Identification of *Naegleria* specific gene products by immunoscreening of a cDNA library

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ABSTRACT

Naegleria fowleri is the causative agent of Primary amoebic Meningoencephalitis, a rapidly fatal disease of the central nervous system. The determinants of pathogenicity and virulence of this organism are poorly undestood. To define the molecular basis of pathogenicity, a cDNA library was constructed from polyA+ mRNA isolated from highly pathogenic *N. fowleri*. cDNA was synthesized using reverse transcriptase. cDNAs greater than 500 bp were ligated directionally into the plasmid vector, pSPORT1, and introduced into *E. coli* by electroporation. Immunoscreening of the *N. fowleri* cDNA expression library with rabbit polyclonal antiserum to *N. fowleri* adsorbed on heat-killed *E. coli* identified five positive clones expressing *N. fowleri* gene products. Plasmids were isolated from two clones, Mp1CL10 and Mp2CL5. Restriction digestion of the plasmid DNA revealed a 2300 bp and 750 bp insert, respectively. Mp1CL10 clone expresses a putative 100 kDa *N. fowleri* protein after IPTG induction, while Mp2CL5 expresses a 23 kDa protein. Northern analysis indicated that cDNA inserts of Mp1CL10 and Mp2CL5 hybridized to RNA of pathogenic *N. fowleri*. Neither insert hybridized to RNA of the nonpathogenic species of *Naegleria*, indicating that the two clones are pathogen specific.

Introduction

The genus *Naegleria* consists of amoeboflagellates found in soil and freshwater. Several species of *Naegleria* have been isolated from environmental sources but only *N. fowleri* is known to cause disease in humans. *Naegleria fowleri* is the causative agent of Primary Amoebic Meningoencephalitis, a rapid fatal disease of the central nervous system in previously healthy children and young adults. Death generally occurs 10-14 days following infection [1].

Both pathogenic and nonpathogenic species of *Naegleria* are known to exist but the determinants of pathogenicity and virulence are not understood. A number of biochemical and biological parameters have been reported which characterize highly-pathogenic *N. fowleri*. As a pathogen, the amoeba gains entry into a host, utilizes host tissues as a nutrient source, and survives attack by the host's immune system. Some of possible determinative factors of pathogenicity include: the ability of the amoebae to attach to and penetrate the nasal mucosa, an increased rate of locomotion with chemotaxis to the brain, an increased rate of cell division, evasion of the host immune system by capping of antibodies and resistance to complement-mediated lysis, and the production of cytolytic factors by the amoebae which lyses nerve cells and other mammalian cells by a contact dependent process [2-7]. Environmental or host signals may control expression of genes encoding virulence-related factors because *N. fowleri* amoebae passaged through the brains of experimental animals or maintained in Cline medium, an enriched growth medium containing hemin as a source of iron, demonstrate increased virulence. *N. fowleri* cultured axenically in minimal medium lacking hemin are less virulent for mice [8, 9].

The objective of this study was to construct a cDNA library from RNA isolated from highly-pathogenic *N. fowleri* amoebae, in order to determine and characterize the genes and gene products responsible for virulence.

Material and methods

Ameobae

N. fowleri Lee strain was isolated from a fatal human case of PAME in 1968 at the Medical College of Virginia Hospital [10]. This amoeba has been cultured axenically in the laboratory since that time and is weakly-pathogenic in mice with an LD_{50} (Lethal Dose) of 10^6. A highly-pathogenic strain, *N. fowleri* Leemp, was developped by passaging the Lee strain through $B_6C_3F_1$ mice at monthly intervals for over one hundred passages. The LD_{50} of Leemp strain is less than 100 [7]. *N. gruberi*

and *N. lovaniensis*, two non pathogenic species of *Naegleria*, were used in select experiments. All amoebae were grown axenically in Nelson-Balamuth medium (50:50) [8].

cDNA library construction

PolyA+ mRNA was isolated from *N. fowleri* Leemp amoebae and the double stranded cDNA (ds cDNA) was synthesized using the BRL Superscript Plasmid System for cDNA and cloning (Bethesda Research Laboratory, Gaithersburg, MD). The ds cDNA was constructed with *Not I* adapters on the 3' end and *Sal I* adapters on the 5'end, to enable directional cloning. The cDNA was subjected to size fractionation using a Sephacryl S-500 column. cDNA (> 500 bp) were ligated into pre-digested *Not I*, *Sal I* cut pSPORT 1 plasmid vector and introduced into Electromax DH10B *E. coli* cells by electroporation. Blue/white screening of colonies on Luria Bertani medium (LB) plates containing Ampicillin (Amp, 100 mg/ml), isopropylthio-b-D-galactoside (IPTG, 1 mM) and 5-bromo-4-chloro-3-indolyl-b-D-galactoside (X-gal, 50 mg/ml) was performed to determine the titer of pSPORT 1 transformants. The library was amplified and frozen at - 70° C.

Immunoscreeing of cDNA library

Expression of *Neagleria* specific proteins by recombinant *E. coli* was induced by growth of *E. coli* in the presence of IPTG. The bacterial cDNA library of highly pathogenic *N. fowleri* Leemp was plated onto sterile nitrocellulose filters which were placed onto LB/Amp plates and incubated at 37° C overnight. Colonies were replica plated and theses filters were incubated overnight on LB/Amp/IPTG. The colonies on the replica filters were lysed with chloroform and incubated in colony lysis buffer (Tris-buffer, Tween 20, 1% bovine serum albumin, 1 mg/ml DNAse, and 40 mg/ml lysozyme) overnight at room temperature. The nitrocellulose filters were blocked with Blot Mix (phosphate buffer saline containing 5% nonfat dry milk, 5 mM EDTA, 0.01% antifoam A, and 0.0001% thimerosal) for several hours at room temperature. The filters were washed and incubated overnight with rabbit anti-*N. fowleri* Leemp antiserum (diluted in Blot Mix) which was adorbed with heat-treated *E. coli*. The antiserum was adsorbed with *E. coli* twice for 2 hours at 37° C to remove antibodies that would react with *E. coli* proteins. The filters were washed and incubated with peroxidase-conjugated goat anti-rabbit antiserum. Antibody reactivity was visualized using 3 mM chloronaphtol, 0.015% H_2O_2, 16.7% methanol in Tris buffer saline, pH 7.4. Positive staining colonies were rescreened until pure cultures were obtained.

Restriction Endonuclease Digestion of plasmid DNA from positive clones

Plasmids containing *N. fowleri* Leemp cDNA inserts were isolated from *E. coli* using the Qiagen system (Qiagen, Chatsworth, CA). The plasmid preparations were digested with *Not I* and *Sal I* to remove the cDNA inserts. The digest was subjected to 0.8% agarose gel electrophoresis at 22 V overnight to determine the size of the cDNA insert. The insert size was determined by extrapolation from 1 kb DNA ladder fragments standards co-electrophoresed in a separate lane.

Northern blot analysis

To determine whether the *Naegleria* cDNA inserts were pathogen specific, Northern analysis was undertaken. Total RNA was isolated from pathogenic and non-pathogenic species of *Naegleria* using the isothiocyanate-cesium chloride gradient method [11]. The RNA samples were loaded onto a 1% agarose-formaldehyde gel and electrophoresed at 30 V overnight. The RNA was transferred to a nylon membrane in 20X SSC overnight, and the RNA crosslinked to the membrane using a UV stratalinker (Stratagene, La Jolla, CA). Plasmid DNA was digested with *Not I* and *Sal I* and subjected to agarose gel electrophoresis. The cDNA insert fragments were cut from the gel and extracted using a QIAEX Gel Extraction Kit (Qiagen, Chatsworth, CA). The extracted cDNA was labeled with ^{32}P-dCTP by nick translation (BRL, Gaithersburg, MD) and used to probe the nylon membrane. Membranes containing RNA were incubated in prehybridization buffer (50% formamide, 0.1% BSA, 0.1% Ficoll 400, 0.1% polyvinyl-pyrrolidone 40, 0.005% SDS, 0.05 M NaHPO4, 5X SSC, 0.025% yeast RNA) for 3-4 h with gentle agitation. Membranes were hybridized for 18 h for 22° C in hybridization buffer (prehybridization buffer containing 5% dextran sulfate) containing 10^7 cpm/ml of radolabeled probe, with gentle agitation. Membranes were washed in 2X SSC/0.2% SDS (4X, 5 min each – 1X, 30 min), and 0.5X SSC/0.2% SDS (1X, 20 min). All washes were performed at 37° C. Membranes were allowed to dry and were exposed to X-ray film at - 70° C for 2-3 days.

Analysis of *Naegleria* recombinant proteins by SDS-PAGE and Western Immunoblot

In order to determine whether the transformed *E. coli* were expressing *Naegleria* specific proteins, SDS-PAGE and Western immunoblot analysis was performed. *E. coli* clones containing *Naegleria* specific cDNA as determined by immunoscreening were induced or un-induced with IPTG for 1 hour. The *E. coli* were harvested and lysates were prepared. The proteins were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membranes were reacted with rabbit polyclonal antiserum prepared against *N. fowleri* whole cell lysate which was adsorbed

with heat-treated *E. coli*. The membranes were incubated with peroxidase-conjugated goat anti-rabbit antiserum and antibody reactivity was visualized using 3 mM chloronaphtol, 0.015% H_2O_2, 16.7% methanol in Tris buffer saline, pH 7.4.

Results

A directional cDNA library was constructed and expressed in *E. coli* DH10B. Transformed colonies containing cDNA inserts were detected using blue/white screening on LB agar containing Amp, X-gal, and IPTG. Expression of *Naegleria* specific proteins was identified on nitrocellulose filters using an anti-*N. fowleri* rabbit polyclonal antiserum adsorbed on *E. coli*. Positive colonies were rescreened until pure colonies were obtained. The pure colonies were amplified. These five clones designated Mp1CL1, Mp1CL3, Mp1CL10, Mp2CL4 and Mp2CL5 were analyzed further. *Table 1* sums up results obtained in the study.

The cDNA inserts obtained after digestion with *Not I* and *Sal I* were analyzed by agarose gel electrophoresis. *Not I* and *Sal I* digestion of Mp1CL10 revealed three distinct bands: one band of vector DNA and two insert bands of appromately 1100 bp and 1200 bp for a total insert size of 2300 bp. Further studies demonstrated that two insert bands were obtained because an internal *Sal I* restriction site was present within the cDNA insert. Restriction digestion of Mp1CL1, Mp1CL3, Mp2CL4 and Mp2CL5 plasmid DNA revealed single cDNA inserts of approximately 1500 bp, 300 bp, 100 bp, and 700 bp.

The cDNA inserts of Mp1CL1, Mp1CL10 and Mp2CL5 were sequenced. Analysis of the sequence revealed that Mp1CL10 shares 20% identity (70% similarity) with myosin II heavy chain gene from the amoeba, *Acanthamoeba castellanii*. Mp1CL10 showed similar identity and similarity to the myosin II heavy chain of *Dictyostelium discoideum*. Analysis of the sequence of Mp2CL5 and Mp1CL1 cDNA inserts did not show any homology to any gene products in the sequence databank.

To determine whether expression of Mp1CL3, Mp1CL10 and Mp2CL5 cDNAs was pathogen specific, Northern analysis was performed. RNA from nonpathogenic *N. lovaniensis* and *N. gruberi*, weakly-pathogenic *N. fowleri* Lee, and highly-pathogenic *N. fowleri* Leemp was probed with radiolabeled cDNA from each of the three clones. The Mp1CL10 hybridized to a 6 kb species RNA in both pathogenic strains tested. However, the intensity of the hybridization signal was 16-fold greater in weakly-pathogenic *N. fowleri* Lee as compared to highly-pathogenic *N. fowleri* Leemp. In contrast, hybridization to RNA from nonpathogenic amoebae was not observed. Mp2CL5 cDNA hybridized to a 1.2 kb species of RNA in both highly-pathogenic and weakly-pathogenic strains of *N. fowleri*, but did not hybridize to

Table 1. Naegleria *clones obtained after immunoscreening.*

Clone	Insert size	Sequence Homology	Northern Analysis	Western Immunoblot Analysis
Mp1CL1	1500 bp	No significant Homology	ND[1]	No protein
Mp1CL3	300 bp	ND	P[2] & NP[3]	No protein
Mp1CL10	2300 bp	Myosin II Heavy chain	P (↓[4]HP[5], ↓[6]WP[7])	100 kDa, 69 kDa
Mp2CL4	100 bp	ND	ND	ND
Mp2CL5	700 bp	No significant homology	P (↑HP, ↓WP)	23 kDa

[1] ND: Not Done
[2] P: Pathogen
[3] NP: Non-Pathogen
[4] ↓: Decreased
[5] HP: Highly-Pathogenic
[6] ↑: Increased
[7] WP: Weakly-Pathogenic.

RNA from non-pathogenic *Naegleria*. The intensity of the hybridization signal was 2-fold greater with RNA of highly-pathogenic *N. fowleri* Leemp when compared to weakly-pathogenic *N. fowleri* Lee. The nick-translated cDNA from Mp1CL3 hybridized to a 1.8 kb species of RNA in both pathogenic and non-pathogenic *Naegleria*.

Lysates of recombinant *E. coli* were prepared and subjected to SDS-PAGE followed by western immunoblot analysis. Nitrocellulose membranes containing the recombinant *E. coli* lysate proteins were incubated with anti-*N. fowleri* antiserum adsorbed on *E. coli*. *E. coli* expressing Mp1CL10 were induced to express a protein of approximate molecular mass of 100 kDa. Also, a second 69 kDa protein species was observed. A 23 kDa protein was identified after *E. coli* Mp2CL5 was induced with IPTG. Neither Mp1CL10 nor Mp2CL5 produced *Naegleria* specific proteins in the absence of IPTG. *Naegleria* specific proteins were not detected when clone Mp1CL1 or Mp1CL3 were induced with IPTG.

Discussion

Investigation of potential virulence markers at the molecular level is necessary in order to understand the complex changes that occur in *N. fowleri* amoebae during transformation from weakly-pathogenic to highly-pathogenic amoebae. The factors

responsible for the pathogenesis of *N. fowleri* induced meningoencephalitis are poorly defined. In the present study, construction of a directional cDNA library prepared from mRNA isolated from virulent mouse-passaged amoebae and expression of *N. fowleri* proteins in *E. coli* has allowed for the successful identification of five positive clones by immunoscreening with *N. fowleri* specific rabbit polyclonal antiserum. Two clones, Mp1CL10 and Mp2CL5, encoding for transcripts which were detected only in pathogenic *Naegleria* amoebae and were undetectable in pathogenic *Naegleria* species by Northern analysis, were identified. As these trancripts may be pathogen specific, clones Mp1CL10 and Mp2CL5 were characterized further.

Mp1CL10 was identified as pathogen specific by Northern analysis and encoded a nonmuscle myosin II heavy chain based on the partial DNA sequence analysis. The greatest similarity of the cDNA sequence was to a nonmuscle myosin II heavy chain gene of two other amoebae, *Acanthamoeba castellanii* and *Dictyostelium discoideum*. Similarity to a variety of other myosin heavy chain gene products, including those of *Entamoeba histolytica*, *Schistosoma mansoni*, human beta heavy chain, and non muscle cellular myosin type A, were noted when comparisons to other eucaryotic genes were undertaken. Moreover, Northern blot analysis demonstrated that the Mp1CL10 transcript is upregulated in weakly-pathogenic *N. fowleri* as compared to highly-pathogenic *N. fowleri*. When clone Mp1CL10 was induced by IPTG, it expressed a *Naegleria* protein of approximate molecular mass of 100 kDa. A lower molecular mass species of approximately 69 kDa was observed also and may represent a proteolytic degradation product of the 100 kDa protein or a truncated protein species produced by the transformed *E. coli*.

When clone Mp2CL5 was induced by IPTG, it expressed a 23 kDa *Naegleria* protein. As Mp1CL10, Mp2CL5, also, was identified as pathogen specific by Northern analysis but the Mp2CL5 message was over expressed in highly-pathogenic *N. fowleri* Leemp. However, Mp2CL5 did not show any significant homology to any gene products previously described.

In this study, although we have shown that the transcripts for Mp1CL10 and Mp2CL5 were pathogen specific, the functions of these proteins are unknown. Therefore, more studies are needed to clarify the role that the gene products of Mp1CL10 and Mp2CL5 play in *Naegleria* amoebae. Large quantities of both recombinant proteins will be produced. Purification of these recombinant proteins will allow for functional studies to be undertaken. Additionally, production of antiserum to the recombinant proteins may help to elucidate the function of the proteins by inhibiting or enhancing specific activities of highly-pathogenic amoebae such as resistance to complement lysis or chemotaxis to brain cells. Also transfection of non-pathogenic *Naegleria* with Mp1CL10 or Mp2CL5 cDNA may allow transformation from the nonpathogenic to the pathogenic form. These approaches

may allow for the identification of virulence factors of highly-pathogenic *Naegleria* amoebae.

Also, Southern analysis will be performed to determine whether Mp1CL10 and Mp2CL5 genes are specific for the pathogenic species, *N. fowleri*. Such studies will help to determine if the sequences of these cDNA inserts can be used to develop tests based on molecular techniques (nucleic probes, PCR) in order to distinguish pathogenic *N. fowleri* among other inoffensive free-living amoebae.

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