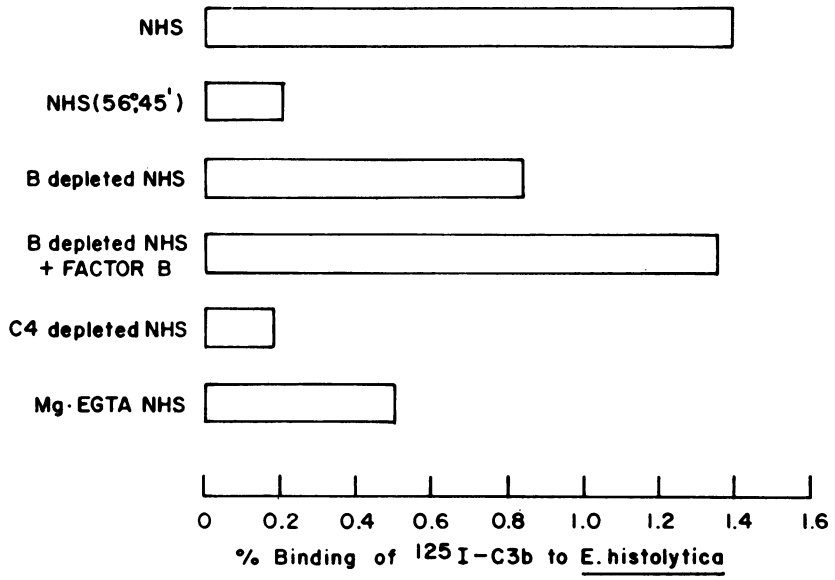

FIG. 3. C3 deposition onto amebae as a result of complement activation through the classical pathway or the alternative pathway. Sera immunochemically depleted of factor B or C4 or in the presence of Mg-EGTA were used as described in Materials and Methods.

TABLE 2. Comparison of classical pathway- and alternative pathway-mediated deposition of C3 onto *E. histolytica* from the same serum[a]

NHS s̄ C1q + D reconstituted with:	C3 Deposition (%) in the following sera		Mean lysis (%) ± SD
	Absorbed	Unabsorbed	
Nothing	0	0	0 ± 3
C1q	0.42	0.48	54 ± 10
D	0.22	0.15	ND[b]
C1q + D	0.67	0.64	ND

[a] NHS s̄ C1q + D (500 μl) was absorbed twice on 5 × 10^6 trophozoites in VBS-A. ^{125}I-C3 deposition and lysis were measured after incubation at 37°C. For reconstitution, 70 μg of C1q, 4 μg of factor D, or a combination of the two per ml of depleted serum was used.
[b] ND, Not determined.

TABLE 3. Time-dependent killing of *E. histolytica* by the classical and alternative complement pathways in the same serum[a]

Serum lacking	Addition or treatment	Mean lysis (%) ± SD at the following times	
		5 min	60 min
Nothing	Nothing	74 ± 5	86 ± 3
C1q + D	Nothing	0 ± 5	5 ± 8
C1q + D	C1q	67 ± 5	64 ± 7
C1q + D	D	0 ± 12	32 ± 7
C1q + D	C1q + D	81 ± 4	89 ± 3
Nothing	Mg-EGTA	9 ± 9	46 ± 6
Nothing	56°C, 45 min	0 ± 12	0 ± 9

[a] Assays contained 4 × 10^5 trophozoites per ml with 40% (vol/vol) serum. For reconstitution, 70 μg of C1q, 4 μg of factor D, or a combination of the two per ml of depleted serum was used.

pathway functions by the removal of C1q and factor D on a Biorex-70 column. Each pathway could then be reconstituted either individually or together by the addition of the appropriate component. In serum lacking both proteins, neither C3 deposition nor cell lysis was observed (Table 2). The addition of C1q to the serum led to C3 deposition and killing of the amebae. The reconstitution of alternative pathway activity with factor D caused some C3 deposition, although to a lower extent than with C1q. The simultaneous addition of both molecules led to a deposition which was comparable to the additive contributions of either individual pathway. These results were unaffected when absorbed or untreated serum was used.

Demonstration that either pathway can lyse *E. histolytica*. The kinetics of lysis of trophozoites by untreated human serum or serum lacking the classical pathway function (EGTA-Mg-NHS) is shown in Fig. 4. The alternative pathway induced killing at a slow rate, effecting 50 and 60% lysis at 10 and 30 min, respectively; while untreated serum produced 100% lysis during the first 2 min. To determine the relative contributions of the classical or alternative pathways to the lytic process, we utilized NHS s̄ C1q + D and quantitated the killing after 5 and 60 min of incubation following reconstitution. After 5 min of incubation, 67% lysis of amebae was observed in the presence of C1q, and no lysis was detectable with the addition of factor D (Table 3). At 1 h there was 64% lysis through classical pathway activation and only 32% by the alternative pathway (in the presence of factor D). Mg-EGTA-treated serum produced 9 and 46% lysis at 5 and 60 min, respectively, while untreated NHS effected 74 and 86% lysis at these respective times. Therefore, the destruction of amebae produced by serum during the first 5 min (Fig. 4) appears to be a function of the classical pathway.

Other studies (7, 12) have shown that the alternative pathway can lyse the whole population of trophozoites when the cells were incubated in low numbers with Mg-EGTA serum. To investigate the influence of cell density on pathway activation, we incubated serum with four different concentrations of trophozoites, ranging from 3.6 × 10^4 to 4.6 × 10^6/ml. Figure 5 shows that complement activated by the alternative pathway is more effective in killing amebae at low cell densities, such as 4 × 10^4 and 2 × 10^5/ml, but only damaged half of the population of microorganisms when higher cell densities were tested. Untreated serum displayed effective killing of trophozoites at any range of cell density, suggesting that the classical pathway activation significantly enhanced cytolysis.

Consumption of C3 in serum incubated with *E. histolytica*.

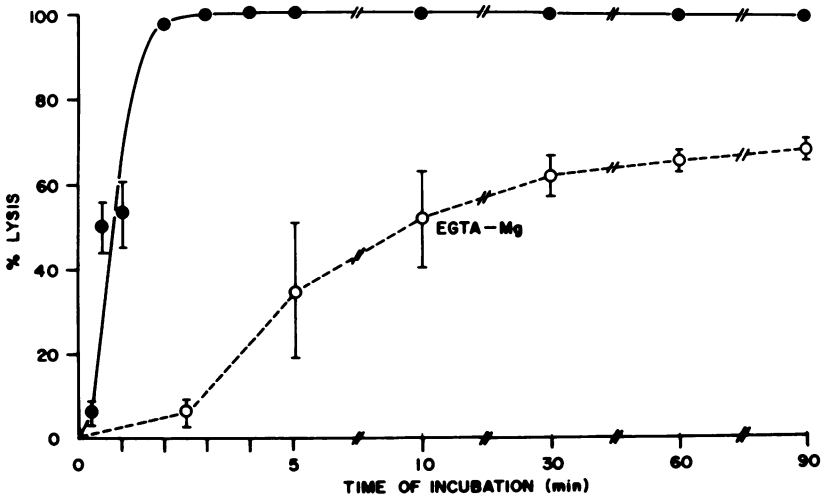

FIG. 4. Kinetics of complement lysis of *E. histolytica* by NHS and serum lacking classical pathway activity. Trophozoites (10^6) in the presence of 40% (vol/vol) NHS (●) or serum treated with 10 mM EGTA–5 mM $MgCl_2$ (○) were incubated at 37°C for various lengths of time.

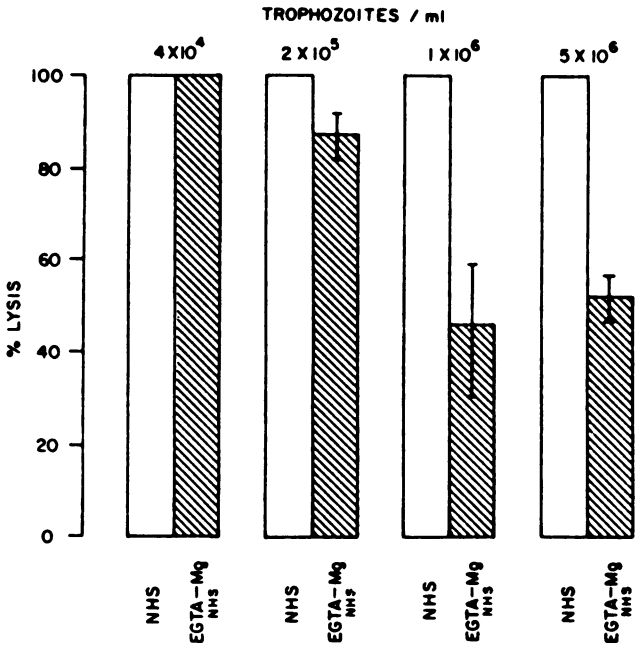

FIG. 5. Killing of trophozoites by complement activated by the alternative pathway as a function of cell density. NHS or EGTA-Mg NHS at 50% (vol/vol) were incubated with various densities of amebae at 37°C for 20 min.

To examine the consumption of C3 by the activation of either complement pathway, trophozoites were mixed with NHS $\bar{s}$ C1q + D in the presence or absence of the reconstituting proteins. After incubation for 1 h at 37°C, the amount of C3 consumed in the serum and the degree of cell lysis were quantitated. Table 4 shows that activation of the alternative pathway led to 46% lysis and 17% C3 consumption, while activation of only the classical pathway effected 84% lysis but only 6% C3 consumption. When both pathways were reconstituted, 93% lysis and 33% C3 consumption occurred.

DISCUSSION

The results of this study indicate that *E. histolytica*, propagated in vitro, in the absence of bacteria (axenic) activated both complement pathways in nonimmune human serum. Activation was assessed by the measurement of C3 deposition onto the trophozoite and cell lysis. The kinetics and magnitude of deposition and lysis indicated that, under the experimental conditions employed, the classical pathway played a dominant role in complement-mediated resistance to the parasite.

TABLE 4. C3 consumption and cell lysis by the alternative and classical complement pathways[a]

NHS $\bar{s}$ C1q + D reconstituted with:	% C3 consumption	% Lysis
Nothing	0	0
D	17	46
C1q	6	84
D + C1q	33	93

[a] Reaction mixtures of 250 μl contained 100 μl of NHS $\bar{s}$ C1q + D, 1.25×10^5 *E. histolytica*, and the appropriate reconstituting protein. Incubation was for 10 min at 37°C.

In part, these studies confirm the earlier observations by Ortiz-Ortiz et al. (12) and Huldt et al. (7), who demonstrated that certain strains of *E. histolytica* (HK9 and NIH 200) activated the alternative complement pathway. However, our studies also demonstrated that *E. histolytica* HM1 activated the classical pathway as well in the apparent absence of specific antibody. These differences are not attributable to the use of different parasite strains, since HK9 was indistinguishable from HM1 in our studies (data not shown). However, they may be a reflection of the use of parasites at low cell density in the former studies. In addition, antibody-independent activation can display species specificity since nonspecific recognition mechanisms are based on host-pathogen differences. Therefore, a comparison cannot be made with the studies of Huldt et al. (7), who utilized C4-deficient guinea pig serum to rule out a role of the classical pathway in ameba-dependent complement activation.

Ortiz-Ortiz et al. (12) reported that, upon incubation of serum with amebae, there was no significant consumption of C1, C4, and C2, but there was 81% consumption of C3 and approximately 25 to 30% conversion of factor B. In our experiments with NHS $\bar{s}$ C1q + D, we observed 6% consumption of C3 and 84% lysis with serum reconstituted with C1q, 17% consumption and 46% lysis in the presence of factor D, and 33% consumption and 93% lysis when both pathways were reconstituted. Therefore, under the proper conditions, lysis and deposition of C3 are more reliable indicators of complement activation than is consumption of components in the fluid phase. The results also indicate that activation of the classical pathway is more efficient on the basis of component utilization since, although less C3 was consumed as compared with alternative pathway activation, more C3 deposition occurred onto the parasite and more lysis occurred both quantitatively and kinetically. A similar situation has been observed in studies which examined the role of properdin in the alternative pathway (15, 16).

The studies in this report employed serum specifically depleted of various complement proteins to identify the relative contributions of the two pathways. Earlier studies have confirmed the validity of this approach in studying complement activation by activators such as bacteria (15), neoplastic cells (16), and virus-infected cells (17). The use of NHS $\bar{s}$ C1q + D allowed reconstitution experiments to be performed to quantitate classical and alternative pathway activity either separately or together in the same serum.

Attempts have been made to rule out the participation of antibody in the classical pathway-dependent activation. The sera used in the experiments were from nonimmune donors and were negative for trophozoite reactivity in an ELISA. The minimum amount of antibody detectable in this assay was 1 to 3 ng. In some cases, these sera were absorbed one to two times with large numbers of parasites at 4°C to remove amounts of antibody which might have gone undetected. No differences were observed between the absorbed and untreated sera.

There exist several precedents for classical pathway activation in the absence of antibody. A number of biological particles have been identified which directly activate the human classical complement pathway. These include certain bacteria and viruses and a number of proteins, carbohydrates, and polyions (reviewed in reference 3). In addition, Musoke and Barbet (11) have shown that a variant specific surface antigen of *Trypanosoma brucei* can effect classical

pathway activation in sera lacking specific antibody. Some antibody-independent classical pathway activators, such as lipopolysaccharide, can also activate the alternative complement pathway in the absence of antibody (reviewed in reference 10). Thus, the dual pathway activation by *E. histolytica* appears to be analogous to these systems.

Although the in vitro experiments indicate that complement can display cytocidal activity toward *E. histolytica*, they do not address the mechanisms utilized by the parasite to escape destruction in vivo. This situation must certainly exist, since a large number of parasitized individuals develop clinical manifestations of amebiasis. No correlation has yet been found which connects the development of the disease to deficiencies of the complement system or other systems of host defense. The factors which influence the persistence of ameba infection are undefined. The compartmentalization of the parasite may effectively protect it from the full cytocidal potential of the host-mediated humoral and cellular defense system. The parasite itself may undergo alteration so as to no longer be recognized as foreign or to become resistant to the cytocidal effects of complement and thereby escape destruction. Evidence has been obtained in the in vitro system to support these latter possibilities. Amebae treated with antibody lose surface components which are recognized by the immune system yet remain viable (2), and parasites grown in the presence of complement lose susceptibility to complement-mediated lysis (J. Calderon, unpublished data). The study of complement-parasite interaction at a molecular level should elucidate the pathobiological processes which give rise to clinical disease.

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