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Activation of the Alternative Complement Pathway by *Trichomonas vaginalis*

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The mechanism underlying the lysis of *Trichomonas vaginalis* by normal human or guinea pig serum was investigated. The involvement of the complement system was demonstrated by the failure of human serum deficient in C3 or C8 to mediate parasite killing and by the ablation of lytic activity observed when fresh sera were heated at 56°C or treated with ethylenediaminetetraacetate. Fixation of human C3 on the parasite surface was demonstrated by indirect immunofluorescence. The involvement of the alternative complement pathway was demonstrated (i) by the inability of properdin-depleted human serum to lyse *T. vaginalis* and (ii) by the normal killing observed with guinea pig serum lacking C4 and with normal human or guinea pig serum treated with ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid and Mg^{2+} to selectively inhibit the classical pathway.

The flagellated protozoan *Trichomonas vaginalis* is a common pathogen of the human urogenital tract (6). It has long been observed that *T. vaginalis* and other trichomonads are lysed by fresh, but not heated, normal human or animal serum (5, 6, 14). Recently, killing of *T. vaginalis* by polymorphonuclear neutrophils from uninfected humans was reported. Fresh serum was required for this reaction (12). These observations suggested that *T. vaginalis* activates the alternative complement pathway and that this activation can lead to parasite killing.

Several parasitic protozoa have been shown to be lysed by complement. For example, *Leishmania donovani* promastigotes, but not amastigotes, evidently bind "natural" antibody, activate the classical complement pathway, and are lysed by the resultant membrane attack complex (11). In contrast, *Entamoeba histolytica* trophozoites (7, 10) and *Trypanosoma cruzi* epimastigotes, but not trypomastigotes (9), activate the alternative complement pathway and are lysed.

Activation of the classical pathway requires antibody, Mg^{2+}, Ca^{2+}, and the early complement components C1, C4, C2, and C3. The alternative pathway is usually not dependent on antibodies and requires Mg^{2+} (but not Ca^{2+}) and factors B and D, properdin, and C3. In the alternative pathway, the slow spontaneous cleavage of C3 is amplified when the major fragments C3b and Bb form a complex with properdin on an activating particle which retards the inactivation of this amplification C3 convertase by the regulatory proteins C3b inactivator and β1H. Activation of either the classical or alternative sequence leads to cleavage of C5 and assembly of the C5-C9 membrane attack complex, which results in lysis (4).

The studies presented here demonstrate that *T. vaginalis* activates the alternative complement pathway and that this reaction is responsible for the lysis of the parasites observed in fresh sera.

MATERIALS AND METHODS

Organisms. *T. vaginalis* JH31A#4, clone 1 (American Type Culture Collection no. 30236), was obtained from C. F. T. Mattern (National Institutes of Health). It was grown axenically in Diamond TYI-S-33 medium (1) with twice weekly transfer. Organisms were enumerated with a Coulter Counter. For experiments, cells from late-log-phase cultures (with 2×10^6 to 5×10^6 cells per ml) were washed by centrifugation in Eagle minimal essential medium without $NaHCO_3$ or serum, but buffered with HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; 20 mM) and supplemented with 6 mM freshly prepared L-cysteine and ascorbic acid (MEM-CH). The reducing agents were included to minimize spontaneous lysis, as *T. vaginalis* is an aerotolerant anaerobe (8).

Sera. Venous blood was collected from normal volunteers of both sexes with no history of trichomoniasis or other sexually transmitted disease and also from two hypogammaglobulinemic patients. The blood was clotted at room temperature for 15 to 60 min and then for 3 h at 4°C. After centrifugation, the serum was decanted. Lyophilized guinea pig complement (GIBCO Laboratories, Grand Island, N.Y.) was reconstituted with distilled water.

Serum from C3-deficient and C8-deficient humans and purified C3 and partially purified C8 were the

generous gifts of Michael Frank (National Institutes of Health). Partially purified properdin (approximately 50% by polyacrylamide gel, apparently free of other complement proteins by immunological and molecular weight criteria) was the generous gift of Carl Hammer (National Institutes of Health). Serum from a patient with active trichomoniasis was the generous gift of Michael R. Spence (Johns Hopkins University).

Normal human serum and guinea pig serum (4 ml) were each absorbed with 5.5×10^8 washed *T. vaginalis* organisms at 4°C for 2 h. The sera were then centrifuged and passed through filters (0.45 μm pore size).

All sera were stored in small volumes at −70°C. Samples of serum were inactivated at 56°C for 30 min where so indicated.

Properdin depletion. Serum from a normal individual was depleted of properdin by immunoabsorption. Serum (2.5 ml) was passed over a column of the immunoglobulin fraction of goat anti-human properdin (20 mg; Miles Laboratories, Inc., Elkhart, Ind.) coupled to activated Sepharose 4B at 4°C. Protein in the effluent was measured continuously by a model 2138 Uvicord B (LKB Instruments, Inc., Rockville, Md.), and the properdin-depleted serum was assayed immediately for its lytic activity against *T. vaginalis*. Serum passed over a control column lacking antibody to human properdin retained full lytic activity, indicating that the columns did not inactivate the complement non-specifically.

Lysis assay. Lysis of *T. vaginalis* was quantitated in vitro in multiwell tissue culture plates with a Zeiss inverted microscope (×250 final magnification). In initial experiments, 90,000 parasites and various concentrations of serum (0.6-ml final volume) were placed in 24-well plates. In later experiments, flat-bottomed 96-well tissue culture trays containing 15,000 to 20,000 cells and serum in 0.1-ml volumes were used. Incubation at 35.5 ± 0.5°C was for 60 min unless otherwise specified. Most of the parasites settled to the bottom of the wells, and many adhered to the plastic. Motile organisms were counted. Organisms were scored as motile if they exhibited movement of either flagella or undulating membrane, regardless of whether they were attached to the plastic. The concentration of organisms was such that there were 50 to 250 per microscope field. Five random fields were enumerated.

Indirect immunofluorescence. (i) Detection of C3 on the surface of *T. vaginalis*. Organisms were sedimented, washed twice with MEM-CH, resuspended at 10^7 cells per ml, and mixed with fresh or heated (control) normal human serum (1:20, vol/vol). Lysis of the parasite by the fresh serum was partially inhibited at high cell concentrations probably because of the depletion of some complement component(s). After incubation at 35.5°C for 20 min, the organisms were chilled, and all subsequent steps were carried out at 4°C. After the organisms were washed twice, they were incubated for 30 min with fluorescein isothiocyanate-goat antibody to human C3 (1:40) (Meloy Laboratories Inc., Springfield, Va.) with 10 mM ethylenediaminetetraacetate. The organisms were washed and observed with a Leitz fluorescence microscope.

(ii) Detection of antibodies to *T. vaginalis*. The procedure for the detection of antibodies to *T. vaginalis* was the same as that described above, except the organisms were incubated at 4°C with heat-inactivated normal human or patient serum. The conjugate used was fluorescein isothiocyanate-rabbit antibody to human 7S globulin, which was the generous gift of Diane Taylor (National Institutes of Health).

RESULTS

In preliminary experiments, *T. vaginalis* was lysed by 10% (vol/vol) fresh serum from 11 normal and 2 hypogammaglobulinemic human volunteers and by guinea pig complement. Heating at either 56 or 52°C for 30 min abrogated the lytic activity, confirming the observations of others (5, 14). The organisms exposed to fresh serum became swollen and rounded, then membrane integrity was lost (Fig. 1), and cellular granules were released.

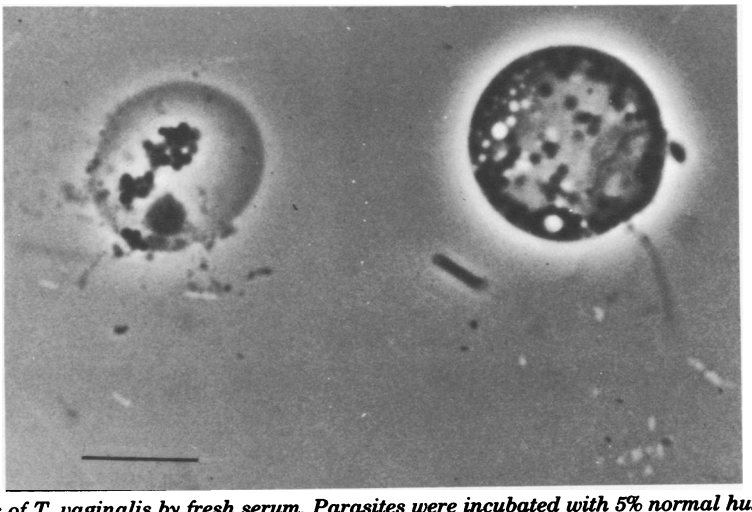

FIG. 1. *Lysis of T. vaginalis by fresh serum. Parasites were incubated with 5% normal human serum for 5 to 10 min. The ghost of one organism appears next to an intact, but swollen, parasite. Bar = 10 μm.*

Serum from a C3-deficient patient or from a C8-deficient patient did not lyse *T. vaginalis*. When the respective deficient sera were reconstituted with purified C3 or a partially purified preparation of C8 in physiological concentrations, lysis was observed (Fig. 2). Thus, lysis of *T. vaginalis* requires the terminal membrane attack complex formed as a result of C3 activation by the classical or alternative complement pathway.

The presence of activated C3 on the surface of *T. vaginalis* cells incubated with fresh human serum was visualized by indirect immunofluorescence (Fig. 3). No fluorescence was observed in cells incubated with heated serum (data not shown).

The lytic activity of C4-deficient guinea pig serum (2) was identical to that of normal guinea pig serum (Table 1), indicating that the classical complement pathway is not required for killing of *T. vaginalis*.

Although the classical complement pathway requires both Ca^{2+} and Mg^{2+}, the alternative pathway requires only Mg^{2+} (4). Human serum or guinea pig complement in which both pathways were blocked by the addition of ethylenediaminetetraacetate to chelate the two metals did not lyse *T. vaginalis*. In contrast, the parasite was lysed by serum to which ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid and Mg^{2+} had been added, removing only Ca^{2+} and leaving the alternative pathway intact (Table 2).

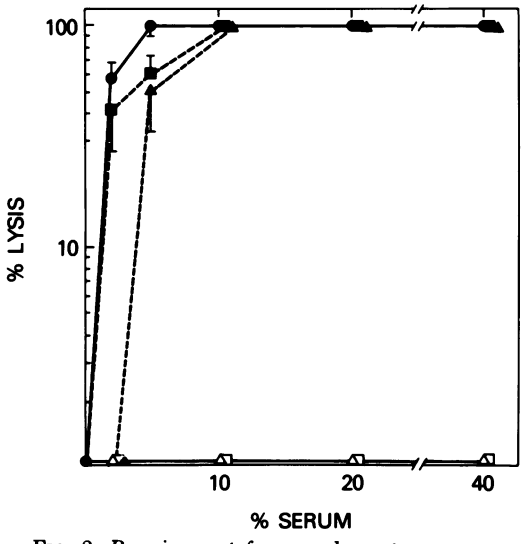

FIG. 2. *Requirement for complement components C3 and C8 for lysis of T. vaginalis. Parasites (200,000/ml) were incubated for 1 h with normal (●), C3-deficient (□), or C8-deficient (△) human serum at the concentrations shown in a final volume of 0.1 ml. The deficient sera were reconstituted with 10 μl of purified C3 (■) or partially purified C8 (▲), respectively. The points represent mean values of five microscope fields; the bars represent standard deviations.*

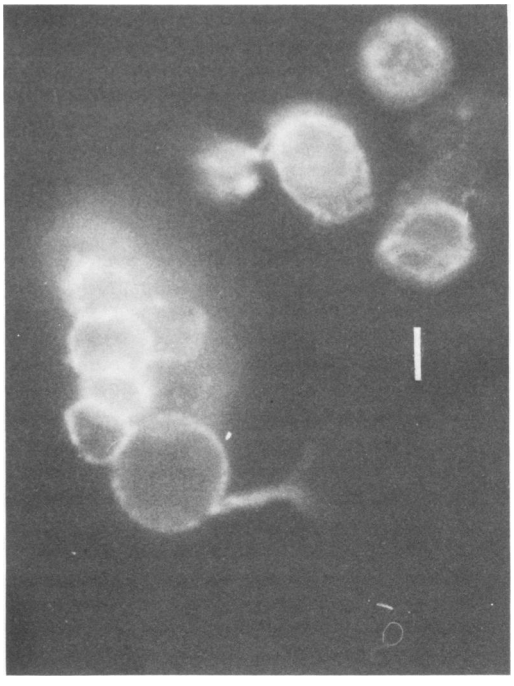

FIG. 3. *Binding of C3 by T. vaginalis demonstrated by indirect immunofluorescence. The organisms were incubated with fresh normal human serum. In controls incubated with heated serum, no immunofluorescence was observed. Bar = 10 μm.*

TABLE 1. *Lysis of T. vaginalis by normal and C4-deficient guinea pig sera*

Serum concn (%, vol/vol)	% Lysis			
	Control serum		C4-deficient serum	
	20 min	60 min	20 min	60 min
0	<5	<5	<5	<5
5	<5	100	<5	100
10	>90	100	>95	100
20	100	100	100	100

The observation that human serum depleted of properdin by immunoabsorption had little or no lytic activity (Table 3) directly supported the concept that the alternative complement pathway was required for killing of *T. vaginalis*. When partially purified properdin or C3-deficient serum was added to the properdin-depleted serum, efficient killing ensued.

In some instances, natural antibody has been

shown to play an auxiliary role in activation of the alternative complement pathway (13). Absorption of guinea pig complement with *T. vaginalis* did not significantly alter its lytic activity (data not shown). Heated normal human serum exhibited very faint reactivity against *T. vaginalis* as assayed by immunofluorescence with fluorescent antibodies to human immunoglobulins (data not shown). In contrast, very bright immunofluorescence was observed with serum from a patient with trichomoniasis (Fig. 4).

Absorption of the normal serum with *T. vaginalis* caused a small reduction in the lytic activity when measured at low concentrations (5 to 15%). However, at higher concentrations (23%), no reduction was observed. The addition of heated normal serum to the absorbed serum increased its lytic activity (Fig. 5).

TABLE 2. *Requirement of Mg^{2+}, but not Ca^{2+}, for lysis of T. vaginalis*

Addition to serum	% Lysis	
	Human serum[a]	Guinea pig complement[a]
None	100	100
EDTA[b] (7 mM)	<2	<2
EGTA[c] (13 mM) + $MgCl_2$ (2 mM)	100	100

[a] 10% (vol/vol).
[b] EDTA, Ethylenediaminetetraacetate.
[c] EGTA, Ethylene glycol-bis(β-aminoethyl ether)-*N,N*-tetraacetic acid.

TABLE 3. *Properdin requirement for lysis of T. vaginalis*

Serum	% Lysis (center)	
	5% Serum	10% Serum
Normal human	100	100
Properdin depleted	<5	<5
Properdin depleted + properdin[a]	67	85
Properdin depleted + C3 deficient[b]	ND[c]	75

[a] Partially purified preparation (80 μg).
[b] 5% (vol/vol).
[c] ND, Not done.

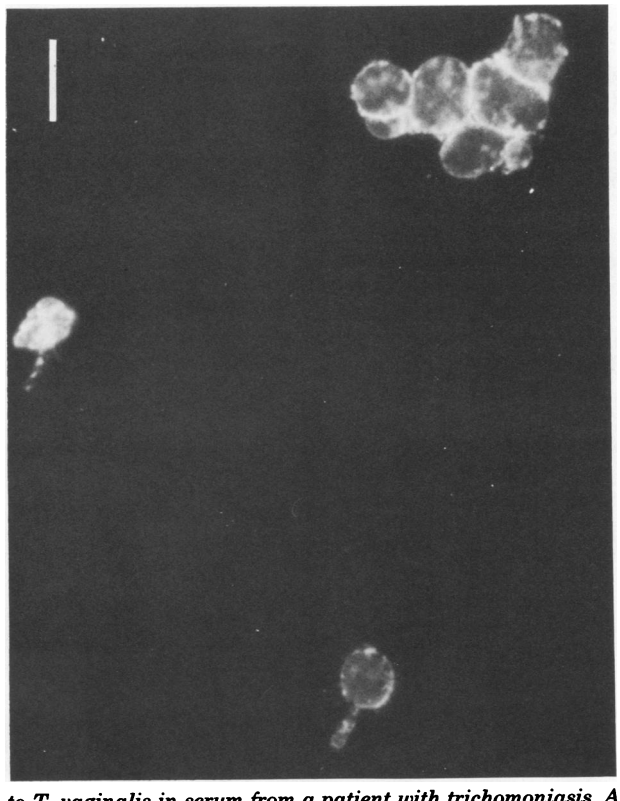

FIG. 4. *Antibodies to T. vaginalis in serum from a patient with trichomoniasis. Antibodies on the surface of living T. vaginalis, which had been incubated at 4°C with heated patient serum, were made visible with fluorescent rabbit antibodies to human 7S immunoglobulins. Bar = 20 μm.*

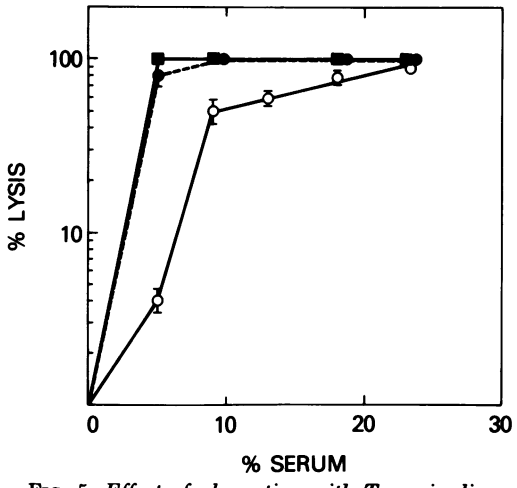

FIG. 5. *Effect of absorption with T. vaginalis on lytic activity of normal human serum. Parasites (200,000/ml) were incubated with control (■) or absorbed (○) fresh normal human serum or with absorbed serum plus 5 μl of heat-inactivated control serum (●). The points represent mean values of five microscope fields; the bars represent standard deviations.*

DISCUSSION

These studies demonstrate that *T. vaginalis* activates the alternative complement pathway and is lysed by the resultant membrane attack complex. Thus, the earlier observations of lysis of *T. vaginalis* by fresh serum (5, 6, 14) are explained.

The lysis observed was shown to be independent of the classical complement pathway as it occurred in C4-deficient (Table 1) and Ca^{2+}-depleted (Table 2) sera and was ablated in serum from which properdin was removed (Table 3).

The role of antibody in the alternative complement pathway has been controversial (4). Recently, specific natural antibody was shown to play an auxiliary or stimulatory role in activation of this pathway by zymosan (13). There are many reports of natural antibody to *T. vaginalis* (5, 6). That absorption with *T. vaginalis* reduces the lytic activity of normal human serum and that the addition of heated unabsorbed serum reconstitutes the activity (Fig. 5) are consistent with the hypothesis that small quantities of natural antibody in human serum may help stimulate the lysis of the parasite by the alternative pathway. However, it should be emphasized that antibody is not essential for killing, since absorption of guinea pig serum with parasites failed to significantly reduce its lytic activity. The question of whether antibody can stimulate lysis of *T. vaginalis* by the classical complement pathway is under investigation.

Rein et al. (12) have shown that *T. vaginalis* is killed by polymorphonuclear neutrophils in the presence of fresh human serum. In their experiments, killing was not observed with serum alone, a result which differs from the observations reported here and in previous studies (5, 6, 14). Since we have observed lysis of the parasites with assay conditions similar to those of Rein et al. (data not shown), it is possible that the discrepancy may be due to differences in the behavior of the strains used.

Host defenses against *T. vaginalis*, a noninvasive mucosal parasite, are not well understood. It is not known whether functional complement is present in cervical or vaginal mucus or discharge (3). It is extremely likely, however, that complement is present in menstrual blood, where it could act against the parasite. If *T. vaginalis* is exposed to complement in vivo, the question of how the organism survives this natural defense is relevant. One possibility is that the surface of *T. vaginalis* may undergo an adaptive change in vivo, enabling it to avoid lysis by complement. This hypothesis is under investigation.

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