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# Alterations in Protein Expression and Complement Resistance of Pathogenic *Naegleria* Amoebae

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**Highly pathogenic strains of *Naegleria fowleri* activate the alternative complement pathway but are resistant to lysis. In contrast, weakly pathogenic and nonpathogenic *Naegleria* spp. activate the complement pathway and are readily lysed. The present study was undertaken to determine whether surface components on amoebae accounted for resistance to complement lysis. Enzymatic removal of surface components from highly pathogenic *N. fowleri* with phosphatidylinositol-specific phospholipase C or with endoglycosidase H increased the susceptibility of these amoebae to complement-mediated lysis. Similar treatment of nonpathogenic amoebae had no effect on susceptibility to complement. Tunicamycin treatment of highly and weakly pathogenic *N. fowleri* increased susceptibility to lysis by complement in a dose-related manner. Tunicamycin treatment did not alter the susceptibility of nonpathogenic amoebae to complement. Proteins of 234 and 47 kDa were detected in supernatant fluid from phosphatidylinositol-specific phospholipase C-treated highly pathogenic amoebae but not in supernatant fluid from phosphatidylinositol-specific phospholipase C-treated weakly pathogenic amoebae. Electrophoretic analysis of iodinated surface proteins of highly pathogenic *N. fowleri* revealed species of 89, 60, 44, and 28 kDa. Western immunoblots of lysates from surface-iodinated amoebae were stained with biotinylated concanavalin A or biotinylated *Ulex europaeus* agglutinin I. Surface proteins, identified in highly pathogenic amoebae by iodination, were shown to be glycoproteins by lectin analysis specific for the detection of mannose and fucose residues.**

The genus *Naegleria* is composed of a distinct group of amoeboflagellates that include nonpathogenic species and species with pathogenic potential. One species, *Naegleria fowleri*, is the causative agent of primary amoebic meningoencephalitis, a fatal disease of the central nervous system in humans and in experiment animals (3, 5, 6). The pathogenesis of this disease is poorly understood. Both weakly pathogenic and highly pathogenic strains of *N. fowleri* are known to exist. The ability to survive and grow at temperatures of 37°C and above does not appear to be a determinative factor in pathogenesis, since thermophilic nonpathogenic species such as *Naegleria lovaniensis* have been described (38).

Hematogenous spread of *N. fowleri* amoebae has not been observed in human cases and is believed to be due to amoebicidal factors present in serum (3). Mice infected intranasally with the amoebae develop a rapidly fatal disease resembling primary amoebic meningoencephalitis in humans. The mouse model has been used extensively to study host resistance to *N. fowleri* (26, 35). Susceptibility to *N. fowleri* infection varies greatly among mouse species. The most susceptible mouse model of primary amoebic meningoencephalitis is the C5 complement-deficient strain A/HeCr (11). Humoral and cell-mediated immunity are not the major lines of defense against *Naegleria* infections (35). Experimental evidence suggests that complement is an important factor in host defense to infection, since complement-deficient mice or mice treated with cobra venom factor to deplete complement are more susceptible than normal mice to *N. fowleri* infections (12, 35).

The pathogenicity of *Naegleria* amoebae in vivo correlates with resistance to complement-mediated lysis in vitro (39). Weakly pathogenic strains of *N. fowleri* and nonpathogenic *Naegleria* spp. activate the alternative complement pathway and are readily lysed by human and guinea pig complement (17, 39, 40). In contrast, a highly pathogenic strain of *N. fowleri* activates the complement pathway but is resistant to lysis (39). Thus, the ability of pathogenic amoebae to escape complement lysis may be an important virulence factor in the pathogenesis of primary amoebic meningoencephalitis. The naeglerial components that activate complement but allow highly pathogenic *N. fowleri* to escape complement-mediated lysis are unknown. Enzymatic removal of surface components from amoebae with trypsin or papain, but no sialidase, converts complement-resistant amoebae to complement-sensitive organisms, indicating that membrane surface proteins play a role in resistance to complement lysis (40).

A number of glycoproteins possessing glycosyl-phosphatidylinositol (GPI) anchors play a significant role in regulating complement-mediated lysis of cells (7, 13, 21, 28). Some protozoa and helminths such as *Trypanosoma cruzi* and *Schistosoma mansoni* have on their surfaces GPI-anchored glycoproteins that regulate the complement pathway (19, 34, 36). In the present study, we investigated the role of surface glycoproteins in resistance of highly pathogenic *N. fowleri* amoebae to complement-mediated lysis through the use of glycoprotein-specific inhibitors, lectin analysis, and endo-β-*N*-acetylglucosaminidase H (endo H) treatment. In addition, we utilized specific enzymatic treatment and surface iodination to examine and compare surface proteins of pathogenic strains of *N. fowleri* to characterize specific components that may play a role in the resistance of pathogenic amoebae to complement-mediated lysis.

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## MATERIALS AND METHODS

**Amoebae.** *N. fowleri* LEE (ATCC 30894), a strain that is weakly pathogenic in mice, was cultured axenically at 37°C in 75-$mm^2$ plastic flasks (Thomas Scientific, Swedesboro, N.J.) in Cline medium, which consists of equal parts of Nelson medium and Balamuth medium (1, 4, 25). *N. fowleri* LEEmpC1, a highly pathogenic strain, was obtained by serially passaging the LEE strain intranasally through $B_6C_3F_1$ mice. After 4 days of exposure to the amoebae, samples of brain from infected mice containing strain LEEmp were cultured axenically at 37°C for not more than 1 month before another mouse passage. After 75 consecutive mouse passages at monthly intervals, the amoebae were cloned by serial dilution in microtiter well plates to obtain one amoeba per well. Wells containing one amoeba were grown to confluency, and serial dilutions of the cultures were continued for 20 growth cycles. The highly pathogenic strain *N. fowleri* LEEmp, which consisted of a homogeneous population, was termed LEEmpC1 (LEE mouse passage clone 1). The strain used in these studies has been passaged through mice a minimum of 90 times. *N. gruberi* $EG_B$, a nonpathogenic soil isolate, was grown in Cline medium at 30°C (37).

**Complement source.** Normal guinea pig complement (NGPC) was purchased from GIBCO Laboratories (Grand Island, N.Y.), dispensed into vials, and stored at −70°C.

**Amoebicidal assay.** Log-phase cultures of *Naegleria* amoebae grown in Cline medium were labeled for 24 h with 50 μCi of [$^3$H]uridine (Dupont, NEN Research Products, Boston, Mass.) at 37°C for both strains of *N. fowleri* or at 30°C for *Naegleria gruberi*. Amoebae were harvested by centrifugation, washed three times in Hanks' balanced salt solution (HBSS) to remove unincorporated [$^3$H]uridine label, and suspended in gelatin-Veronal buffer ($GVB^{2+}$). Amoebae were counted with a hemacytometer and adjusted to a cell density of $10^6$ amoebae per ml. NGPC was diluted with $GVB^{2+}$ in 96-well microtiter plates (Thomas Scientific) and mixed with $10^5$ [$^3$H]uridine-labeled amoebae for 1 h at 37°C. After incubation, the supernatant fluid was harvested and the counts per minute were determined. The percent specific release of radiolabel from the amoebae was determined and used as an index of lysis (39). All data were analyzed statistically by using the two-tailed Student *t* test.

**Enzymatic treatments.** Phosphatidylinositol-specific phospholipase C (PIPLC) purified from *Bacillus thuringiensis* was purchased from ICN Biochemicals (Cleveland, Ohio). Log-phase cultures of pathogenic and nonpathogenic *Naegleria* spp. were radiolabeled with [$^3$H]uridine for 24 h at 37 and 30°C, respectively. Trophozoites were harvested by centrifugation, washed free of excess radiolabel, and adjusted to a cell density of $10^6$ amoebae per ml. Aliquots of diluted amoebae were placed in 1.5-ml polypropylene microfuge tubes (American Scientific, Columbia, Md.) and treated with 500 mU of PIPLC for 1 h at 37°C with gentle agitation. After the enzymatic treatment, PIPLC-treated amoebae were removed and washed free of PIPLC. Radiolabeled PIPLC-treated or untreated amoebae were then incubated with NGPC in 96-well microtiter plates, and the percent specific release of radiolabel was determined by the amoebicidal assay. In addition, supernatant fluids from PIPLC-treated *Naegleria* spp. and from untreated amoebae were obtained and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (20) and then Western immunoblot analysis. [$^3$H]uridine-labeled *Naegleria* spp. ($10^5$ amoebae) were incubated with 40 mU of endo H from *Streptomyces lividans* (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) diluted in a mixture of phosphate-buffered saline and HBSS (pH 7.0) for 4 h at 37°C. After incubation, the enzyme was removed and amoebae were washed twice with the phosphate-buffered saline–HBSS mixture, suspended in $GVB^{2+}$, and incubated with NGPC for 1 h at 37°C. The percent specific release of radiolabel was determined by the amoebicidal assay.

**Tunicamycin treatments.** Log-phase cultures of both strains of pathogenic *N. fowleri* and nonpathogenic *N. gruberi* amoebae were grown in [$^3$H]uridine-containing Cline medium in the absence or presence of 2.0 or 3.0 μg of tunicamycin (Sigma Chemical Co., St. Louis, Mo.) per ml for 18 h at 37 or 30°C. After incubation, amoebae were harvested and washed in HBSS to remove excess tunicamycin. Amoebae were counted, and $10^5$ amoebae were incubated in NGPC for 1 h at 37°C. Parallel cultures of amoebae were grown in Cline medium in the absence or presence of tunicamycin for 18 h; then the drug was removed, and the amoebae were incubated in tunicamycin-free growth medium for 24 h. Amoebae were harvested as described above and incubated in NGPC for 1 h at 37°C. The percent specific release of radiolabel from the amoebae was determined.

**Determination of protein synthesis inhibition.** *Naegleria* spp. were radiolabeled with 30 μCi of [$^{35}$S]methionine (Dupont, NEN) per ml in Cline medium. Labeling of cells with [$^{35}$S]methionine in the presence of tunicamycin was performed either immediately or after appropriate periods of preincubation with the drug. After radiolabeling, the amoebae were washed twice with cold HBSS and subjected to lysis with a cocktail of 2 mM Tris, 100 mM sodium chloride, 2% (vol/vol) Triton X-100, 0.5% (wt/vol) sodium deoxycholate, and 4 mM sodium azide containing the protease inhibitors 0.2 U/ml aprotinin, 0.1 mM phenylmethylsulfphonyl fluoride, and 5 mM iodoacetamide (Sigma). Incorporation of [$^{35}$S]methionine into newly synthesized proteins was determined by precipitation with 5% (vol/vol) trichloroacetic acid at 0°C. The precipitates were collected onto 0.45-μm-pore-size nitrocellulose filters (Millipore Corp., Bedford, Mass.), washed with 10 ml of 5% trichloroacetic acid containing 2 mg of methionine per ml, dried, and counted in 4 ml of Beckman Ready Protein scintillation cocktail (Beckman Instruments Inc., Fullerton, Calif.). The protein concentration of the pellets was determined by a modified Bradford protein microassay (2). The specific activity (counts per minute per microgram of protein) was determined by using the formula (counts per minute of trichloroacetic acid-precipitated protein per microliter)/(microgram of protein/microliter of sample). Precipitates were analyzed by SDS-PAGE, and the polypeptide bands were detected by staining with Coomassie brilliant blue R-250 (Bio-Rad, Rockville Centre, N.Y.) at a final concentration of 0.125%. After staining, the gels were dried on Whatman paper (Bio-Rad) under vacuum and heat and then exposed at −70°C to RP X-Omat XRP-5 diagnostic film (Eastman Kodak Co., Rochester, N.Y.) for 14 days.

**Surface iodination of *Naegleria* amoebae.** Sterile glass vials were coated with 100 μg of 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril (iodogen; Pierce Chemical Co., Rockford, Ill.) by dissolving iodogen in chloroform and drying under a stream of $N_2$. Vials were stored dessicated at 4°C. Amoebae were washed in HBSS to remove the bovine serum albumin present in the amoeba growth medium. Amoebae were harvested by centrifugation and incubated at room temperature for 15 min in iodogen-coated vials in the presence or absence of 500 μCi of $Na^{125}I$ with intermittent swirling. After incubation, amoebae were washed in cold HBSS

containing 5 mM KI and subjected to lysis with a cocktail of Triton X-100 (Sigma), sodium deoxycholate (Sigma), and protease inhibitors as described above for amoebae labeled with [$^{35}$S]methionine. Incorporation of $^{125}$I into protein was determined by precipitation with a mixture of 5% TCA containing 0.3 mg of KI per ml and 150 mM NaCl containing 0.5% fetal calf serum at 0°C. Precipitates were pelleted by centrifugation, and the counts per minute present in the supernatant fluids and pellets were quantitated with a gamma counter. The concentration of protein in the pellets was determined as described above. Samples of the precipitated protein were analyzed by SDS-PAGE and Coomassie brilliant blue staining. The gels were dried on Whatman paper under vacuum and heat and exposed to RP X-Omat XRP-5 diagnostic film at room temperature for 28 h.

**Trypan blue exclusion.** The viability of amoebae after surface iodination was assessed by mixing equal volumes of amoebae with a 4% trypan blue (Sigma) solution after iodination in iodogen-coated vials. The amoebae were examined for exclusion of dye by using a hemacytometer and phase-contrast microscopy. The ratio of live to dead amoebae was determined, and the percent viability was calculated.

**Biotinylated lectin analysis.** Lysates of amoebae subjected to surface iodination were used also, for glycoprotein determination. Amoebic lysates (25 μg) were separated by SDS-PAGE, and proteins were transferred to nitrocellulose. Glycoproteins were detected by using VECTASTAIN ABC reagents (Vector Laboratories, Burlingame, Calif.). Nitrocellulose membranes were incubated in Tris-buffered saline (pH 7.5) containing 0.1% Tween-20 (Sigma) (TTBS) for 30 min. Nitrocellulose membranes were incubated for 45 min with 20 μg of either biotinylated concanavalin A or biotinylated *Ulex europaeus* agglutinin I per ml suspended in TTBS with gentle agitation. After incubation, the membranes were washed three times with TTBS and incubated for 30 min in horseradish peroxidase-conjugated avidin D (10 μg/ml). Membranes were washed as before and developed with a substrate solution containing diaminobenzidine-hydrochloric acid, hydrogen peroxide, and nickel chloride (Sigma). After development, the nitrocellulose blots were subjected to autoradiography at −70°C for 4 days.

## RESULTS

Three *Naegleria* strains were treated with either PIPLC or endo H. An in vitro lytic assay was used to confirm that enzymatic removal of surface-associated membrane components from complement-resistant amoebae enhanced their susceptibility to complement-mediated lysis. Treatment of complement-resistant *N. fowleri* LEEmpC1 with PIPLC or endo H increased the susceptibility of highly pathogenic amoebae to lysis by complement. In contrast, neither enzymatic treatment had a significant effect on the susceptibility of weakly pathogenic or nonpathogenic *Naegleria* amoebae to the lytic effects of complement (Table 1). Supernatant fluid from PIPLC-treated amoebae was collected and subjected to SDS-PAGE and then Western immunoblot analysis. Proteins transferred to nitrocellulose were detected with polyclonal rabbit antiserum prepared against freeze-thawed extracts of *N. fowleri* LEEmpC1 and peroxidase-conjugated goat anti-rabbit immunoglobulin G. Polypeptides from LEEmpC1 cleaved by PIPLC treatment (Fig. 1, lane 2) were compared with those released by PIPLC treatment from *N. fowleri* LEE (Fig. 1, lane 3). Proteins with relative molecular masses of 234 and 47 kDa were detected in supernatant fluid from PIPLC-treated LEEmpC1; these proteins were not detected in supernatant fluid from untreated LEEmpC1 amoebae or from untreated or PIPLC-treated LEE amoebae.

TABLE 1. Effect of enzymatic treatment on lysis of *Naegleria* amoebae

| Treatment | % Specific release[a] | | |
|---|---|---|---|
| | *N. fowleri* LEEmpC1 | *N. fowleri* LEE | *N. gruberi* $EG_B$ |
| None | 7.4 ± 1.6 | 65.8 ± 0.2 | 84.4 ± 0.7 |
| PIPLC[b] | 14.1 ± 0.6[c] | 58.0 ± 1.2 | 83.0 ± 0.2 |
| None | 9.4 ± 4.4 | 52.3 ± 9.7 | 83.6 ± 1.2 |
| Endo H[d] | 39.1 ± 9.7[e] | 63.6 ± 7.6[e] | 79.2 ± 0.5 |

[a] [$^3$H]uridine-labeled *Naegleria* organisms were incubated with NGPC (1:2). Each value represents the percent specific release of radiolabel ± the standard error of the mean from a representative experiment. Similar data were obtained each time the full experiment was repeated.
[b] Treatment of $10^6$ amoebae with 500 mU of PIPLC per ml for 1 h at 37°C.
[c] $P < 0.05$ versus untreated amoebae for triplicate determinations from a single representative experiment.
[d] Treatment of $10^5$ amoebae with 40 mU of endo H for 4 h at 37°C.
[e] $P < 0.2$ versus untreated amoebae for duplicate determinations from a single representative experiment.

To assess the role of glycoproteins in resistance of *N. fowleri* to complement-mediated lysis, *Naegleria* amoebae were grown in Cline medium containing various concentrations of tunicamycin for 18 h before NGPC was added. The susceptibility of highly pathogenic *N. fowleri* LEEmpC1 and weakly pathogenic *N. fowleri* LEE amoebae to lysis by complement increased in a dose-related manner after growth in medium containing 2.0 or 3.0 μg of tunicamycin per ml. In contrast, growth of complement-sensitive, nonpathogenic *N. gruberi* amoebae in the presence of tunicamycin did not alter the susceptibility of the amoebae to complement-mediated lysis (Table 2). To ensure that tunicamycin was not

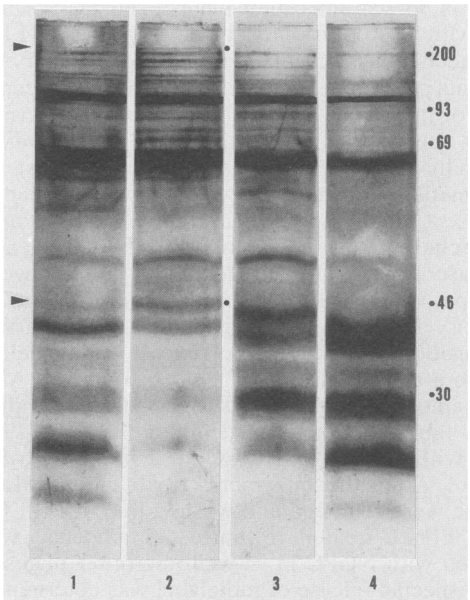


FIG. 1. Western immunoblots of supernatant fluid harvested from *N. fowleri* amoebae. Lanes: 1, LEEmpC1 amoebae incubated in HBSS alone; 2, LEEmpC1 amoebae treated with PIPLC; 3, LEE amoebae treated with PIPLC; 4, LEE amoebae incubated in HBSS alone. Arrows indicate the unique or overexpressed peptides in highly pathogenic LEEmpC1 amoebae.

TABLE 2. Effect of tunicamycin on the susceptibility of *Naegleria* amoebae to complement-mediated lysis

| Pretreatment of amoebae[a] | % Specific release[b] | | |
|---|---|---|---|
| | *N. fowleri* LEEmpC1 | *N. fowleri* LEE | *N. gruberi* $EG_B$ |
| None | 4.7 ± 0.4 | 34.7 ± 1.6 | 84.4 ± 0.9 |
| Tunicamycin (2 μg/ml) | 21.4 ± 2.0[c] | 63.1 ± 0.9[d] | 83.2 ± 0.3 |
| Tunicamycin (3 μg/ml) | 33.6 ± 2.2[c] | 64.3 ± 0.8[d] | 82.9 ± 0.8 |

[a] *Naegleria* spp. were grown in the presence of 2 or 3 μg of tunicamycin per ml for 18 h at 37°C (*N. fowleri*) or at 30°C (*N. gruberi*) before the assay.
[b] [$^3$H]uridine-labeled *Naegleria* spp. were incubated with NGPC (1:2). Each value represents the percent specific release of radiolabel ± the standard error of the mean from a representative experiment.
[c] $P < 0.01$ versus untreated amoebae for triplicate determinations from a single representative experiment.
[d] $P < 0.001$ versus untreated amoebae for triplicate determinations from a single representative experiment.

inhibiting protein synthesis, *Naegleria* spp. were radiolabeled with [$^{35}$S]methionine in the presence or absence of tunicamycin. Inhibition of protein synthesis was determined by comparing the amount of [$^{35}$S]methionine incorporated into tunicamycin-treated amoebae with the amount incorporated into untreated control amoebae. Treatment of amoebae with concentrations of 2 or 3 μg of tunicamycin per ml for 18 h resulted in less than 6% inhibition of protein synthesis in *N. fowleri* LEEmpC1 or LEE amoebae relative to that in untreated control amoebae. Treatment of *N. fowleri* amoebae with a higher concentration (4 μg/ml) of tunicamycin or increased incubation periods with the drug inhibited protein synthesis by 24%. In comparison, nonpathogenic *N. gruberi* amoebae were more sensitive to tunicamycin treatment. A minimal inhibition of protein synthesis (13%) occurred in the presence of 3 μg of the drug per ml. Higher concentrations of tunicamycin were toxic to nonpathogenic *Naegleria* amoebae.

To determine whether the effect of tunicamycin is reversible, [$^3$H]uridine-labeled LEEmpC1 and LEE amoebae were treated with tunicamycin for 18 h and then incubated in tunicamycin-free medium for 24 h. This resulted in an increased ability to resist complement-mediated damage relative to that of untreated control amoebae (Table 3).

TABLE 3. Effect of tunicamycin treatment followed by removal of the drug on the susceptibility of *Naegleria* amoebae to complement-mediated lysis

| Pretreatment of amoebae[a] | % Specific release[b] | | |
|---|---|---|---|
| | *N. fowleri* LEEmpC1 | *N. fowleri* LEE | *N. gruberi* $EG_B$ |
| None | 15.0 ± 2.0 | 40.0 ± 2.4 | 86.3 ± 0.3 |
| Tunicamycin | 53.9 ± 1.3[c] | 68.5 ± 0.7[d] | 84.2 ± 0.5 |
| Tunicamycin + removal | 0.3 ± 0.1 | 13.9 ± 1.0 | 86.2 ± 0.3 |

[a] *Naegleria* amoebae were grown in the presence of 3 μg of tunicamycin per ml for 18 h at 37°C or 30°C before the assay. Parallel cultures were treated with 3 μg of tunicamycin per ml for 18 h; then the drug was removed, and amoebae were incubated in tunicamycin-free medium for an additional 24 h before the assay.
[b] [$^3$H]uridine-labeled *Naegleria* amoebae were incubated with NGPC (1:2). Each value represents the percent specific release of radiolabel ± the standard error of the mean from a representative experiment.
[c] $P < 0.001$ versus untreated amoebae for triplicate determinations from a single representative experiment.
[d] $P < 0.01$ versus untreated amoebae for triplicate determinations from a single representative experiment.

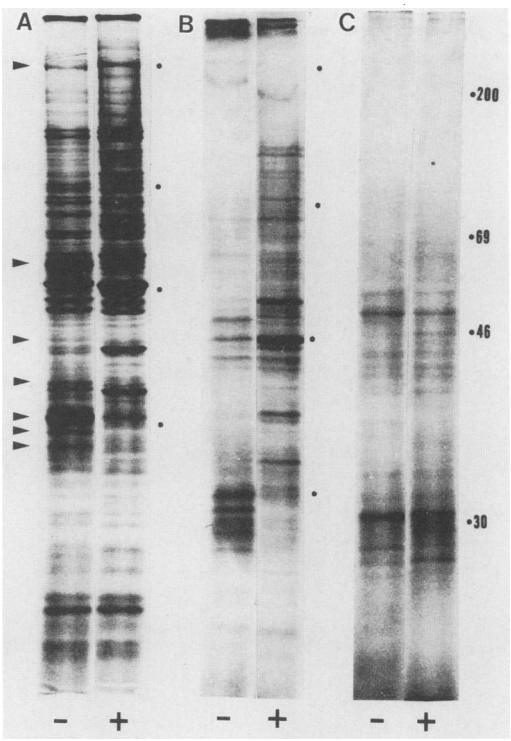


FIG. 2. Effect of tunicamycin on newly synthesized *Naegleria*-specific proteins. Autoradiographs of SDS-PAGE gels containing 25 μg of [$^{35}$S]methionine-labeled proteins of *N. fowleri* LEEmpC1 (A), *N. fowleri* LEE (B), and *N. gruberi* $EG_B$ (C) amoebae were incubated in the presence (+) or absence (−) of 3 μg of tunicamycin per ml for 18 h at 37°C (*N. fowleri*) or 30°C (*N. gruberi*). Arrows indicated proteins showing altered expression after tunicamycin treatment.

Treatment with tunicamycin and removal of the drug had no effect on the susceptibility of nonpathogenic *N. gruberi* amoebae to complement.

To examine the effect of tunicamycin treatment on protein expression in pathogenic and nonpathogenic amoebae, we employed [$^{35}$S]methionine metabolic labeling. Amoebae were radiolabeled in the presence or absence of 3 μg of tunicamycin per ml. Cell lysates were prepared and analyzed by SDS-PAGE and autoradiography. The autoradiograms of both strains of *N. fowleri* amoebae (LEEmpC1 and LEE) treated with tunicamycin in the presence of [$^{35}$S]methionine demonstrate increases in the quantity or concentration of de novo-synthesized proteins after tunicamycin treatment relative to that of untreated control amoebae (Fig. 2A and B). In contrast, an increased concentration of de novo-synthesized proteins was not detected in nonpathogenic *N. gruberi* amoebae treated with tunicamycin (Fig. 2C). In addition to an accumulation of proteins within the amoebae, the synthesis of several glycoproteins with relative molecular masses ranging from 18 to 73 kDa in pathogenic LEEmpC1 and LEE amoebae was decreased or altered after tunicamycin treatment (Fig. 2). In particular, the synthesis of proteins of 182, 50, 39, 34, 32, 30, and 29 kDa was decreased or altered in both LEEmpC1 and LEE amoebae.

Autoradiograms of iodinated amoeba surface proteins from the highly pathogenic *N. fowleri* LEEmpC1 and weakly pathogenic LEE detected proteins of similar molecular masses in the two strains. Proteins of 89, 60, 44, and 28 kDa were identified in greater quantity in the highly pathogenic

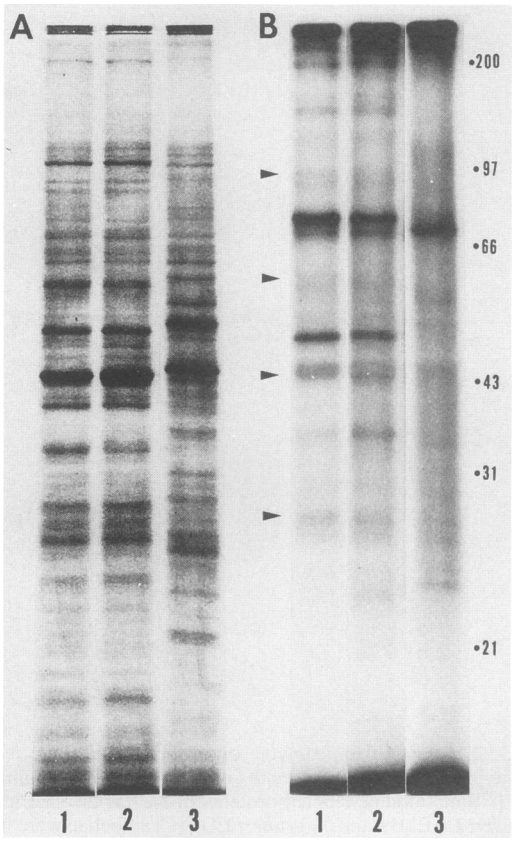


FIG. 3. Autoradiographic patterns of $^{125}$I-labeled cell surface proteins of pathogenic and nonpathogenic *Naegleria* spp. separated by SDS-PAGE. Iodinated cell surface proteins of *N. fowleri* LEEmpC1, *N. fowleri* LEE, and *N. gruberi* $EG_B$ amoebae were separated by SDS-PAGE, and bands were identified by Coomassie blue staining (A). Gels were dried and exposed to film for 28 h (B). Lanes; 1, LEEmpC1; 2, LEE; 3, $EG_B$. Arrows indicate differences between the proteins of LEEmpC1 amoebae and those of LEE amoebae.

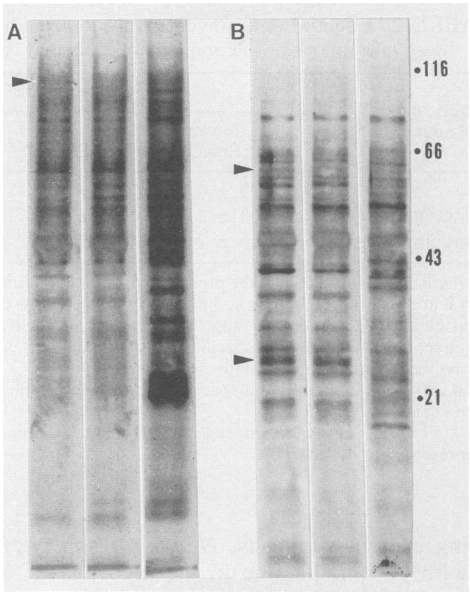


FIG. 4. Biotinylated lectin stain of lysates from surface-iodinated *Naegleria* spp. Lysates of surface-iodinated amoebae were separated by SDS-PAGE and transferred to nitrocellulose. Bands were identified by using biotinylated concanavalin A (A) or biotinylated *U. europaeus* agglutinin I (B). Lanes: 1, LEEmpC1; 2, LEE; 3, $EG_B$. Arrows indicate lectin staining of bands corresponding to surface-iodinated proteins identified in highly pathogenic LEEmpC1 amoebae.

LEEmpC1 strain of *N. fowleri* (Fig. 3). These proteins were either absent or detected to a lesser extent by surface iodination in the weakly pathogenic LEE strain. Few similarities in surface-iodinated proteins from pathogenic and nonpathogenic amoebae were detected. Trypan blue exclusion and phase-contrast microscopy were used to determine whether amoebae were viable after iodination. Greater than 93% of the amoebae were viable as determined by the exclusion of dye.

To further characterize the specific nature of these surface proteins, lysates from surface-iodinated amoebae were separated by SDS-PAGE, transferred to nitrocellulose, and stained with biotinylated lectins specific for either mannose (concanavalin A) or fucose (*U. europaeus* agglutinin I). A comparison of duplicate immunoblots stained with the two lectins indicated that *Naegleria* proteins stain more intensely with concanavalin A than with *U. europaeus* agglutinin I (Fig. 4). Although concanavalin A recognized a number of glycoproteins in nonpathogenic *N. gruberi* amoebae, the molecular masses of these glycoproteins differed from those of the glycoproteins recognized by concanavalin A in pathogenic strains of *N. fowleri*. Autoradiograms of the lectin-stained immunoblots indicate that three proteins identified in highly pathogenic LEEmpC1 amoebae by surface iodination are glycoproteins and that different types of sugar residues are associated with the proteins. The 89-kDa surface protein was recognized by concanavalin A, suggesting that mannose residues were associated with this protein. Surface 60- and 28-kDa proteins stained with *U. europaeus* agglutinin I, suggesting the presence of fucose residues. The arrows in Fig. 4 indicate the bands stained by each lectin; these bands correspond to those detected by surface iodination in greater quantities in highly pathogenic LEEmpC1 amoebae.

## DISCUSSION

The membrane attack complex of the complement system comprises terminal complement components C5b through C9, which damage cell surface membranes by the formation of transmembrane channels or pores within the membrane, ultimately resulting in osmotic lysis of the cell (29, 30). A number of microorganisms and eucaryotic cells have developed mechanisms to evade complement lysis. Regulatory proteins present on the surface of cells interfere either by preventing activation of the complement pathway or by preventing lysis of the cell once complement has been activated (18, 29).

We showed previously that enzymatic treatment to remove surface components from highly pathogenic *N. fowleri* amoebae with papain or trypsin, but not sialidase, increases the susceptibility of complement-resistant amoebae to complement-mediated lysis. Enzymatic treatment to remove surface polypeptides from nonpathogenic or weakly pathogenic amoebae had no effect on complement-mediated lysis, indicating that surface components of highly pathogenic amoebae play a role in preventing complement-mediated lysis (40). To determine whether the membrane components responsible for conferring resistance to complement lysis are

GPI-anchored proteins or glycoproteins, enzymes with specific cleavage sites were utilized. Both PIPLC, which removes GPI-anchored proteins, and endo H, which removes high-mannose residues present on glycoproteins, increased the susceptibility of resistant amoebae to complement-mediated lysis, but neither significantly effected complement-mediated lysis of weakly pathogenic or nonpathogenic strains of *Naegleria* spp. The increased susceptibility after PIPLC treatment of highly pathogenic *Naegleria* suggests that *N. fowleri* amoebae have in their membranes GPI-anchored proteins that may play a role in regulating the complement pathway. GPI-anchored proteins that confer resistance to complement lysis include decay-accelerating factor (DAF) (8, 28), homologous restriction factor or C8-binding protein (13), and CD59 (7, 32). The slight increase in susceptibility of *Naegleria* amoebae to complement after PIPLC treatment may be due to the fact that many GPI-anchored proteins are relatively resistant to PIPLC release (9, 21, 22). Two peptides that were cleaved by PIPLC treatment were identified by Western immunoblotting. Weakly pathogenic amoebae may have the same proteins but to a lesser extent. Increased concentrations of PIPLC enzyme or prolonged treatment with the enzyme can overcome the resistance of certain proteins to release by PIPLC. In the present study, high concentrations of PIPLC or increased treatment periods were found to be toxic to *Naegleria* amoebae (data not shown).

DAF, a regulatory membrane-bound glycoprotein, protects cells from lysis by autologous complement (15, 16, 23). DAF binds to complement cleavage fragments C3b and C4b and either prevents or dissociates classical and alternative pathway C3-C5 convertase formation (15, 16, 27, 31). DAF or a DAF-like protein does not appear to be involved in resistance of *N. fowleri* amoebae to complement-mediated lysis. Earlier studies in our laboratory of electrophoretic patterns of serum after incubation with amoebae demonstrated the conversion of complement components C3 and C5 to C3b and C5b, respectively (40). The generation of C3b and C5b cleavage products indicates that the complement pathway is activated beyond the point of DAF regulation and that DAF alone cannot be responsible for resistance of *N. fowleri* amoebae to complement lysis.

A number of complement-regulatory proteins are membrane-bound glycoproteins. To analyze the role of carbohydrate moieties on membrane glycoproteins, we used endo H, which specifically cleaves N-linked high-mannose residues of glycoproteins. Treatment of highly pathogenic LEEmpC1 amoebae with endo H increased the susceptibility of these amoebae to complement lysis. Treatment of weakly pathogenic or nonpathogenic strains of *Naegleria* did not significantly alter susceptibility to complement-mediated lysis. These data indicate that the proteins involved in resistance to complement-mediated lysis are glycoproteins. Removal of N-linked carbohydrates may affect the ability of amoebae to resist lysis by altering the conformation of specific proteins or by removing a functional portion of the protein that is required for inhibition of complement-mediated lysis.

Tunicamycin, a naturally occurring antibiotic, specifically blocks the formation of N-linked protein-carbohydrate linkages and allows for an analysis of the functional role for specific carbohydrates of glycoproteins. Heterogeneous preparations of tunicamycin can significantly inhibit protein synthesis in addition to inhibiting protein glycosylation at asparagine residues (24). Tunicamycin treatment of *Naegleria* amoebae for 18 h at a concentration that inhibits glycosylation but not protein synthesis increases susceptibility of both highly pathogenic and weakly pathogenic amoebae to complement lysis but has no effect on nonpathogenic *N. gruberi* amoebae. These studies support the hypothesis that glycoproteins are important in the ability of pathogenic amoebae to resist complement-mediated damage. Increased susceptibility to complement lysis after tunicamycin treatment is reversible, since the amoebae completely recover their ability to resist complement damage after removal of the drug and subsequent incubation in tunicamycin-free medium. A slight increase in the ability of drug-treated amoebae to resist complement-mediated damage is detected after removal of the drug. Analysis of de novo-synthesized proteins in the presence of tunicamycin (Fig. 2) indicates an accumulation of proteins within the amoebae after an 18-h treatment with the drug. Carbohydrate residues present on proteins are known to dictate proper protein folding and configuration, which are necessary signals for secretion (10). Glycoproteins that lack carbohydrates may aggregate in the endoplasmic reticulum with subsequent degradation, whereas other proteins may remain unaffected (14, 33). Increased resistance to lysis after removal of the drug may indicate that tunicamycin treatment results in the accumulation of proteins within the amoebae. Removal of tunicamycin allows these proteins to become rapidly processed and shuttled to the surface of the amoebae, resulting in a net increase in the concentration of proteins on the surface of the amoebae and enabling the amoebae to more efficiently resist complement-mediated damage and lysis.

Enzymatic treatment to remove surface components increases the susceptibility of highly pathogenic amoebae to complement, whereas inhibition of protein glycosylation by tunicamycin increases the susceptibility of both highly pathogenic and weakly pathogenic strains of *N. fowleri*. These results indicate that glycoproteins present on both strains may play a role in protecting the amoebae from complement but that resistance may be the result of a quantitative difference in the glycoproteins expressed on the surfaces of the different strains. Such glycoproteins are absent from nonpathogenic amoebae.

To compare surface proteins on complement-resistant amoebae that may be involved in resistance to lysis, we employed surface iodination in combination with autoradiography. The use of this technique with highly pathogenic, complement-resistant amoebae revealed four proteins that were either absent or reduced in quantity in the weakly pathogenic LEE strain. Lectin staining of surface proteins determined that these unique proteins were glycoproteins. The attachment of terminal sugars is a major source of structural diversity for N-linked glycoproteins. Mannose and fucose are known to be typical sugar residues associated with glycoproteins (33). In the present study, lectins specific for the detection of mannose and fucose were used to differentiate the carbohydrate residues associated with these surface proteins. Surface proteins detected in highly pathogenic LEEmpC1 amoebae contain the carbohydrate residues fucose and mannose. Studies are in progress to analyze these specific glycoproteins and determine their role in conferring complement resistance. Few similarities were detected between the surface proteins of pathogenic *N. fowleri* and those of nonpathogenic *N. gruberi* amoebae.

The increased susceptibility of highly pathogenic *N. fowleri* after tunicamycin and endo H treatment confirms the importance of N-linked surface glycoproteins in resistance of highly pathogenic LEEmpC1 amoebae to complement lysis. The glycoproteins that are important in resistance to complement are absent from nonpathogenic amoebae. A

functional association of a particular tunicamycin-sensitive glycoprotein that confers resistance to complement is under investigation. Although these proteins were unique to pathogenic amoebae, further studies are needed to assess their role in the resistance of pathogenic amoebae to complement.

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