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# Resistance of Highly Pathogenic *Naegleria fowleri* Amoebae to Complement-Mediated Lysis

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**Weakly pathogenic and nonpathogenic *Naegleria* spp. are readily lysed by human and guinea pig complement. Highly pathogenic *Naegleria fowleri* are resistant to complement-mediated lysis. Electrophoretic analysis of normal human serum (NHS) incubated with pathogenic or nonpathogenic *Naegleria* spp. demonstrates that amoebae activate the complement cascade, resulting in the production of C3 and C5 complement cleavage products. To determine whether surface constituents play a role in resistance to complement lysis, trophozoites of *Naegleria* spp. were subjected to enzymatic treatments prior to incubation in NHS. Treatment of trophozoites with papain or trypsin for 1 h, but not with neuraminidase, increased susceptibility of highly pathogenic *Naegleria fowleri* to complement lysis. Treatment of trophozoites with actinomycin D or cycloheximide during incubation with NHS or pretreatment with various protease inhibitors for 4 h did not increase the susceptibility of *N. fowleri* amoebae to lysis. Neither a repair process involving de novo protein synthesis nor a complement-inactivating protease appears to account for the increased resistance of *N. fowleri* amoebae to complement-mediated lysis.**

*Naegleria fowleri* is the causative agent of primary amoebic meningoencephalitis, a rare disease of the central nervous system. Host defense mechanisms against this opportunistic pathogen are still unclear; however, the complement system may play a significant role in resistance to infection. Activation of the alternative complement pathway alone is sufficient to kill susceptible *Naegleria* amoebae. Nonpathogenic and weakly pathogenic *Naegleria* spp. are readily lysed by human and guinea pig complement (33). In contrast, highly pathogenic strains of *N. fowleri* are resistant to complement-mediated lysis. Thus, the ability of pathogenic amoebae to escape lysis may be an important factor in its pathogenicity.

Both the classical and alternative complement pathways, in the presence or absence of antibodies, are activated in vitro by a number of protozoan parasites (3, 6, 7, 33). Complement activation results in either elimination of the parasite by complement-mediated lysis or evasion of complement-mediated lysis by changes occurring within the parasite. The classical and alternative complement pathways can be activated in vitro by trophozoites or antigenic fractions of *Entamoeba histolytica* (3, 11, 22, 26). Pathogenic and nonpathogenic strains of *E. histolytica* activate complement equally well, but pathogenic strains are resistant to complement-mediated lysis (28). The African trypanosome, *Trypanosoma brucei* subsp. *gambiense* activates the alternative complement pathway; however, the organisms escape lysis because the cascade does not continue beyond the establishment of the C3 convertase on the trypanosome surface (7).

In this study, we have demonstrated that both pathogenic and nonpathogenic *Naegleria* spp. activate the complement cascade, resulting in the production of C3 and C5 complement cleavage fragments, but highly pathogenic amoebae remain resistant to complement-mediated lysis. The presence of *Naegleria*-specific antibody enhances the amoebicidal activity of serum for nonpathogenic amoebae but not for highly pathogenic amoebae. Treatment of pathogenic amoebae with trypsin or papain increased their susceptibility to lysis, indicating that surface components may play a role in resistance to complement-mediated lysis.

## MATERIALS AND METHODS

**Amoebae.** *N. fowleri* LEE (ATCC 30894), a weakly pathogenic strain in mice, was cultured axenically and maintained in 75-cm$^2$ plastic flasks (Becton Dickinson Labware, Oxnard, Calif.) in Cline medium at 37°C (5, 17). *N. fowleri* LEEmp, a highly pathogenic strain, was obtained by serially passaging the LEE strain through 6-week-old female B6C3F$_1$ mice at monthly intervals. All challenges were given intranasally. Samples of brain from infected mice containing LEEmp were cultured axenically at 37°C for a period of not more than 1 month prior to another mouse passage. These studies were performed with passages 60 to 75. *N. gruberi* EG$_B$, a nonpathogenic species, was grown axenically in Cline medium at 30°C (17, 32).

**Complement source.** Normal human serum (NHS) samples from healthy adult volunteers were allowed to clot for 1 h at room temperature and were centrifuged at 850 × *g* for 10 min, dispensed into vials, and stored at −70°C. Fresh normal guinea pig serum samples were obtained from adult female Hartley guinea pigs, each weighing 800 g. Hyperimmune guinea pig serum samples were prepared for each *Naegleria* strain by immunizing guinea pigs with the appropriate amoebic lysate (two animals per lysate). Sera from the two animals injected with a given lysate were pooled. Amoebic lysates were prepared as follows. Amoebae were harvested by centrifugation, washed in Hanks balanced salt solution, and subjected to sequential freezing and thawing in an ethanol-dry ice slush. The protein concentration of the amoebic lysates was determined by the Kalb-Bernlohr assay (12). Guinea pigs were immunized by intraperitoneal injections of 1 mg of amoebic lysate per ml twice at weekly intervals. Immune guinea pig serum samples were collected 14 days after the second injection.

**Cytopathic effect of NHS for *Naegleria* spp.** *N. fowleri*

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LEEmp and *Naegleria gruberi* $EG_B$ amoebae were incubated with a 1:8 dilution of NHS for 0, 15, 30, 45, and 60 min at 37°C and fixed in situ by removing the serum and adding 2.5% glutaraldehyde. Samples containing amoebae incubated in NHS were prepared for transmission electron microscopy as previously described (19).

**Immunoelectrophoretic analysis of NHS incubated with *Naegleria* spp.** *N. fowleri* LEE or LEEmp and *N. gruberi* $EG_B$ ($10^6$ amoebae per ml) were washed in gelatin Veronal buffer ($GVB^{2+}$) (30) and incubated with fresh human serum for 1 h at 37°C. Conversion of C3 and C5 was assessed by immunoelectrophoresis with antiserum specific for C3, C5, or their activation cleavage products (ICN ImmunoBiologicals, Lisle, Ill.). Serum was incubated in $GVB^{2+}$ alone at 37 or 4°C to serve as a control for spontaneous complement activation. Purified complement components C3 and C5 and zymosan-activated human serum were a gift from Shaun Ruddy (Medical College of Virginia). Electrophoresis was carried out in a layer of 1% agarose A (Pharmacia LKB Biotechnology, Inc., Piscataway, N.J.) on Gelbond. The buffer was Tris-barbiturate buffer with 0.01 M EDTA (pH 8.6). Samples of undiluted absorbed sera or control sera (10 μl) were dispensed into 4-mm wells. Electrophoresis was carried out at 20 V/cm for 2 h. After the run, the agarose from the center trough was removed and the trough was filled with 100 μl of specific antiserum. The gel was incubated overnight at room temperature, washed in physiological saline, and stained in Coomassie brilliant blue R250 (Bio-Rad, Richmond, Calif.).

**Amoebicidal assays.** Log-phase cultures of *Naegleria* amoebae were labeled for 24 h with 25 μCi of [$^3$H]uridine at 37°C for both strains of *N. fowleri* or at 30°C for *N. gruberi*. Human or guinea pig sera were diluted with $GVB^{2+}$ in microdilution wells and mixed with [$^3$H]uridine-labeled amoebae. At the end of the incubation period, supernatant fluid was harvested and the percent specific release of radiolabel from the amoebae, which was used as the index of lysis, was determined as previously described (33). All data were analyzed statistically by using the Student *t* test.

**Effect of antibody on amoebicidal activity of guinea pig serum.** Hyperimmune antisera to *N. fowleri* LEE, LEEmp, or *N. gruberi* $EG_B$ were obtained from female Hartley guinea pigs as described above. Prebleeds were obtained from all animals to serve as the normal control sera. The titers of *Naegleria*-specific immunoglobulin M (IgM) and IgG antibody in all sera were quantitated with an immunodot blot assay (Bio-Rad) (18). Briefly, amoebic lysates, serially diluted from 5 to 0.002 μg, were blotted onto a nitrocellulose membrane (Schleicher & Schuell, Inc., Keene, N.H.) and reacted with a 1:50 dilution of test guinea pig serum containing *Naegleria*-specific antibodies. The blots were reacted with goat anti-guinea pig IgG (Organon Teknika-Cappel, West Chester, Pa.) or with rabbit anti-guinea pig IgM followed by the appropriate peroxidase-conjugated anti-immunoglobulin. The highest dilution of *Naegleria* antigen giving a positive reaction was used as the antibody titer. The amoebicidal assay was conducted on [$^3$H]uridine-labeled *Naegleria* spp. with a 1:8 dilution of normal or hyperimmune guinea pig serum.

**Effect of enzymatic treatment of *Naegleria* spp. on complement lysis.** Trypsin-EDTA (10×), papain type III (twice crystallized, from papaya latex), or neuraminidase type X from *Clostridium perfringens* (EC 3.2.1.18) were obtained from Sigma Chemical Co. (St. Louis, Mo.). *Naegleria* spp. were radiolabeled with [$^3$H]uridine. Trophozoites ($5 \times 10^6$) were treated with 10× trypsin-EDTA for 1 h at 37°C. Trophozoites ($2 \times 10^6$) were treated with 2 U of neuraminidase or 20 U of papain for 1 h at 37°C. After treatment, the amoebae were incubated for 10 min either with Hanks balanced salt solution containing fetal calf serum to inactivate trypsin or iodoacetamide to inactivate papain and washed in HBSS. Radiolabeled enzyme-treated amoebae and untreated amoebae ($5 \times 10^4$) were then incubated with a 1:2 dilution of normal guinea pig serum, and the percent specific release of [$^3$H]uridine was determined by the amoebicidal assay.

**Effect of RNA and protein synthesis inhibitors on susceptibility to complement lysis.** *Naegleria* spp. ($10^6$) labeled with [$^3$H]uridine were incubated with a 1:8 dilution of NHS for 1 h at 37°C in the presence or absence of 5 μg of actinomycin D or 5 μg of cycloheximide (Sigma Chemical Co.) per ml. The release of radiolabel from the amoebae was determined by the amoebicidal assay. In separate experiments, labeled *N. fowleri* LEEmp amoebae were pretreated with 5 μg of actinomycin D or 5 to 7 μg of cycloheximide per ml for 6 or 18 h before use in the amoebicidal assay. In addition, inhibition of RNA synthesis by actinomycin D and the inhibition of protein synthesis by cycloheximide were assessed. *N. fowleri* LEEmp amoebae were labeled with [$^3$H]uridine or [$^{35}$S]methionine in the presence of actinomycin D or cycloheximide, respectively. The incorporation of radiolabel was determined in the presence or absence of drug, and the percent inhibition of RNA or protein synthesis was calculated as follows: percent inhibition = 100 − [(average counts per minute in the presence of drug/average counts per minute in the absence of drug) × 100].

**Effect of protease inhibitors on *N. fowleri* susceptibility to complement lysis.** *N. fowleri* LEEmp amoebae ($10^6$) labeled with [$^3$H]uridine were pretreated for 4 or 24 h at 37°C with different protease inhibitors or medium alone. Phenylmethanesulfonyl fluoride, pepstatin A, phenanthroline, leupeptin, antipain, and iodoacetamide were obtained from Sigma Chemical Co. After incubation with the inhibitors, the amoebae were washed in Hanks balanced salt solution to remove the protease inhibitors and were suspended in $GVB^{2+}$ buffer. Radiolabeled protease inhibitor-treated amoebae and untreated amoebae ($5 \times 10^4$) were then incubated with a 1:4 dilution of normal human serum, and the percent specific release of label was determined by the amoebicidal assay.

## RESULTS

The cytopathic effect of NHS for pathogenic and nonpathogenic *Naegleria* spp. was evaluated by transmission electron microscopy. The micrographs of untreated amoebae revealed healthy, intact, amoeboid cells (Fig. 1A and C). Within 30 min of incubation, the nonpathogenic *N. gruberi* amoebae showed cytopathic changes and damage to the plasma membrane (data not shown). After a 60-min incubation with NHS, the nonpathogenic amoebae revealed extensive cytopathic changes, including complete disruption of the cells and extrusion of nuclei and other cellular components (Fig. 1D). Although the complement-resistant *N. fowleri* LEEmp appear round after incubation in NHS containing *Naegleria*-specific antibodies (18), the highly pathogenic amoebae showed little cytopathic change after incubation in NHS for 60 min (Fig. 1B).

To rule out the possibility that highly pathogenic *N. fowleri* amoebae are resistant to complement-mediated lysis because they fail to activate the lytic pathways, we assessed NHS preincubated with each *Naegleria* strain for the pres-

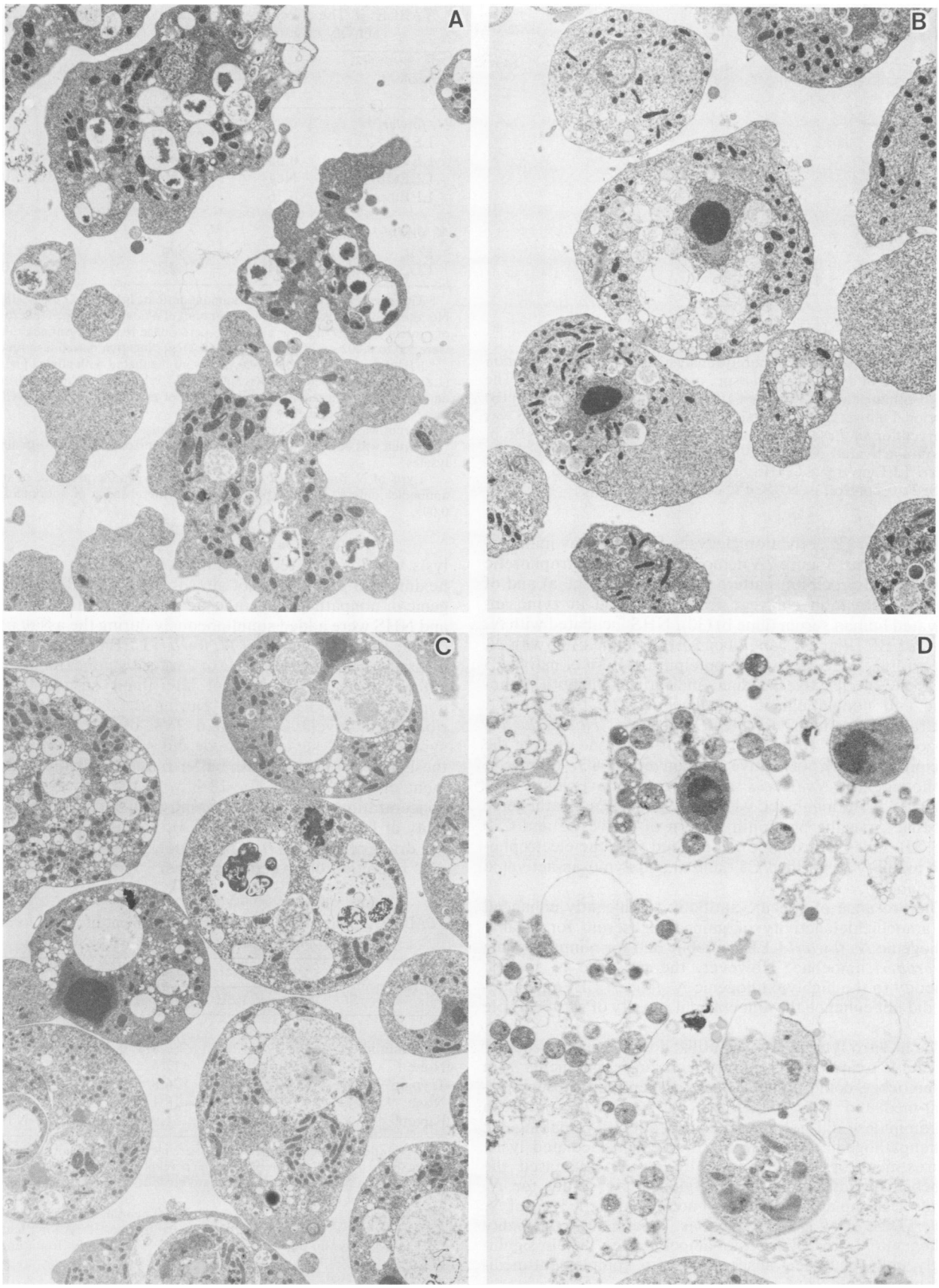


FIG. 1. Transmission electron microscopy of *Naegleria* trophozoites treated with NHS (1:8 dilution) for 0 or 60 min at 37°C. (A) *N. fowleri* (LEEmp), 0 min; (B) *N. fowleri* (LEEmp), 60 min; (C) *N. gruberi* ($EG_B$), 0 min; (D) *N. gruberi* ($EG_B$), 60 min.

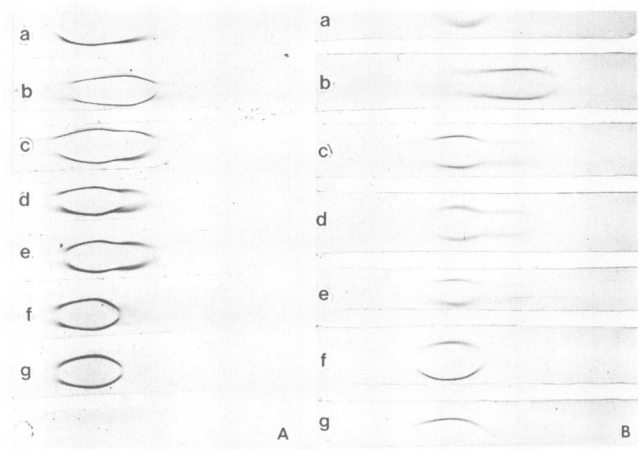


FIG. 2. Immunoelectrophoretic analysis of NHS after incubation with trophozoites of *Naegleria* spp. or $GVB^{2+}$ for 1 h at 37°C. The trough contains anti-C3 (diluted 1:5) (A) or anti-C5 (diluted 1:2) (B) polyclonal antiserum. Conversion of C3 to C3b and C5 to C5b was observed for all three strains. Lanes: a, native C3 (A) or C5 (B); b, zymosan-activated NHS; c, *N. fowleri* LEE-treated NHS; d, *N. fowleri* LEEmp-treated NHS; e, *N. gruberi* $EG_B$-treated NHS; f, NHS, 37°C control; g, NHS, 4°C control.

ence of C3 or C5 activation cleavage fragments by immunoelectrophoresis. Figure 2A demonstrates the electrophoretic mobility and precipitin pattern of native C3 (lane a) and of the C3b activation cleavage fragment found in zymosan-activated human serum (lane b) (30). NHS incubated with *N. fowleri* LEE (Fig. 2A, lane c) or LEEmp (lane d) or with *N. gruberi* $EG_B$ (lane e) gave the precipitin pattern of native C3 and the C3b cleavage product, indicating that both pathogenic and nonpathogenic amoebae activate complement. Control serum incubated in the absence of *Naegleria* trophozoites at 37°C (Fig. 2A, lane f) or 4°C (lane g) gave the precipitin pattern of native, unconverted C3. Similarly, trophozoites of *Naegleria* spp. induced conversion of C5 (Fig. 2B). NHS incubated with pathogenic or nonpathogenic amoebae gave the precipitin pattern of native C5 and C5b (Fig. 2B, lanes c through e), compared with the electrophoretic mobility of purified C5 (lane a) and zymosan-activated C5b (lane b).

The presence of specific antibody significantly enhanced the amoebicidal activity of guinea pig serum for weakly pathogenic *N. fowleri* LEE amoebae and for nonpathogenic *N. gruberi* amoebae. However, the presence of specific antibody to the highly pathogenic *N. fowleri* LEEmp amoebae did not enhance the amoebicidal activity of serum (Table 1).

The in vitro lytic assay was utilized to determine whether enzymatic treatment to remove membrane components from the amoebae could enhance their susceptibility to complement-mediated lysis. Treatment of *Naegleria* spp. with neuraminidase did not alter the susceptibility of pathogenic or nonpathogenic amoebae to complement-mediated lysis. Pretreatment of trophozoites with trypsin increased the amoebicidal activity of normal guinea pig serum for *N. fowleri* LEEmp amoebae but did not enhance the lysis of *N. fowleri* LEE or *N. gruberi* amoebae. Treatment of the highly pathogenic *N. fowleri* LEEmp amoebae with papain significantly enhanced their susceptibility to complement-mediated lysis (Table 2).

The effect of inhibitors of RNA and protein synthesis on the susceptibility of *Naegleria* spp. to complement-mediated lysis was investigated. Actinomycin D (5 μg/ml) or cycloheximide (5 μg/ml) did not alter the susceptibility of pathogenic or nonpathogenic amoebae to lysis when the inhibitors and NHS were added simultaneously during the assay period (Table 3). Pretreatment of *N. fowleri* LEEmp amoebae for 6 h with 5 μg of actinomycin D or 7 μg of cycloheximide per ml before adding NHS did not alter their susceptibility to complement-mediated lysis. During the 6-h treatment period, actinomycin D inhibited 36% of the cellular RNA synthesis, while cycloheximide did not inhibit protein synthesis when compared with buffer-treated controls. Pretreatment of the amoebae for 18 h with the above-mentioned concentrations of inhibitors greatly enhanced the susceptibility of LEEmp to lysis (Table 4). The 18-h treatment with the drugs resulted in 91% inhibition of RNA synthesis and 45% inhibition of protein synthesis.

TABLE 1. The effect of specific antibody on the amoebicidal activity of guinea pig serum for *Naegleria* spp.

| Organism | Dot blot titer for[a]: | | Mean % specific release ± SEM[b] |
|---|---|---|---|
| | IgM | IgG | |
| *N. fowleri* | | | |
| LEE | Neg | Neg[c] | 25.1 ± 1.4 |
| LEE | 0.08 | 0.63[d] | 44.5 ± 1.0[e] |
| LEEmp | Neg | Neg[c] | 8.5 ± 0.6 |
| LEEmp | 0.16 | 0.63[d] | 7.1 ± 1.6 |
| *N. gruberi* | | | |
| $EG_B$ | Neg | Neg[c] | 31.4 ± 2.0 |
| $EG_B$ | 0.04 | 1.25[d] | 79.2 ± 0.5[e] |

[a] Titers were determined by an immunodot blot IgM or IgG assay (18). Dot blot titers are expressed as the least amount of *Naegleria* antigen (micrograms of protein per dot) yielding a positive peroxidase reaction. Antigen dilutions were 5.0 to 0.002 μg of protein per dot. Neg, Negative peroxidase reaction.

[b] [$^3$H]uridine-labeled *Naegleria* spp. were incubated with guinea pig serum (1:8). Values represent the percent specific release of radiolabel from the amoebae plus or minus the standard error of the mean from four wells in a representative experiment.

[c] Serum was obtained from normal guinea pigs.

[d] Serum was obtained from guinea pigs immunized two times with amoebic lysates.

[e] Lysis of *N. fowleri* LEE and *N. gruberi* $EG_B$ in the presence of specific antibodies differs significantly from lysis in the absence of antibodies; $P < 0.005$.

TABLE 2. The effect of enzymatic treatment of *Naegleria* spp. on susceptibility to complement-mediated lysis

| Treatment | Mean % specific release ± SEM[a] | | |
|---|---|---|---|
| | *N. fowleri* LEE | *N. fowleri* LEEmp | *N. gruberi* $EG_B$ |
| None | 74.6 ± 2.4 | 5.7 ± 1.5 | 80.3 ± 0.8 |
| Neuraminidase[b] | 68.3 ± 1.8 | 6.8 ± 0.3 | 81.4 ± 0.3 |
| None | 58.6 ± 0.7 | 12.5 ± 0.8 | 79.5 ± 1.0 |
| Trypsin[c] | 59.3 ± 3.0 | 26.8 ± 2.6[d] | 75.7 ± 0.2 |
| None | 72.9 ± 1.4 | 18.1 ± 1.3 | NT[e] |
| Papain[f] | 70.2 ± 0.9 | 51.9 ± 1.0[g] | NT |

[a] [$^3$H]uridine-labeled *Naegleria* spp. were incubated with normal guinea pig serum (diluted 1:2). Values represent the percent specific release of radiolabel from the amoebae plus or minus standard error of the mean from four wells in a representative experiment.

[b] Amoebae ($2 \times 10^6$) were treated with 2 U of neuraminidase for 1 h at 37°C.

[c] Amoebae ($5 \times 10^6$) were treated with 10× trypsin-EDTA for 1 h at 37°C.

[d] Lysis of trypsin-treated LEEmp differs significantly from untreated LEEmp; $P < 0.01$.

[e] NT, Not tested.

[f] Amoebae ($2 \times 10^6$) were treated with 20 U of papain for 1 h at 37°C.

[g] Lysis of papain-treated LEEmp differs significantly from untreated LEEmp; $P < 0.005$.

TABLE 3. Effect of RNA and protein synthesis inhibition on susceptibility of *Naegleria* spp. to complement lysis

| Treatment[b] | Mean % specific release ± SEM[a] | | |
|---|---|---|---|
| | *N. fowleri* LEE | *N. fowleri* LEEmp | *N. gruberi* $EG_B$ |
| $GVB^{2+}$ | 42.7 ± 0.5 | 2.0 ± 0.3 | 69.6 ± 0.6 |
| Actinomycin D | 43.3 ± 1.2 | 2.8 ± 0.4 | 68.3 ± 0.6 |
| Cycloheximide | 34.3 ± 1.6 | 1.9 ± 0.5 | 74.2 ± 0.5 |

[a] [$^3$H]uridine-labeled amoebae ($5 \times 10^4$) were incubated with a 1:8 dilution of NHS for 1 h at 37°C. Values represent the percent specific release of radiolabel from the amoebae plus or minus standard error from four wells in a representative experiment.

[b] Amoebae were incubated with NHS in the presence or absence of 5 μg of actinomycin D or cycloheximide per ml dissolved in $GVB^{2+}$ buffer.

The spontaneous release values for the pretreatment incubation periods and the 1-h lytic assay indicated that the drugs were not toxic for the amoebae after short-term exposures. Visual observation by light microscopy for cell viability indicated that an 18-h incubation with 7 μg of cycloheximide did not disrupt the cell, although the spontaneous release values were elevated over the control. Concentrations of actinomycin D and cycloheximide greater than 5 and 7 μg/ml, respectively, were injurious for the amoebae, as indicated by high spontaneous release values and decreased cell viability (data not shown).

To determine whether amoebic proteases play a role in resistance to complement-mediated lysis, pathogenic *N. fowleri* LEEmp amoebae were incubated for 4 and 24 h with protease inhibitors prior to incubation in NHS (Table 5). Amoebae preincubated for 4 h with the irreversible serine protease inhibitor phenylmethanesulfonyl fluoride or with the aspartic protease inhibitor pepstatin A were as resistant to complement-mediated lysis as the buffer-treated controls were. In addition, treatment with leupeptin and antipain, which reversibly inhibit serine and cysteine proteases, did not alter the susceptibility of the amoebae to lysis. Phenanthroline, a metalloprotease inhibitor, and iodoacetamide, a cysteine protease inhibitor, were toxic for the amoebae after a 4-h incubation period. After 24 h of treatment with pepstatin A and antipain, sensitivity of *Naegleria* amoebae to complement-mediated lysis doubled, while phenylmethanesulfonyl fluoride treatment resulted in an eightfold enhancement of lysis.

TABLE 4. Effect of long-term RNA and protein synthesis inhibition on susceptibility of *N. fowleri* LEEmp to complement-mediated lysis

| Preincubation time and treatment[a] | Mean % specific release ± SEM[b] | % Spontaneous release during[c] | | % Inhibition[d] |
|---|---|---|---|---|
| | | 1 h of lytic assay | 6 or 18 h preincubation | |
| 6 h | | | | |
| $GVB^{2+}$ | 6.0 ± 0.81 | 5.2 | 22.7 | |
| Actinomycin D | 4.8 ± 1.6 | <5.2 | 15.9 | 36.0 |
| Cycloheximide | 8.2 ± 2.9 | <5.2 | 13.2 | 0.0 |
| 18 h | | | | |
| $GVB^{2+}$ | 15.5 ± 0.66 | 14.7 | 45.5 | |
| Actinomycin D | 49.4 ± 0.19[e] | 25.0 | 38.9 | 91.4 |
| Cycloheximide | 55.9 ± 1.5[e] | 18.5 | 68.6 | 44.6 |

[a] [$^3$H]uridine-labeled amoebae were incubated with 5 μg of actinomycin D or 7 μg of cycloheximide per ml for 6 or 18 h before use in the amoebicidal assay.

[b] Amoebae were incubated with NHS (1:8) for 1 h at 37°C. Values represent the percent specific release of radiolabel from the amoebae ± standard error of the mean from four wells in a representative experiment.

[c] Values represent the percent spontaneous release of label from the amoebae during the 1 h of the lytic assay or the 6 or 18 h of preincubation with either drug.

[d] Actinomycin D data represent the percent inhibition of [$^3$H]uridine incorporation. Cycloheximide data represent the percent inhibition of [$^{35}$S] methionine incorporation.

[e] Lysis of amoebae pretreated for 18 h with drugs differed significantly from untreated ($GVB^{2+}$) amoebae; $P < 0.005$.

TABLE 5. Effect of protease inhibitors on susceptibility of *N. fowleri* LEEmp to complement-mediated lysis

| Treatment (conc) | Mean % specific release ± SEM[a] | |
|---|---|---|
| | 4 h | 24 h |
| $GVB^{2+}$ | 6.8 ± 0.7 | 11.6 ± 0.4 |
| Phenylmethanesulfonyl fluoride (1 mM) | 5.0 ± 0.4 | 84.5 ± 1.0[b] |
| Pepstatin A (20 μg/ml) | 7.0 ± 0.6 | 29.7 ± 2.2[b] |
| Leupeptin (1 mM) | 6.2 ± 0.5 | 16.0 ± 1.6 |
| Antipain (20 μg/ml) | 8.7 ± 0.3 | 25.0 ± 0.8[b] |

[a] [$^3$H]uridine-labeled *N. fowleri* LEEmp amoebae were incubated with NHS (diluted 1:8). Values represent the percent specific release of radiolabel from the amoebae plus or minus standard error of the mean. Amoebae were pretreated for 4 h at 37°C with protease inhibitor or $GVB^{2+}$ buffer before incubation with NHS, or they were incubated overnight at 37°C with protease inhibitor or $GVB^{2+}$ buffer and then incubated for an additional 4 h with fresh inhibitor on the day of the assay before incubation in NHS.

[b] Lysis of amoebae treated with protease inhibitors for 24 h differed significantly from untreated control ($GVB^{2+}$); $P < 0.005$.

## DISCUSSION

Nonpathogenic and weakly pathogenic species of *Naegleria* amoebae are susceptible to lysis by complement, while highly pathogenic species of *Naegleria* are complement resistant. Plasma membrane disruption and extrusion of intracellular components were observed within 15 min of incubation of nonpathogenic amoebae with NHS. In contrast, highly pathogenic *N. fowleri* LEEmp amoebae showed little cytopathic change after incubation in NHS for 60 min. The presence of *Naegleria*-specific antibodies enhanced the amoebicidal activity of guinea pig serum for the nonpathogenic amoebae *N. gruberi* and for the weakly pathogenic *N. fowleri* LEE. Specific antibody had no effect on enhancing the lytic activity of serum for *N. fowleri* LEEmp. Highly pathogenic *Naegleria* amoebae are not susceptible to killing by complement alone or by the combination of specific antibody and complement. Similar results have been reported for pathogenic strains of *E. histolytica* (28). In contrast, the trophozoite stage of *Giardia lamblia* and bloodstream forms of *Trypanosoma cruzi*, both of which are resistant to complement, are lysed in the presence of specific antibody (9, 15, 16). The mechanism(s) of resistance of the highly pathogenic *N. fowleri* LEEmp amoebae to lysis is not due to a block in complement activation. The presence of C3 and C5 activation products is a sensitive index of complement activation, as these factors are involved in both the classical and alternative pathways. Both pathogenic and nonpathogenic *Naegleria* spp. activate complement, resulting in the conversion of C3 to C3b and C5 to C5b.

Membrane-associated inhibitory molecules of the complement pathway have been described for a number of eucaryotic cells (10, 21, 25, 31). Inactivation of the complement cascade can occur at any stage in the complement pathway because of regulatory proteins which modulate the efficiency of complement. Decay accelerating factor, isolated from erythrocyte membranes, regulates the early complement components by binding to C3b and C4b, causing dissociation

of the convertase systems (21, 25). Recently, Rimoldi et al. (29) have proposed that a surface glycoprotein on *T. cruzi* trypomastigotes analogous to mammalian decay accelerating factor accelerates the intrinsic decay of the classical and alternative pathway C3 convertases and regulates resistance to complement lysis. Conversion of C5 to C5b in NHS after incubation at 37°C with trophozoites of *N. fowleri* LEEmp indicates that the cascade continues beyond the establishment of C3/C5 convertase on the surface of the amoebae. Furthermore, previous studies assessing total hemolytic activity of serum before and after incubation with amoebae have shown that complement-resistant and complement-sensitive *Naegleria* spp. are equally capable of consuming complement (33).

Complement-mediated lysis of target cells by the alternative pathway is inhibited by the presence of sialic acid on the surface of plasma membranes. Sialic acid acts to prevent alternative pathway activation by increasing the affinity of the serum inhibitory protein factor H for C3b (13, 27). Treatment of nonactivating cell types, such as sheep erythrocytes or trypomastigotes of *T. cruzi*, with neuraminidase to remove sialic acid residues transformed these cells into activators of the alternative complement pathway (8, 14). Treatment of complement-resistant *N. fowleri* LEEmp with neuraminidase did not increase susceptibility to complement, indicating that sialic acid residues do not play a role in resistance to complement-mediated lysis. Similarly, trophozoites of *G. lamblia*, which fail to activate the alternative pathway, are not converted to activators after neuraminidase treatment (6).

Treatment of *T. cruzi* trypomastigotes with trypsin converts these cells from serum-resistant to serum-sensitive organisms (14). Treatment of *N. fowleri* LEEmp with trypsin or papain increased the susceptibility to lysis, indicating that surface components sensitive to proteolytic activity function to limit complement-mediated lysis.

In order to determine whether highly pathogenic amoebae counteract the lytic activity of complement by synthesizing repair proteins, *Naegleria* amoebae were treated with inhibitors of RNA or protein synthesis. Treatment of pathogenic or nonpathogenic *Naegleria* spp. with actinomycin D or cycloheximide during exposure to complement did not alter resistance to the amoebicidal activity of serum. Complement-induced release of [$^3$H]uridine from highly pathogenic *N. fowleri* amoebae was enhanced by actinomycin D and cycloheximide only after 18 h of pretreatment with the drugs. The expression of complement-inhibitory molecules on the surface or secreted by *N. fowleri* LEEmp amoebae is not in response to exposure of the amoebae to complement but rather may be constitutively synthesized during cellular metabolic processes.

Parasite proteases have been shown to modulate the lytic activity of the complement system. The major surface glycoprotein of *Leishmania mexicana* is believed to cleave C3b into the inactive C3bi form, thereby inhibiting further activation of the lethal complement cascade (1, 2, 4, 24). Protease activity among amoebic fractions of *N. fowleri* has been demonstrated (17, 20). Highly pathogenic *N. fowleri* LEEmp amoebae were treated with protease inhibitors to determine whether these amoebic proteases play a role in resistance to complement-mediated lysis. The extensive treatment period (18 h) with protease inhibitors required to alter the susceptibility of *Naegleria* amoebae to complement-mediated lysis indicates that the inhibitors cross the plasma membrane and interact with internal proteases. Alterations in the susceptibility to lysis would have been expected after a 4-h treatment period if surface-associated proteases played a role in resistance to complement-mediated lysis. Inhibition of intracellular proteases may enhance the susceptibility of the amoebae to lysis directly or indirectly by interfering with intracellular proteolysis, thereby making the cell vulnerable to attack by complement.

To date, the naeglerial components which cause complement activation and which modulate the lytic process to allow highly pathogenic *N. fowleri* LEEmp to escape the amoebicidal activity of serum are unknown. Unique trypsin- and papain-sensitive components on the membrane of highly pathogenic *Naegleria* appear to play a role in resistance to lysis. This study indicates that pathogenic amoebae activate complement, but pathogenic amoebae may evade lysis by inhibiting the cascade at the terminal stages of complement action, the membrane attack complex, since C5 activation products were detected in human serum incubated with *Naegleria* amoebae. Resistance of mammalian cells to complement-mediated lysis at the level of the membrane attack complex has been demonstrated. Homologous restriction factor, an erythrocyte membrane protein, controls the final assembly of the terminal complement complex by binding to C8 or C9, inhibiting transmembrane channel formation (31). Neutrophils are able to recover from homologous complement attack by removal of the membrane attack complex through both exocytic and endocytic processes (23). Similarly, highly pathogenic *Naegleria* amoebae may be resistant to complement-mediated lysis by shedding the membrane attack complex or by internalizing and degrading the terminal complement components. Neither a repair process involving de novo protein synthesis nor a complement-specific protease appear to account for the increased resistance of *N. fowleri* amoebae to complement-mediated lysis.

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## LITERATURE CITED

1. **Bordier, C.** 1987. The promastigote surface protease of *Leishmania*. Parasitol. Today **3**:151–153.
2. **Bouvier, J., R. Etges, and C. Bordier.** 1987. Identification of the promastigote surface protease in seven species of *Leishmania*. Mol. Biochem. Parasitol. **24**:73–79.
3. **Calderon, J., and R. D. Schreiber.** 1985. Activation of the alternative and classical complement pathway by *Entamoeba histolytica*. Infect. Immun. **50**:560–565.
4. **Chaudhuri, G., and K. Chang.** 1988. Acid protease activity of a major surface membrane glycoprotein (gp 63) from *Leishmania mexicana* promastigotes. Mol. Biochem. Parasitol. **27**:43–52.
5. **Cline, M., F. Marciano-Cabral, and S. G. Bradley.** 1983. Comparison of *Naegleria fowleri* and *Naegleria gruberi* cultivated in the same nutrient medium. J. Protozool. **30**:387–391.
6. **Deguchi, M., F. D. Gillin, and I. Gigli.** 1987. Mechanism of killing of *Giardia lamblia* trophozoites by complement. J. Clin. Invest. **79**:1296–1302.
7. **Devine, D. V., R. J. Falk, and A. E. Balber.** 1986. Restriction of the alternative pathway of human complement by intact *Tryanosoma brucei* subsp. *gambiense*. Infect. Immun. **52**:223–229.
8. **Fearon, D. T.** 1978. Regulation by membrane sialic acid of β1H-dependent decay-dissociation of amplification C3 convertase of the alternative complement pathway. Proc. Natl. Acad. Sci. USA **75**:1971–1975.
9. **Hill, D. R., J. J. Burge, and R. D. Pearson.** 1984. Susceptibility of *Giardia lamblia* trophozoites to the lethal effect of human serum. J. Immunol. **132**:2046–2052.

10. **Hoffman, E. M.** 1969. Inhibition of complement by a substance isolated from human erythrocytes. I. Extraction from human erythrocyte stroma. Immunochemistry **6**:391–404.
11. **Huldt, G., P. Davies, A. C. Allison, and H. U. Schorlemmer.** 1979. Interaction between *Entamoeba histolytica* and complement. Nature (London) **277**:214–216.
12. **Kalb, U. F., and R. W. Bernlohr.** 1977. A new spectrophotometric assay for protein in cell extracts. Anal. Biochem. **82**: 362–371.
13. **Kazatchkine, M. D., D. T. Fearon, and K. F. Austen.** 1979. Human alternative complement pathway: membrane-associated sialic acid regulates the competition between B and β1H for cell-bound C3b. J. Immunol. **122**:75–81.
14. **Kipnis, T. L., J. R. David, C. A. Alper, A. Sher, and W. Dias da Silva.** 1981. Enzymatic treatment transforms trypomastigotes of *Trypanosoma cruzi* into activators of the alternative complement pathway and potentiates their uptake by macrophages. Proc. Natl. Acad. Sci. USA **78**:602–608.
15. **Kipnis, T. L., A. U. Krettli, and W. Dias da Silva.** 1985. Transformation of trypomastigote forms of *Trypanosoma cruzi* into activators of alternative complement pathway by immune IgG fragments. Scand. J. Immunol. **22**:217–226.
16. **Kipnis, T. L., M. Sucupira, and W. Dias da Silva.** 1987. Transformation of *Trypanosoma cruzi* bloodstream forms by immune IgM and its fab fragment into activators of the alternative complement pathway. Braz. J. Med. Biol. Res. **20**:105–114.
17. **Marciano-Cabral, F.** 1988. The biology of *Naegleria* spp. Microbiol. Rev. **52**:114–133.
18. **Marciano-Cabral, F. M., M. Cline, and S. G. Bradley.** 1987. Specificity of antibodies from human sera for species of the amoeboflagellate *Naegleria*. J. Clin. Microbiol. **25**:692–697.
19. **Marciano-Cabral, F. M., M. Patterson, D. T. John, and S. G. Bradley.** 1982. Cytopathogenicity of *Naegleria fowleri* and *Naegleria gruberi* for established mammalian cell cultures. J. Parasitol. **68**:1110–1116.
20. **Marciano-Cabral, F., S. Stanitski, V. Radhakrishna, and S. G. Bradley.** 1987. Characterization of a neutral aminoacyl-peptidase hydrolase from *Naegleria fowleri*. J. Protozool. **34**:146–149.
21. **Medof, M. E., T. Kinoshita, and V. Nussenzweig.** 1984. Inhibition of complement activation on the surface of cells after incorporation of decay-accelerating factor into their membranes. J. Exp. Med. **160**:1558–1578.
22. **Meri, S., G. Richaud, and E. Linder.** 1985. Complement activation by antigenic fractions of *Entamoeba histolytica*. Parasite Immunol. **7**:153–164.
23. **Morgan, B. P., J. R. Dankert, and A. F. Esser.** 1987. Recovery of human neutrophils from complement attack: removal of the membrane attack complex by endocytosis and exocytosis. J. Immunol. **138**:246–253.
24. **Mosser, D. M., and P. J. Edelson.** 1985. The mouse macrophage receptor for C3bi (CR3) is a major mechanism in the phagocytosis of *Leishmania* promastigotes. J. Immunol. **135**:2785–2789.
25. **Nicholson-Weller, A., J. Burge, D. T. Fearon, P. F. Weller, and K. F. Austen.** 1982. Isolation of a human erythrocyte membrane glycoprotein with decay-accelerating activity for C3 convertases of the complement system. J. Immunol. **129**:184–189.
26. **Ortiz-Ortiz, L., R. Capin, N. R. Capin, B. Sepulveda, and G. Zamacona.** 1978. Activation of the alternative pathway of complement by *Entamoeba histolytica*. Clin. Exp. Immunol. **34**: 10–18.
27. **Pangburn, M. K., and H. J. Muller-Eberhard.** 1978. Complement C3 convertase cell surface restriction of β1H control and generation of restriction on neuraminidase-treated cells. Proc. Natl. Acad. Sci. USA **75**:2416–2420.
28. **Reed, S. L., J. G. Curd, I. Gigli, F. D. Gillin, and A. I. Braude.** 1986. Activation of complement by pathogenic and nonpathogenic *Entamoeba histolytica*. J. Immunol. **136**:2265–2270.
29. **Rimoldi, M. T., A. Sher, S. Heiny, A. Lituchy, C. H. Hammer, and K. Joiner.** 1988. Developmentally regulated expression by *Trypanosoma cruzi* of molecules that accelerate the decay of complement C3 convertase. Proc. Natl. Acad. Sci. USA **85**: 193–197.
30. **Schenkein, H. A., and S. Ruddy.** 1981. The role of immunoglobulins in alternative complement pathway activation by zymosan. I. Human IgG with specificity for zymosan enhances alternative pathway activation by zymosan. J. Immunol. **126**: 7–10.
31. **Schonermark, S., E. W. Rauterberg, M. L. Shin, S. Loke, D. Roelcke, and G. M. Hansch.** 1986. Homologous species restriction in lysis of human erythrocytes: a membrane-derived protein with C8-binding capacity functions as an inhibitor. J. Immunol. **136**:1772–1776.
32. **Schuster, F. L.** 1969. Intranuclear virus-like bodies in the amoeboflagellate *Naegleria gruberi*. J. Protozool. **16**:724–727.
33. **Whiteman, L. Y., and F. Marciano-Cabral.** 1987. Susceptibility of pathogenic and nonpathogenic *Naegleria* spp. to complement-mediated lysis. Infect. Immun. **55**:2442–2447.