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Antibody Enhances Killing of *Tritrichomonas foetus* by the Alternative Bovine Complement Pathway

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The role of bovine antibody and complement in host defense against *Tritrichomonas foetus* was measured by using an assay of trichomonad viability based on protozoal uptake of tritiated adenine. Moderate killing was measured in the absence of antibody only with high concentrations of complement-preserved hypogammaglobulinemic bovine serum. However, very low concentrations of hyperimmune serum promoted significant enhancement ($P < 0.05$) of killing by complement. Heat inactivation of complement (56°C for 30 min) eliminated antibody-dependent and -independent killing. Similarly, depletion of bovine factor B in serum by heat treatment (50°C for 45 min) abolished antibody-dependent and -independent killing. However, selective inactivation of the classical complement pathway with magnesium ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid did not affect antibody-dependent or -independent killing by complement. These findings demonstrate antibody enhancement of complement-mediated killing of *T. foetus* by the alternative pathway of bovine complement.

Tritrichomonas foetus, a venereal pathogen of cattle, causes infertility, abortion, and pyometritis and results in economic loss to the cattle industry in the United States and worldwide (1, 6, 9, 12, 21, 28).

An important feature of the pathogenesis in *T. foetus* infections in cattle is the persistence of the organism in the reproductive tracts of both cows and bulls (5, 24, 37, 38). Infection in the cow generally persists despite the presence of inflammation and a local immune response (26, 37, 38), a feature implying that *T. foetus* is capable of evading host defenses in the bovine genital tract. However, there is little documentation of the interaction between *T. foetus* and host defenses in the bovine reproductive tract.

Complement-mediated lysis of *T. foetus* with rabbit and guinea pig complement (7) and of *Trichomonas vaginalis* with human, rabbit, and guinea pig complement (20, 31) has been described previously and involves both the classical and alternative pathways of complement activation (7, 20, 31). However, the concentration of both complement and antibody necessary for killing has not been titrated in these investigations.

Since complement and antibody are important elements of host defenses against extracellular pathogens such as *T. foetus*, we investigated complement-mediated lysis in the presence or absence of specific bovine antibody and determined the role of the classical and alternative pathways of bovine complement in killing.

Further, the assay described in the present study facilitates accurate quantitation of trichomonacidal activity, with results independent of antibody-mediated agglutination. By the use of this assay, the role of complement and antibody in promoting the killing of *T. foetus* has been characterized.

MATERIALS AND METHODS

Organism. A strain of *T. foetus* was obtained from samples of an infected cow which were submitted to the Washington Animal Disease Diagnostic Laboratory, Pullman, Wash. Organisms were cultured at 37°C for 18 to 24 h in an atmosphere containing 10% Co_2, in screw-capped glass tubes containing 10 ml of Diamond medium (13) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Flow Laboratories, Inc., McLean, Va.), 1,000 IU of penicillin G per ml, 1,000 μg of streptomycin (GIBCO Laboratories, Grand Island, N.Y.) per ml, and 10 μg of gentamicin (GIBCO Laboratories) per ml. The organisms were washed three times in 0.01 M phosphate-buffered saline (PBS), pH 7.2, containing 20 mM glucose (Sigma Chemical Co., St. Louis, Mo.) and 1% heat-inactivated FBS (PBS-G-FBS) by centrifugation at 350 × *g* for 12 min, and were resuspended in the same solution.

Sera. Complement-preserved hypogammaglobulinemic bovine serum (CNBS) was collected from a 3-day-old calf deprived of colostrum and was frozen in aliquots at −70°C within 8 h of collection.

One heifer was immunized three times at 2-week intervals by intramuscular injection of 10^6 heat-killed (56°C for 30 min) *T. foetus* cells emulsified in incomplete Freund adjuvant (Difco Laboratories, Detroit, Mich.). Blood was collected 12 days after the final injection, and hyperimmune serum (HIS) was stored in aliquots at −20°C. This serum was heat inactivated (56°C for 30 min) prior to use in all assays. In an agglutination assay of living *T. foetus* (11), this HIS had a titer of 1/64.

Quantitation of viable trichomonads. The concentration of viable *T. foetus* was estimated by measuring protozoal incorporation of [8-^{3}H]adenine (27 Ci/mmol) (Amersham Corp., Arlington Heights, Ill.) by using a modification of the method of Meingassner et al. (30). Assay conditions were optimized for incubation time and [^{3}H]adenine concentration. Suspensions were incubated at 37°C, and the reaction was stopped by the addition of formaldehyde to a final concentration of 1%. The labeled suspensions were harvested onto glass fiber filters (Filtermats; Skatron Inc., Sterling, Va.) by using a semiautomatic 12-well cell harvester (Skatron Inc.). Filter discs were dried, scintillant was added (Aqueous Counting Scintillant; Amersham Corp.), and vials were counted in a liquid scintillation counter (model LS 7800; Beckman Instruments, Inc., Irvine, Calif.). Formalin- and heat-killed trichomonads were included among the controls.

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Hemolytic activity of complement. To determine the effect of calcium chelation on complement activity, hemolytic activity was measured by using a modification of the method of Barta and Hubbert (4). Sheep erythrocytes (SRBCs) were collected in Alsever solution at a 1:1 ratio and allowed to age for 5 days at 4°C. Before the experiment, SRBCs were washed three times in PBS by centrifugation at 350 × *g* for 10 min. Sixty μl of a 2.5% suspension of SRBCs was added to 60 μl of PBS or serially diluted rabbit immunoglobulin G antibodies against SRBC (Cordis Laboratories, Miami, Fla.) in 96-well U-bottom polystyrene plates (Costar, Cambridge, Mass.), and the plates were incubated for 30 min at 37°C. Then, 120 μl of normal CNBS, CNBS which was heat inactivated, or CNBS which was treated with magnesium ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetra-acetic acid (EGTA) was added to the mixture, and the plates were incubated for 60 min at 37°C. Following incubation, the plates were centrifuged at 750 × *g* for 15 min and the degree of hemolysis was determined by reading the A_{410} of the supernatants (10) with an enzyme-linked immunosorbent assay reader (Microplate reader, MR 600; Dynatech Instruments, Inc., Princeton, N.J.).

Complement killing assay. The organisms were washed and resuspended as described above. One hundred fifty μl of CNBS, diluted to various concentrations in PBS-G-FBS, was added to 5×10^5 trichomonads in a 50-μl solution and incubated at 37°C. After incubation, the reaction mixtures were brought up to 1 ml with PBS-G-FBS, pulsed by the addition of 5 μCi of [^{3}H]adenine, and incubated for 60 min at 37°C. The reaction was stopped, and the labeled suspensions were harvested as described above. All treatments were done in duplicate, and heat-inactivated CNBS was included as a control.

Trichomonad killing was calculated by the following formula: percent killing = $[cpm_{c-} - cpm_{c+})/cpm_{c-}] \times 100$, where cpm_{c-} is counts per minute in suspensions with heat-inactivated CNBS and cpm_{c+} is counts per minute in suspensions with CNBS.

Effect of antibody on complement killing. The effect of heat-inactivated HIS on trichomonad viability was titrated in the presence of bovine complement. The percentage of *T. foetus* killed was calculated by the above formula, with both the cpm_{c-} and cpm_{c+} assay mixtures containing the same concentration of HIS.

Serum treatments. CNBS was depleted of bovine factor B by heat inactivation at 50°C for 45 min (36). After heat treatment, serum was cooled immediately in an ice-water bath, rewarmed to 37°C, and used in complement killing assays in the presence and absence of HIS. The treatment of bovine serum at 50°C for 45 min has been shown to be effective in depleting factor B as determined by hemolytic titration with unsensitized human erythrocytes (36).

For some experiments, CNBS and HIS were treated with EGTA (Eastman Kodak Co., Rochester, N.Y.) containing an equimolar concentration of Mg^{2+} to specifically inhibit the classical pathway of the complement system (17). Sera were incubated with magnesium EGTA at a final concentration of 43 mM for 25 min at 20°C and then used immediately in the assay of complement killing. This concentration of magnesium EGTA has been shown to inhibit the classical pathway of bovine complement (29).

Statistical method. Statistical significance of differences between group means was determined by the Student *t* test.

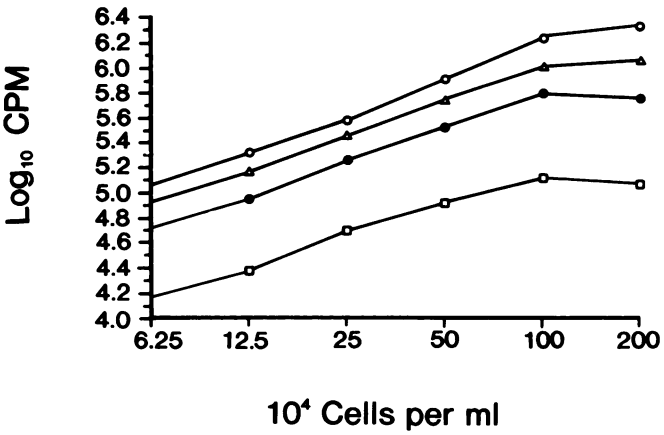

FIG. 1. Relationship between [^{3}H]adenine incorporation and the number of viable *T. foetus* cells. Protozoa were incubated for 60 min with 1 (□), 5 (●), 10 (△), and 20 (○) μCi of [^{3}H]adenine and harvested onto filters, and the radioactivity of the filters was counted by liquid scintillation spectroscopy.

RESULTS

Radiolabeling. At trichomonad concentrations up to 10^6/ml, there was a linear relationship between [^{3}H]adenine incorporation and the number of viable *T. foetus* cells (Fig. 1). [^{3}H]adenine uptake was starting to plateau after 60 min of incubation at 37°C (Fig. 2), as determined by using a pulse of 5 μCi of [^{3}H]adenine. Formalin- or heat-killed protozoa did not take up significant levels of [^{3}H]adenine ($P < 0.05$). On the basis of these results, subsequent assays included *T. foetus* at a concentration of 5×10^5/ml and reaction mixtures were pulsed with 5 μCi of [^{3}H]adenine for 60 min.

Hemolytic activity of complement. Significant hemolysis of antibody-coated SRBCs was observed following incubation with CNBS (Fig. 3). However, this hemolytic activity was markedly diminished by treatment of serum with 43 mM magnesium EGTA (Fig. 3). In the absence of specific anti-

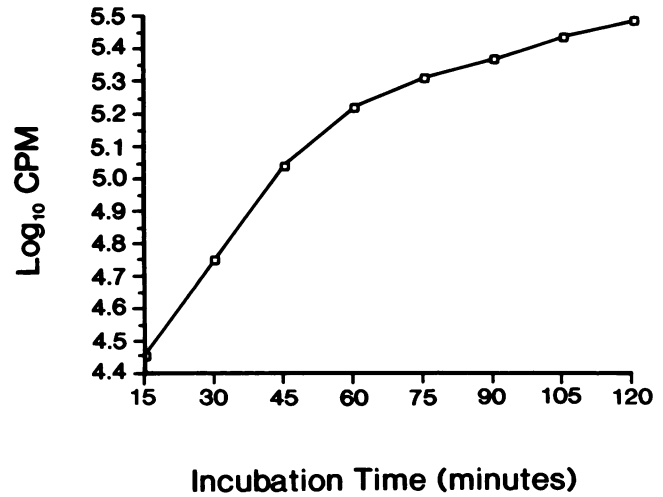

FIG. 2. Time-dependent [^{3}H]adenine uptake of *T. foetus*. Protozoa (5×10^5) were incubated for different time periods with 5 μCi of [^{3}H]adenine. After each incubation time, samples were harvested onto filters and the radioactivity of the filters was counted by liquid scintillation spectroscopy.

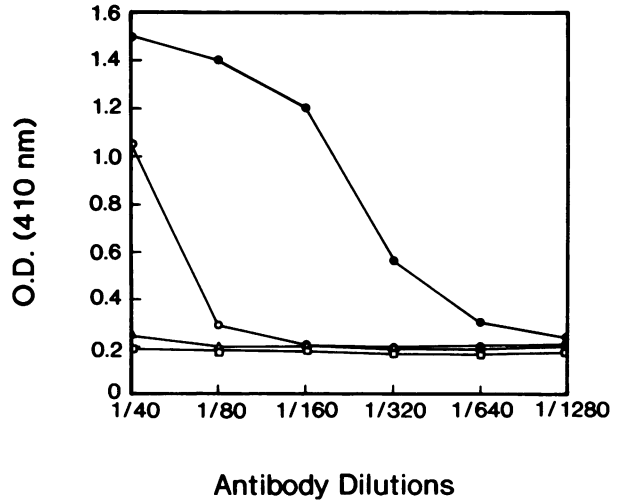

FIG. 3. Hemolytic activity of complement. Antibody-coated SRBCs were incubated for 60 min with normal (●), heat-inactivated (56°C for 30 minutes) (△), or magnesium EGTA-treated (○) CNBS, and the degree of hemolysis was determined by reading the A_{410} of the supernatants. Heat-inactivated and magnesium EGTA-chelated CNBS (□) was also included as a control. O. D., Optical density.

body to SRBCs, no SRBC lysis was observed. Similarly, heat inactivation of serum (56°C for 30 min) abolished the antibody-dependent lysis of SRBCs (Fig. 3).

Complement killing. Moderate killing of *T. foetus* was measured following incubation with CNBS. However, killing activity was rapidly decreased after CNBS was diluted in PBS-G-FBS (Fig. 4). Time course studies showed that in our assay system, peak killing was measured after 30 min of incubation (data not shown).

Role of antibody in killing. Complement-mediated killing was increased significantly ($P < 0.05$) in the presence of HIS (Fig. 4). In the absence of complement, HIS did not inhibit adenine incorporation by *T. foetus*, and at high concentrations of HIS, adenine incorporation was slightly increased (data not shown).

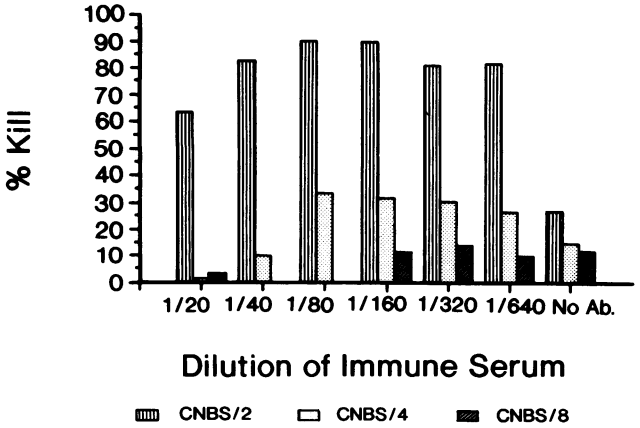

FIG. 4. Complement-mediated killing of *T. foetus* in the presence and absence of HIS. Protozoa were incubated for 30 min with dilutions of CNBS in the presence or absence of dilutions of HIS, and the viability was determined by [^{3}H]adenine labeling. Values represent the means for two separate experiments done in duplicate. Addition of antibody, at a 1/2 dilution of CNBS (CNBS/2), significantly increased trichomonad killing ($P < 0.05$).

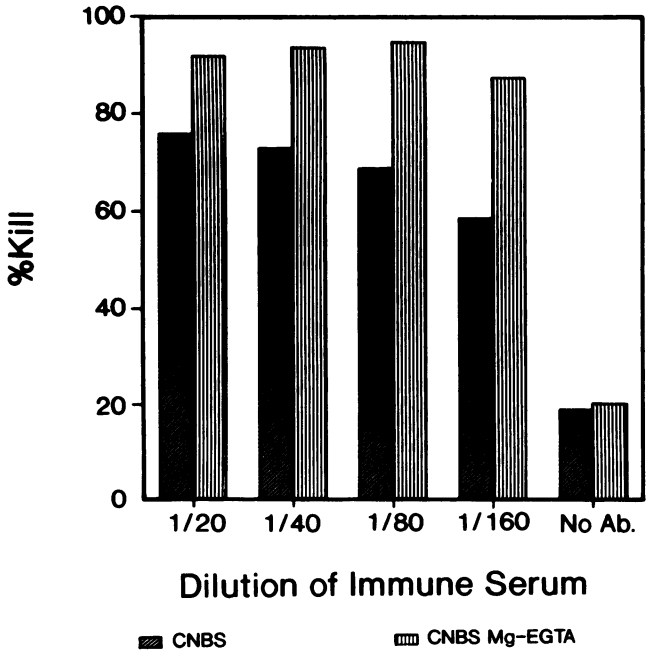

FIG. 5. Effect of magnesium EGTA treatment of complement on antibody-dependent and -independent complement killing of *T. foetus*. Protozoa were incubated for 30 min with normal and magnesium EGTA-chelated CNBS in the presence or absence of dilutions of HIS, and viability was determined by [^{3}H]adenine labeling. Values represent the means for two separate experiments done in duplicate. At each dilution of HIS, percent killing is not significantly different from its corresponding untreated control value.

Chelation and factor B depletion. The different requirements for activation of the alternative and the classical complement pathways have been exploited to investigate the role of both pathways in killing trichomonads. Activation of the classical pathway generally requires specific antibody together with Mg^{2+}, Ca^{2+}, and complement components C1, C4, C2, and C3 (16, 40). The alternative pathway requires Mg^{2+} (but not Ca^{2+}) and factors B and D, properdin, and C3 (16, 40).

Calcium chelation of serum by magnesium EGTA selectively blocks the classical pathway, leaving the alternative pathway intact (17). Depletion of factor B in serum blocks the alternative pathway (36) and allows expression of the classical pathway in human serum (22).

Treatment of serum with magnesium EGTA at a final concentration of 43 mM did not modify antibody-dependent or -independent killing of *T. foetus* (Fig. 5). Treatment of serum with 20 mM magnesium EGTA gave the same result. However, antibody-dependent and -independent killing activity was lost when serum was depleted of factor B by heating at 50°C for 45 min.

DISCUSSION

We have shown that *T. foetus* is susceptible to killing by bovine complement and that specific antibody to *T. foetus* greatly enhances complement-mediated killing. We have demonstrated that enhanced killing in the presence of specific antibody is mediated by the alternative pathway of bovine complement.

The relative lack of information defining the interaction

between trichomonads and host effector mechanisms is due in large part to technical problems in accurately measuring trichomonad viability. The methods used to determine interaction between host defenses and trichomonads, namely microscopic (7) and CFU (39) assays, are tedious, time-consuming, applicable only to small sample sizes, and subject to operator error. More importantly, the accuracy of these methods is limited in the presence of agglutinating concentrations of antibody, as trichomonad viability cannot be determined accurately where organisms are clumped. Since antibody is likely to be an important component in the host defense against *T. foetus* infection, the deficiency in these assay systems limits their value for analyzing the interaction between T. foetus and host defenses.

The assay used in this study, based on inhibition of protozoal incorporation of tritiated adenine, has been previously characterized to monitor the activity of antiprotozoal agents. The assay has been shown to provide precise and readily quantifiable results (30). Our results have supported these findings, since we have shown a linear relationship between [^{3}H]adenine incorporation and the number of viable *T. foetus*. Further, the uptake of tritiated adenine by nonliving protozoa was not significant ($P < 0.05$). Importantly, agglutinating concentrations of antibody did not inhibit adenine incorporation. In fact, at high concentrations of heat-inactivated HIS, adenine incorporation by *T. foetus* was slightly increased. Polyclonal antibody, in the absence of complement, did not compromise the viability of *T. foetus*, in contrast to the effect of some monoclonal antibodies which mediated complement-independent lysis of *T. vaginalis* (2), *Trypanosoma cruzi* (3), and *Giardia lamblia* (33).

Use of CNBS from a colostrum-deprived calf was an important component in our work, as such sera are essentially devoid of circulating immunoglobulins (8). This serum did not agglutinate *T. foetus* and, therefore, enabled us to measure the susceptibility of *T. foetus* to complement in the absence of specific antibody. Bovine complement was only moderately effective at killing *T. foetus*, with 25% killing at the highest CNBS concentration tested. This activity was rapidly lost by dilution of the CNBS. In an earlier study, neither rabbit nor guinea pig complement was able to promote killing of *T. foetus* (7).

To evaluate the complement pathway involved in killing *T. foetus*, trichomonacidal activity was quantitated in untreated and magnesium EGTA-treated CNBS. We confirmed that magnesium EGTA treatment effectively blocked the classical pathway of bovine complement (29). However, treatment of CNBS with magnesium EGTA failed to inhibit killing of *T. foetus*, a feature which indicated that the classical pathway of complement activation was not involved in antibody-independent killing by bovine serum. Similarly, bovine conglutinin was not involved in the killing, because activation of conglutinin is dependent on the presence of Ca^{2+} (27). Destruction of factor B in CNBS eliminated trichomonacidal activity, suggesting that killing was mediated by the alternative pathway of bovine complement. These findings parallel those reported for *T. vaginalis*, where antibody-independent killing was mediated by the alternative pathway (20). In that study, the trichomonacidal activity of normal human serum was abolished on depletion of properdin or by chelation of Mg^{2+} and Ca^{2+} (20). Use of a C4-deficient reagent or Ca^{2+} chelation did not reduce the lytic activity of normal human serum (20).

Addition of antibody, in the form of HIS, to CNBS significantly enhanced the trichomonacidal activity of bovine complement. Monoclonal antibodies that reacted with the protozoal surface promoted similar enhancement of *T. foetus* killing by rabbit and guinea pig complement (7), while antibodies present in normal human serum apparently participated in complement-mediated killing of *T. vaginalis* (20). The increased killing of *T. foetus* by specific antibody was due to activation of the alternative pathway of bovine complement, since destruction of factor B in bovine serum eliminated the killing activity while inactivation of the classical pathway by magnesium EGTA treatment did not inhibit antibody-dependent complement-mediated killing. Our results parallel those obtained by using monoclonal antibodies, as these antibodies induced lysis of *Toxoplasma gondii* through activation of the alternative pathway of human complement (19). Antibody has been found to promote alternative pathway activation in assay systems as diverse as zymosan (41), rabbit erythrocytes (34), SRBCs (35), *E. coli* (23), group B streptococci (14), *Haemophilus infuenzae* (43), and measles virus-infected cells (42). In most cases, the mechanism for this antibody-dependent effect is unknown. Antibody specific for the capsule of *H. influenzae* type b apparently altered the bacterial surface to promote alternative pathway killing (43). Similarly, specific antibody promoted killing of *E. coli* by the alternative pathway (25), possibly by directing C5b-C9 deposition around sites of antibody and C3 deposition. Antibody specific for the O side chain of *E. coli* promoted bacteriolysis even without increasing the uptake of C3 and C9 on the organism (18). It was proposed that antibody binding to the O-polysaccharide side chains resulted in exposure of outer membrane residues, thus rendering the bacteria susceptible to lysis (18). Other possible mechanisms for antibody-mediated enhancement of alternative pathway activity include binding of specific antibody to chemical groups, such as sialic acid, which inhibit alternative pathway activation (15). Similarly, antibody binding may increase deposition of C3b for conversion of C3 convertases to C5 convertases (32).

The results of this study, demonstrating antibody-independent and -dependent complement-mediated killing of *T. foetus*, imply that the surface of this organism is capable of activating the alternative pathway of bovine complement. The protozoal surface structures that promote alternative pathway activation are unknown. However, the relative resistance of *T. foetus* to bovine complement in the absence of specific antibody is an important determinant in the pathogenesis of bovine trichomoniasis. Furthermore, the ability of specific antibody to promote killing of *T. foetus* in vitro suggests that antibody and complement can promote protection against infection with *T. foetus* in the bovine reproductive tract.

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