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Identification of a *Naegleria fowleri* Membrane Protein Reactive with Anti-Human CD59 Antibody

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***Naegleria fowleri*, the causative agent of primary amebic meningoencephalitis, is resistant to complement lysis. The presence of a complement regulatory protein on the surface of *N. fowleri* was investigated. Southern blot and Northern blot analyses demonstrated hybridization of a radiolabeled cDNA probe for CD59 to genomic DNA and RNA, respectively, from pathogenic *N. fowleri*. An 18-kDa immunoreactive protein was detected on the membrane of *N. fowleri* by Western immunoblot and immunofluorescence analyses with monoclonal antibodies for human CD59. Complement component C9 immunoprecipitated with the *N. fowleri* "CD59-like" protein from amebae incubated with normal human serum. In contrast, a gene or protein similar to CD59 was not detected in nonpathogenic, complement-sensitive *N. gruberi* amebae. Collectively, our studies suggest that a protein reactive with antibodies to human CD59 is present on the surface of *N. fowleri* amebae and may play a role in resistance to lysis by cytolytic proteins.**

Naegleria fowleri, an ameboflagellate found in freshwater lakes and ponds, is the causative agent of primary amebic meningoencephalitis, a rapidly fatal disease of the central nervous system (1, 7). The determinants of virulence for this ameba are unknown, but resistance to complement lysis appears to play a major role in its pathogenicity (13, 30). Both pathogenic and nonpathogenic *Naegleria* species activate the alternative pathway of complement (16). However, pathogenic *N. fowleri* amebae are resistant to the lytic effect of complement (36). Immunoelectrophoretic studies have established that complement components C3 and C5 are converted to C3b and C5b, respectively, following incubation of complement-resistant *N. fowleri* in normal human serum (NHS) (37). These results suggest that complement regulation by *N. fowleri* occurs at the stage of membrane attack complex (MAC) formation.

CD59 is an 18- to 20-kDa glycosyl-phosphatidylinositol/inositol-anchored glycoprotein found on the surface of a variety of cell types which functions to inhibit complete formation of the MAC of complement. CD59 inhibition of complement lysis occurs by binding complement components C8 and C9, ultimately preventing C9 insertion into and polymerization in the cell membrane (9). The purpose of the present study was to determine whether complement-regulatory protein CD59, which is present on mammalian cells, could be detected on pathogenic *N. fowleri* amebae.

Molecular and immunology-based assays were used to test the hypothesis. Southern blot analysis was performed to determine whether *N. fowleri* possesses the CD59 gene. Northern blot analysis was utilized to identify the presence of CD59 transcripts expressed by complement-resistant *N. fowleri* amebae. With monoclonal antibodies to human CD59, an immunoreactive protein was detected in the membrane fraction of *N. fowleri* by Western immunoblot analysis. Immunoprecipitation studies were used to establish whether the ameba "CD59-like" protein was able to associate with human complement component C9. In addition, an anti-CD59 monoclonal antibody was used to detect the presence of a reactive antigen in *N. fowleri* on and near serum-induced membrane vesicles.

Our data demonstrate the presence of a CD59-like protein on the surface of pathogenic *N. fowleri* amebae. More importantly, the ability of a pathogenic species of *Naegleria* to synthesize a protein which protects the amebae from lytic molecules, such as the MAC of complement (C5b-C9), may serve as an important virulence factor.

MATERIALS AND METHODS

Amebae. *N. fowleri* LEE (ATCC 30894) amebae, obtained from the American Type Culture Collection (ATCC), were originally isolated from a patient with a fatal case of primary amebic meningoencephalitis (10). A highly virulent mouse-passaged strain of *N. fowleri* (LEEmp) was obtained by continuous passage of *N. fowleri* (LEE) through 8-week-old female $B_6C_3F_1$ mice at monthly intervals. The mice were used in compliance with current federal regulations. Amebae used in these experiments were cultured in ameba growth medium for no longer than 1 month after removal from mouse brain tissue as described previously (34). The LEEmp (mouse-passaged) strain of *N. fowleri* was maintained in Cline growth medium supplemented with heat-inactivated donor calf serum and hemin at 37°C (8). *N. gruberi* EG_B, a nonpathogenic, complement-sensitive soil isolate, was maintained in Cline medium at 30°C or 37°C (31) and used in selected experiments.

Mammalian cells. The human chronic myelogenous leukemia cell line K562 (ATCC CCL-243) and human red blood cells (hRBCs) known to express CD59 were used as controls (20). K562 cells were cultured at 37°C in an atmosphere of 5% $C0_2$ and maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (BioWhittaker, Walkersville, MD), 1.5% sodium bicarbonate, 25 mM HEPES buffer, 1% L-glutamine, 1% nonessential amino acids, 1% minimal essential medium vitamins, 100 U/ml penicillin, and 100 μg/ml streptomycin. hRBCs lysed in distilled water were used as whole-cell lysates in a Western immunoblot analysis. The protein concentration was determined by performing a Bradford assay (4). Samples were aliquoted and stored at −20°C until used.

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Serum. NHS was used as the source of complement and was stored at −80°C.

Antibodies. Monoclonal antibodies for human CD59 used in Western immunoblot and immunofluorescence analyses included mouse immunoglobulin G1κ (IgG1κ) anti-human CD59 antibody (BRA-10G; Ancell, Bayport, MN), mouse IgG2b anti-human CD59 antibody (BRIC 229; International Blood Group Laboratory, Bristol, United Kingdom), and mouse IgG2a anti-human CD59 antibody (MEM-43; Biomeda, Foster City, CA). Horseradish peroxidase (HRP)-linked rabbit anti-mouse IgG (whole molecule; Sigma, St. Louis, MO) was used as the secondary antibody in a Western immunoblot analysis. Polyclonal goat anti-human C9 antibody (Calbiochem, San Diego, CA) and an HRP-linked rabbit anti-goat antibody were used in a Western immunoblot analysis for immunoprecipitation studies. Fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG (whole molecule) antibody (Cappel, ICN Pharmaceuticals, Aurora, OH) was used in immunofluorescence assays.

cDNA probes. The following human CD59 cDNA, from the myelogenous leukemia cell line K562, was provided by Alfred Bothwell, Yale University School of Medicine, New Haven, CT: GGGGGCTGAG CGCAGAAGCG GC TCGAGGCT GGAAGAGGAT CCTGGGCGCC GCAGGTTCTG TGGACA ATCA CAATGGGAAT CCAAGGAGGG TCTGTCCTGT TCGGGCTGCT GCTCGTCCTG GCTGTCTTCT GCCATTCAGG TCATAGCCTG CAGTG CTACA ACTGTCCTAA CCCAACTGCT GACTGCAAAA CAGCCGTCAA TTGTTCATCT GATTTTGATG CGTGTCTCAT TACCAAAGCT GGGTTA CAAG TGTATAACAA GTGTTGGAAG TTTGAGCATT GCAATTTCAA CGACGTCACA ACCCGCTTGA GGGAAAATGA GCTAACGTAC TACT GCTGCA AGAAGGACCT GTGTAACTTT AACGAACAGC TTGAAA ATGG TGGGACATCC TTATCAGAGA AAACAGTTCT TCTGCTGGTG ACTCCATTTC TGGCAGCAGC CTGGAGCCTT CATCCCTAAG TC (28).

Glyceraldehyde 3-phosphate dehydrogenase cDNA was obtained from Geoffrey Krystal, Virginia Commonwealth University, Richmond (33).

gDNA isolation and Southern blot analysis. To determine whether a human CD59 probe hybridizes to genomic DNA (gDNA) from *Naegleria* species, Southern blot analysis was performed. Genomic DNA was isolated with the Easy-DNA kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Ten micrograms of gDNA was digested with EcoRI, BamHI, or HindIII (Invitrogen) overnight at 37°C, separated by electrophoresis through a 0.8% agarose gel, and transferred to a positively charged nylon membrane (Roche Applied Science, Indianapolis, IN). The DNA was cross-linked to the nylon membrane with a UV Stratalinker (Stratagene, La Jolla, CA). A human CD59 cDNA probe was labeled with the RadPrime DNA labeling system (Invitrogen). The membrane was incubated with the labeled CD59 probe at 60°C in ExpressHyb hybridization solution (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. The membrane was washed two times with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate (SDS) (25°C for 15 min) and two times with 0.5× SSC containing 0.1% SDS (20 min at 50°C). The membrane was exposed to Kodak Biomax film overnight.

Total RNA isolation and Northern blot analysis. Total RNA was isolated from pathogenic and nonpathogenic *Naegleria* amebae and K562 cells with 4 M guanidine isothiocyanate and purified through a 5.7 M CsCl step gradient. Total RNA was electrophoresed at 30 V for 18 h. RNA was transferred to a QiaBRANE 0.45-μm nitrocellulose membrane (QIAGEN Inc., Chatsworth, CA) by capillary transfer. The RNA was cross-linked to the nitrocellulose membrane with a UV Stratalinker (Stratagene, La Jolla, CA). Nitrocellulose membranes containing RNA were incubated in prehybridization buffer containing 0.2% (wt/vol) bovine serum albumin, 0.2% (wt/vol) Ficoll-400, 0.2% (wt/vol) PVP-40, 0.2% (vol/vol) SDS, 0.1 M $NaPO_4$ (pH 6.5), 10× SSC, and 0.5 mg/ml total yeast RNA (Sigma Chemical Co., St. Louis, MO), followed by hybridization for 18 h at 22°C in 1× buffer containing 5% dextran sulfate and 0.4% (vol/vol) formamide containing a nick-translated, ^{32}P-labeled human CD59 cDNA probe. Washed membranes were subjected to autoradiography with Kodak X-AR diagnostic film (Eastman Kodak Co., Rochester, NY). Membranes were washed with 2× SSC containing 0.2% SDS and 0.5× SSC containing 0.2% SDS at various stringencies at temperatures ranging from 37 to 42°C. Membranes were dried and exposed to diagnostic film at −70°C. Membrane-bound probes were removed by incubation at 95°C for 5 min in 0.01× SSC–0.01% SDS, and membranes were rehybridized with ^{32}P-labeled glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA to confirm equivalent loading of RNA in each lane. Hybridization levels of CD59 and GAPDH probes were quantitated with a phosphorimager, and the CD59 hybridization values were normalized against GAPDH.

Preparation of cytosolic and membrane fractions of *N. fowleri* and *N. gruberi* for Western immunoblot analysis. Pathogenic *N. fowleri* and nonpathogenic *N. gruberi* amebae were treated with Hanks balanced salt solution (HBSS) for 60 min at 37°C. Following treatment, amebae were harvested, suspended in 50 mM Tris-HCl (pH 7.4) containing protease inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 2.1 mM pepstatin A, and 1.5 mM leupeptin), and freeze-thawed three times by alternating cycles in liquid nitrogen and 37°C water. In a separate experiment, *N. fowleri* amebae (1.5×10^7) were harvested and incubated in serum-free medium for 3 h at 37°C. Amebae were washed and incubated with a 1:4 dilution of NHS for 10 min at 37°C. At the end of the incubation period, ice-cold HBSS (Mediatech, Herndon, VA) was added and the amebae pelleted by centrifugation. Amebae were suspended in buffer (50 mM HEPES [pH 7.4], 100 mM NaCl, 2 mM EDTA, 2 mM EGTA, 100 mM NaF, 5 mM β-glycerol phosphate, 20 μM calpain inhibitor 1, 10 mM benzamidine, 7×10^4 U/ml trypsin inhibitor, 1 mM PMSF, 0.05 mg/ml leupeptin, 0.03 mg/ml pepstatin A) and lysed by homogenization. Whole-cell lysates from both experiments were subjected to ultracentrifugation at 100,000 × *g* for 1 h at 4°C to generate cytosolic (supernatant) and membrane (pellet) fractions. Protein determinations for all experiments were performed by the Bradford assay (4).

Western immunoblot analysis. Cytosolic and membrane fractions (80 μg) of *N. fowleri* amebae and whole-cell lysates of hRBCs (5 μg) were resolved by 12% nonreducing SDS-polyacrylamide gel electrophoresis (PAGE) as described by Laemmli, with modifications (18). Briefly, a nonreducing sample buffer (400 mM Tris Cl [pH 6.8], 6% [wt/vol] SDS, 20% [vol/vol] glycerol, 2 mM EDTA [pH 6.8], 0.01% [wt/vol] bromphenol blue) was used as described by Braakman and Hebert (3). Separated proteins were transferred to a nitrocellulose membrane overnight. The membrane was incubated in blocking buffer with 5% nonfat dry milk in 1× TBS/T (10 mM Tris [pH 7.6], 50 mM NaCl, 0.1% Tween 20) for 4.5 h at 4°C. The membrane was incubated with CD59 monoclonal antibody BRA-10G diluted 1:50 or BRIC 229 diluted 1:25 overnight at 4°C. The membrane was washed in TBS/T and incubated in an HRP-linked rabbit anti-mouse secondary antibody (Sigma, St. Louis, MO) at 1:15,000 for 1 h at room temperature. The membrane was washed in TBS/T and then in TBS for 10 min at room temperature, and proteins were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom).

Membrane fractions (200 μg) of *N. fowleri* amebae were subjected to two-dimensional gel electrophoresis (2% pH 4 to 8 ampholines–10% acrylamide slab gel) performed by Kendrick Laboratories, Inc. (Madison, WI), according to the method of O'Farrell (25). Resulting Coomassie brilliant blue R-250 (Bio-Rad, Hercules, CA)-stained polyvinylidene difluoride (PVDF) membranes were used in a Western immunoblot analysis in conjunction with CD59 monoclonal antibodies as described above.

Immunoprecipitation of the CD59-like protein. Pathogenic *N. fowleri* amebae (5×10^6) were seeded in 60-mm petri dishes and allowed to adhere for 1 h at 37°C. The amebae were treated with prewarmed HBSS or NHS diluted in HBSS at 1:4 for 3, 5, 10, or 15 min. After each time period, the treatment was removed and the cells washed twice with HBSS. The amebae were lysed in ice-cold RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1× phosphate-buffered saline [PBS]) containing protease and phosphatase inhibitors (100 μg/ml PMSF, 1 μg/ml pepstatin A, 10 μg/ml leupeptin, 100 mM sodium orthovanadate, 100 mM sodium fluoride, and 10 mM sodium pyrophosphate). Cell lysates (350 μg) were precleared with EZview Red protein A affinity gel (Sigma, St. Louis MO) for 2 h at 4°C with gentle rocking. The samples were then centrifuged at 2,300 × *g* for 10 min at 4°C. The lysates were removed and incubated with 2.5 μg of mouse anti-human CD59 (BRA-10G) overnight at 4°C with gentle rocking. The lysates were then incubated with the protein A affinity gel for 1.5 h at 4°C with gentle rocking. The samples were centrifuged at 4,500 × *g* at 4°C for 5 min and the supernatant discarded. RIPA buffer was used to wash the lysates four times. Equal volumes of RIPA buffer and reducing buffer were added to the samples, which were then vortexed and boiled at 95°C for 5 min as described by the manufacturer (Sigma, St. Louis, MO). The lysates were vortexed and centrifuged for 30 s at 8,200 × *g* to pellet the protein A beads. The supernatants were subjected to 10% SDS-PAGE and Western immunoblot analysis in conjunction with polyclonal goat anti-human C9 diluted 1:50 and HRP-linked rabbit anti-goat secondary antibody diluted 1:10,000. Purified human C9 (10 ng; Sigma, St. Louis, MO) was used as the positive control in the Western blot analysis.

Immunofluorescence microscopy. Pathogenic *N. fowleri* amebae were plated on glass coverslips and allowed to adhere for 1 h at 37°C. The amebae were treated with prewarmed NHS diluted 1:4 in HBSS for 15 to 30 min in order to induce membrane vesiculation. The coverslips were rinsed in prewarmed PBS and air dried before fixation with acetone for 5 min. Coverslips were rehydrated in PBS for 10 min and then incubated in Blocker Casein in TBS (Pierce, Rockford, IL) for 1 h at room temperature. Coverslips containing amebae were incubated in mouse anti-human CD59 (MEM-43) monoclonal antibody at 1:5 or in Blocker Casein in TBS (control) for 1 h at room temperature. Coverslips were washed in PBS four times and then incubated in FITC-labeled rabbit anti-mouse IgG secondary antibody at 1:100 for 1 h at room temperature in the dark.

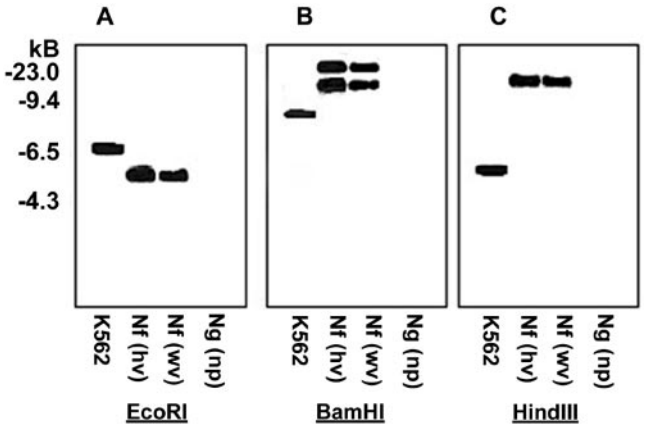

FIG. 1. Southern blot analysis of pathogenic and nonpathogenic *Naegleria* genomic DNAs (10 μg) hybridized with human CD59 cDNA. Genomic DNA was digested overnight with restriction enzyme EcoRI (A), BamHI (B), or HindIII (C); separated by gel electrophoresis; transferred to a nylon membrane; and hybridized with a radiolabeled human CD59 cDNA. Lane 1, K562 erythroleukemic cells; lane 2, highly virulent *N. fowleri* (Nf hv); lane 3, weakly virulent *N. fowleri* (Nf wv); lane 4, nonpathogenic *N. gruberi* (Ng). The human CD59 cDNA probe hybridized with the K562 control and highly virulent and weakly virulent *N. fowleri* but not with nonpathogenic, complement-sensitive *N. gruberi*.

Coverslips were washed with PBS twice and distilled water twice before being mounted on a glass slide with Gel/Mount (Biomeda, Foster City, CA). Results were photographed with Kodak Elite Chrome 100 film.

RESULTS

Detection of the CD59 gene in *N. fowleri*. Southern blot analysis was used to determine whether a gene for CD59 is present in *N. fowleri*. gDNA was digested with restriction enzymes, electrophoresed, transferred to a nylon membrane, and incubated with a radiolabeled CD59 cDNA probe. Restriction enzyme digestion of *N. fowleri* LEE and LEEmp gDNA with EcoRI and HindIII showed a single band when hybridized with human CD59 cDNA (Fig. 1A and C). Restriction enzyme digestion with BamHI showed two bands when hybridized with human CD59 cDNA (Fig. 1B). Hybridization of CD59 cDNA to *N. gruberi* gDNA was not observed.

Detection of a CD59 transcript in *N. fowleri*. Northern blot analysis with a cDNA probe for human CD59 from K562 cells was used to determine whether a CD59 transcript is present in *N. fowleri*. Total RNA extracted from *Naegleria* amebae and the K562 cell control was electrophoresed, transferred to a nitrocellulose membrane, and incubated with a radiolabeled CD59 cDNA probe. The radiolabeled human CD59 cDNA probe hybridized to an approximately 2-kb RNA transcript from the K562 cells and complement-resistant *N. fowleri* LEE and LEEmp. When levels of hybridization were normalized against GAPDH, no significant difference in CD59 expression was observed between weakly and highly virulent *N. fowleri*. CD59 cDNA hybridization to RNA from complement-sensitive *N. gruberi* amebae was not detected (Fig. 2A). Hybridization of the stripped membrane with a GAPDH cDNA probe verified approximately equivalent amounts of RNA in each of the lanes (Fig. 2B).

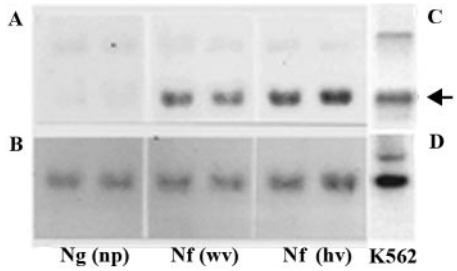

FIG. 2. Northern blot analysis of CD59 expression by pathogenic and nonpathogenic *Naegleria* amebae. Total RNA was isolated from pathogenic and nonpathogenic *Naegleria* amebae (A and B) or K562 cells (C and D) and separated by formaldehyde agarose gel electrophoresis. RNA was transferred to nylon membranes and hybridized with a ^{32}P-radiolabeled human K562-CD59 cDNA probe (A and C) or with a glyceraldehyde 3-phosphate dehydrogenase cDNA probe (B and D). Membranes were washed under low- or mild-stringency conditions and subjected to autoradiography. The arrow denotes a 2-kb RNA transcript detected in *N. fowleri* amebae. Lanes: Ng (np), *N. gruberi* (nonpathogenic); Nf (wv), *N. fowleri* (weakly virulent strain); Nf (hv), *N. fowleri* (highly virulent strain).

Membrane expression of the CD59-like protein in *N. fowleri*. Western immunoblot analysis of *N. fowleri* and *N. gruberi* was performed with a monoclonal antibody specific for the BRIC 229 epitope of human CD59 (Fig. 3A) or the BRA-10G-specific epitope (Fig. 3B) in conjunction with an HRP-linked rabbit anti-mouse secondary antibody. Pathogenic *N. fowleri* amebae were treated with NHS diluted 1:4 or HBSS alone. An immunoreactive protein with an approximate molecular mass of 18 kDa was detected in the membrane fraction of *N. fowleri* by both monoclonal antibodies (Fig. 3). No reactivity was detected with either membrane or cytosolic fractions from *N.*

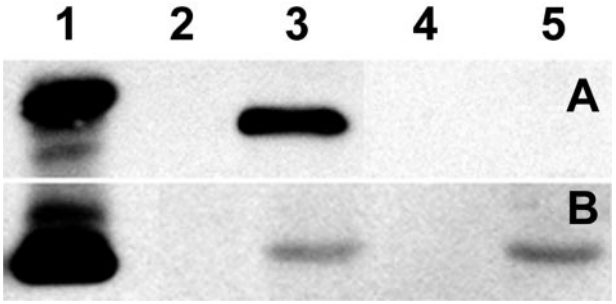

FIG. 3. Western immunoblot analyses of pathogenic and nonpathogenic *Naegleria* species with two CD59 monoclonal antibodies. (A) *N. fowleri* (Nf) and *N. gruberi* (Ng) amebae were treated with HBSS for 60 min. Cytosolic (c) and membrane (m) fractions (80 μg) were separated via 12% nonreducing SDS-PAGE. Western blot analysis was performed with a mouse anti-human CD59 (BRIC 229) monoclonal antibody (1:25) and an HRP-linked rabbit anti-mouse antibody (1:15,000). Lane 1, hRBCs (5 μg); lane 2, Nf plus HBSS, 60 min (c); lane 3, Nf plus HBSS, 60 min (m); lane 4, Ng plus HBSS, 60 min (c); lane 5, Ng plus HBSS, 60 min (m). (B) *N. fowleri* amebae were treated with HBSS or NHS for 10 min. Cytosolic and membrane fractions (80 μg) of *N. fowleri* were resolved by 12% nonreducing SDS-PAGE. Western blot analysis was performed with a mouse anti-human CD59 (BRA-10G) monoclonal antibody (1:50) and an HRP-linked rabbit anti-mouse antibody (1:15,000). Arrows denote 18-kDa protein. Lane 1, hRBCs (5 μg); lane 2, HBSS, 10 min (c); lane 3, HBSS, 10 min (m); lane 4, NHS, 10 min (c); lane 5, NHS, 10 min (m).

gruberi (Fig. 3A). Limited studies were performed to address whether adjusting the culture temperature of *N. gruberi* from 30°C to 37°C would induce expression of an 18-kDa immunoreactive protein. Under the conditions examined, no difference was observed (data not shown).

To confirm that the monoclonal antibodies used in the above analyses reacted with a single protein, the *N. fowleri* membrane fraction was subjected to two-dimensional gel electrophoresis. A Coomassie brilliant blue-stained PVDF membrane demonstrated the presence of numerous proteins in the *N. fowleri* membrane extract (Fig. 4A). Western immunoblot analysis was performed with the BRA-10G and BRIC 229 anti-CD59 antibodies. The results demonstrated reactivity of both antibodies with only one *N. fowleri* membrane protein with an approximate molecular mass of 18 kDa and an approximate isoelectric point (pI) of 8 (Fig. 4B and C, arrows).

Association of the CD59-like protein with human C9. Immunoprecipitation analysis of the CD59-like protein was performed to determine whether the protein was associated with human C9. *N. fowleri* amebae were treated with NHS at 1:4 for 3, 5, 10, or 15 min. After each incubation period, the amebae were washed and lysed. An equal amount of protein from each lysate generated was then used in an immunoprecipitation assay with anti-human CD59 (BRA-10G). The precipitated proteins were separated via 10% reducing SDS-PAGE, and Western immunoblot analysis was performed in conjunction with goat anti-human C9. The results demonstrated immunoreactivity with a 71-kDa protein and the purified human C9 control and in *N. fowleri* samples treated with NHS (Fig. 5). More reactivity was observed in samples generated after 3 and 5 min of treatment with NHS than in samples generated after 10 and 15 min of incubation with NHS, with the greatest reactivity at 5 min and the least at 15 min (Fig. 5). No immunoreactivity was detected with anti-human C9 in the untreated *N. fowleri* control or with *N. fowleri* samples treated with the HBSS diluent alone.

Localization of the CD59-like protein on membrane vesicles in *N. fowleri*. Pathogenic *N. fowleri* amebae were treated with NHS diluted 1:4 to induce membrane vesiculation and fixed with acetone. Immunofluorescence analysis was conducted to confirm localization of the CD59-like protein on the membrane of *N. fowleri* amebae with an anti-CD59 monoclonal antibody (MEM-43) and an FITC-labeled secondary antibody. Amebae incubated in the FITC-labeled secondary antibody alone yielded no areas of immunoreactivity. The expected level of autofluorescence of *N. fowleri* amebae was observed (Fig. 6A). Clusters of immunoreactivity were observed on and near membrane vesicles with anti-CD59 antibody (Fig. 6B).

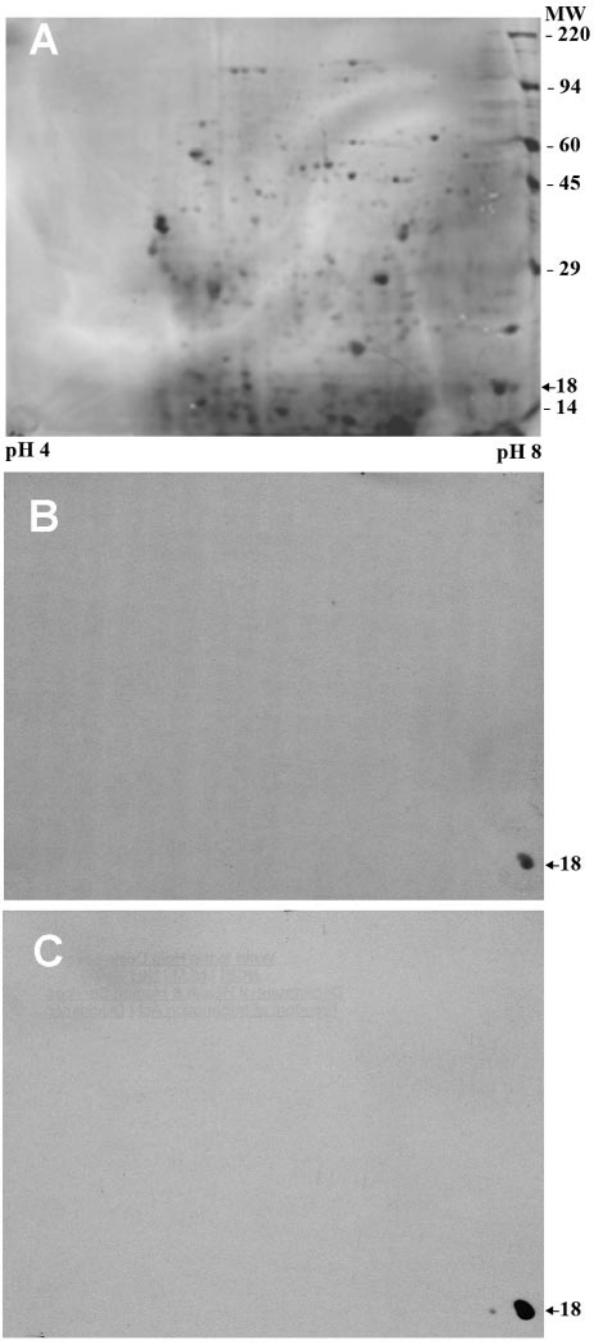

FIG. 4. Two-dimensional gel electrophoresis and Western immunoblot analysis of pathogenic *N. fowleri* membrane extract. (A) *N. fowleri* membrane proteins (200 μg) were electrophoresed with a 10% two-dimensional gel (Kendrick Laboratories) and transferred to a PVDF membrane that was subsequently stained with Coomassie brilliant blue. (B) The PVDF membrane from panel A was used in a Western immunoblot analysis in conjunction with a mouse anti-human CD59 (BRA-10G) monoclonal antibody (1:50) and an HRP-linked rabbit anti-mouse antibody (1:15,000). (C) The membrane was stripped in accordance with the Amersham Biosciences protocol and reprobed with a mouse anti-human CD59 (BRIC 229) monoclonal antibody (1:25) and an HRP-linked rabbit anti-mouse antibody (1:15,000). MW, molecular masses in kilodaltons.

DISCUSSION

Previous studies in our laboratory have suggested that resistance to complement-mediated lysis in *N. fowleri* is regulated at the level of MAC formation (37); thus, the presence of a surface protein similar to mammalian complement-regulatory protein CD59 was investigated. Molecular analyses were used initially to detect CD59 in *N. fowleri*. Southern blot analysis indicates that there is one gene that encodes CD59 in *N. fowleri*. Similarly, the human and rat genomes also contain one gene for CD59 (28, 29). In contrast, the mouse genome con-

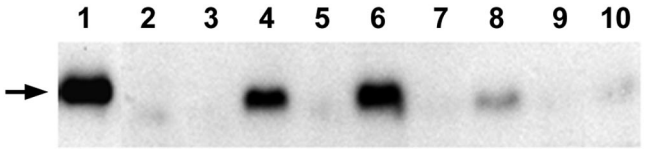

FIG. 5. Western immunoblot analysis of C9 immunoprecipitated with the CD59-like protein of *N. fowleri* incubated in NHS. Pathogenic *N. fowleri* amebae were incubated with HBSS or NHS (1:4) for 3, 5, 10, or 15 min. *N. fowleri* incubated in growth medium was used as a control. The treatment was removed and the amebae harvested. Equal amounts of the lysates (350 μg) were incubated with mouse anti-human CD59 (BRA-10G) overnight at 4°C to immunoprecipitate the CD59-like protein and associated molecules. The lysates were incubated with EZview Red protein A affinity gel for 1.5 h at 4°C. The protein A-bound proteins were then subjected to 10% SDS-PAGE and subsequent Western immunoblot analysis with polyclonal goat anti-human C9 (1:50) and an HRP-linked rabbit anti-goat secondary antibody (1:10,000). Purified human C9 (10 ng) was used as the positive control. Lane 1, human C9; lane 2, untreated; lane 3, HBSS, 3 min; lane 4, NHS, 3 min; lane 5, HBSS, 5 min; lane 6, NHS, 5 min; lane 7, HBSS, 10 min; lane 8, NHS, 10 min; lane 9, HBSS, 15 min; lane 10, NHS, 15 min.

tains two CD59 genes (29). Further studies are necessary to determine the exact structure of the *N. fowleri* CD59 gene since two bands were present in the BamHI digest, and the DNA sequence and restriction sites of the ameba CD59 are unknown. Northern analysis of RNA from pathogenic *N. fowleri* amebae revealed the presence of a predominate 2-kb transcript which hybridized to a human CD59 cDNA probe. It is well recognized that human CD59 possesses four RNA transcripts ranging from 0.7 to 5.8 kb, one of which is also 2 kb (28, 32).

Western immunoblot studies demonstrated the presence of a protein reactive with monoclonal antibodies to human CD59 in the membrane fraction of *N. fowleri*. Two-dimensional gel electrophoresis was utilized to establish reactivity of the CD59 BRA-10G and BRIC 229 monoclonal antibodies with a single *N. fowleri* membrane protein. Immunoreactivity was demonstrated with one protein at an approximate molecular mass of 18 kDa and an approximate pI of 8. These data confirm the results obtained by one-dimensional SDS-PAGE. Human CD59 is an 18- to 20-kDa membrane-expressed protein (9, 20) that exhibits a wide range of pIs, 5.1 to 7.2, while the theoretical pI is approximately 6.0, according to information found at the Swiss Institute of Bioinformatics expert protein analysis system website (http://kr.expasy.org/tools/pi_tool.html). Proteins that exhibit functional and antigenic similarity to human CD59 have been demonstrated in a variety of pathogenic organisms, including *Schistosoma mansoni* (26), *Entamoeba histolytica* (5), and *Borrelia burgdorferi* (27). However, despite the characteristics shared by these proteins with human CD59, a large disparity exists among their molecular masses (94 kDa, 260 kDa, and 80 kDa, respectively).

Immunoprecipitation analysis was performed to determine whether the CD59-like ameba protein associates with pore-forming complement component C9. Western immunoblot analysis demonstrated the presence of a 71-kDa protein in the CD59-like protein immunoprecipitates of amebae treated with NHS. These results suggest that the CD59-like protein in *N. fowleri* amebae may bind C9, as previously demonstrated in mammalian cells (9). The results demonstrated that less C9 was detected in amebae treated with NHS for 15 min compared to the 5-min time period. At 15 min of incubation in

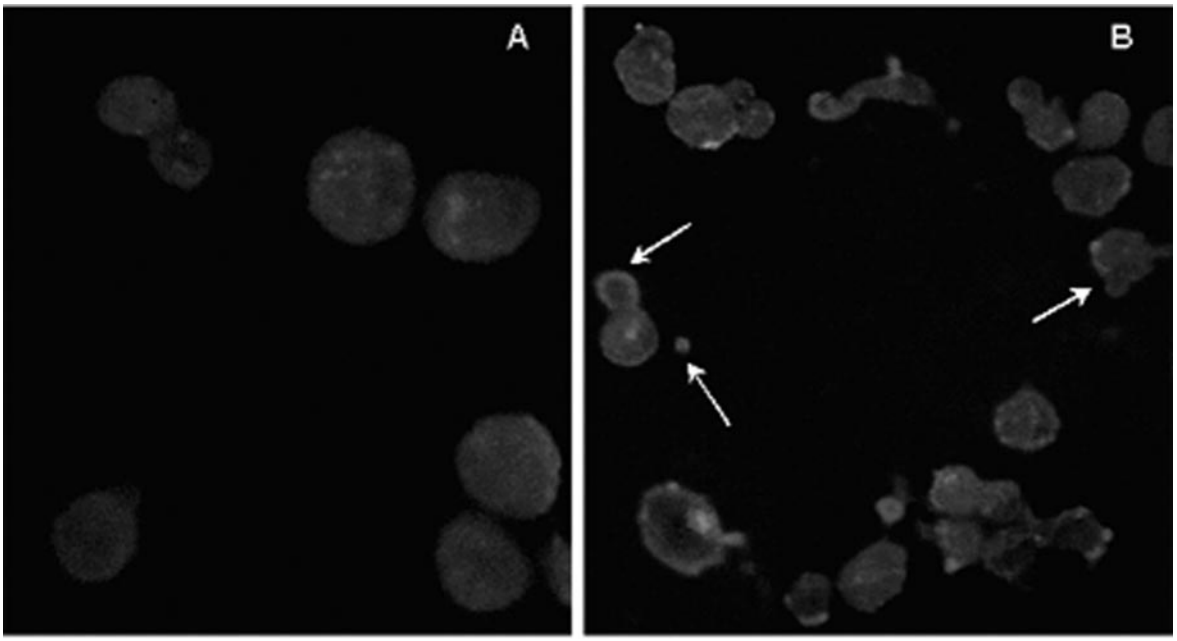

FIG. 6. Immunofluorescence analysis with anti-CD59 on *N. fowleri* amebae. *N. fowleri* amebae were incubated with NHS (1:4) for 30 min and subsequently fixed in acetone. The amebae were incubated with FITC-labeled goat anti-mouse secondary antibody at 1:100 alone (A) or mouse anti-human CD59 monoclonal primary antibody (MEM-43) at 1:5 and FITC-labeled goat anti-mouse secondary antibody at 1:100 (B). Fluorescent areas indicate regions of immunoreactivity of the MEM-43 antibody with the amebae. Clusters of protein were observed on and near membrane vesicles (arrows). Magnification, ×400.

NHS, vesiculation was observed. It has been established that *N. fowleri* amebae undergo vesiculation as a means to remove C5b-C9 from the cell surface (34). Additionally, we have shown that the CD59-like protein is shed on serum-induced membrane vesicles, as observed in mammalian cells (6, 12). Therefore, it is likely that the CD59-like protein is associated with the C9 complement component and both are shed on membrane vesicles which are released into the medium, resulting in less CD59-like protein-bound C9 remaining on the ameba surface.

Immunofluorescence studies on other cell types with antibodies for CD59 demonstrated a correlation between CD59 clustering on the cell surface and membrane vesiculation (6, 14, 17, 22, 24, 38). Immunofluorescence analysis revealed clustering of the protein on the membrane of *N. fowleri* incubated with a sublytic dose of serum complement, as demonstrated in other cell types. Clustering and cross-linking of CD59 have been correlated previously with signaling events occurring through protein tyrosine kinases (21, 23, 24, 35). Thus, the CD59-like protein on *N. fowleri* amebae may function to signal vesiculation.

Recently, it has been demonstrated that *N. fowleri* amebae synthesize and secrete pore-forming proteins, termed naegleriapores, to permeabilize bacterial membranes and lyse eukaryotic cells (15). However, while in the presence of their own cytolytic factors, the amebae remain intact, suggesting that the amebae have a means of protecting themselves (data not shown). We have shown previously that *N. fowleri* incubated with the secreted cytotoxic factors from a clinical isolate of *Pseudomonas aeruginosa* resulted in increased expression of the CD59-like protein (12). Our results, coupled with this evidence, suggest that the amebae are able to shield themselves from harmful stimuli by expressing a protective membrane protein, the CD59-like protein, which in this capacity could possibly function as protectin functions in T cells (19).

Antibodies specific for the BRA-10G and BRIC 229 epitopes of CD59 were effective in a Western immunoblot analysis and yielded strong signals. Notwithstanding this, it was necessary to utilize a 1:25 dilution of the antibodies. Bodian et al. illustrated that the BRIC 229 epitope on human cells is very small compared to the other characterized epitopes of CD59 and therefore may necessitate the use of a high concentration of antibody for detection of the protein (2). The BRA-10G and BRIC 229 specific monoclonal antibodies did not react as strongly as the MEM-43 antibody when used in immunofluorescence studies. The antibodies used were generated to bind specific epitopes of the CD59 protein in particular assays. Recognition of the epitopes by specific antibodies may be dependent on the conformation of the protein, which can be modified during preparation of the samples for analysis, i.e., denaturation or reduction of disulfide bonds. Furthermore, these observations may be indicative of fundamental differences in amino acid sequences and/or conformations between the ameba protein and mammalian CD59.

Collectively, our data suggest the presence of a protein on the surface of pathogenic *N. fowleri* amebae that is cross-reactive with monoclonal antibodies for human CD59. Since the gene, RNA transcript, and CD59-like protein were detected in pathogenic *N. fowleri* and because the protein can bind human C9, we suggest that the CD59-like ameba protein functions to protect the cells from lysis by pore-forming proteins such as the MAC of complement. It is not clear how *N. fowleri* exhibits sequence and protein similarity to human CD59. It is plausible that the observed hybridization occurred within a conserved domain.

Studies are ongoing to determine the precise function of the CD59-like protein. Initial attempts to study the function of the protein with regard to protection from complement lysis were performed with anti-CD59 antibody and live amebae. However, these experiments were problematic. The lack of functional inhibition of the protein is currently attributed to the ability of the amebae to endocytose antibody-bound proteins and actively replace the proteins on the cell surface, since this endocytic activity of antibody has been previously observed in *N. fowleri* (11).

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