Cell Biology of the Philippine Amoeboflagellate, *Naegleria philippinensis*

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INTRODUCTION

Naegleria is a free-living amoeboflagellate whose trophozoite stage may differentiate reversibly into either a non-reproductive flagellate stage or a resistant cyst (Page 1967). A species of *Naegleria* known as *N. fowleri* causes fatal meningoencephalitis in humans (Julio-Martinez et al. 1983). Cell morphology, culture medium preference, temperature tolerance, lecting sensitivity, isoenzyme patterns, DNA restriction patterns, mouse pathogenicity and immunology are the criteria used to differentiate the various *Naegleria* species, as well as pathogenic from non-pathogenic species (De Jonckheere 1987; Milligan and Band 1988; Graham- Clark et al. 1989; Pernin and Cariou 1989).

Naegleria can be isolated from soil, freshwater habitats, as well as from natural or industrial thermally-polluted water systems (Marciano-Cabral 1988). An amoeboflagellate has been isolated in the Philippines by Enriquez et al. (1984). One isolate was obtained from a thermally-polluted stream, another from the cerebrospinal fluid of a young patient with encephalitis and the third from a heated swimming pool. Initial investigations place these isolates in the genus *Naegleria*. The present study further characterizes the isolates.

MATERIALS AND METHODS

Cultivation and Maintenance of Organisms. The Philippine *Naegleria* isolate was first cultured in non-nutrient agar plates lawned with *Escherichia coli.* The plates were incubated at 37°C

and microscopically examined daily for amoeba growth. Axenic cultures were prepared by transferring amoebae into Serum-Casein- Glucose-Yeast-Extract (SCGYEM) medium supplemented with 10% FBS.

Naegleria fowleri (Kul ATCC 30808), *N. lovaniensis* (Aq/8/1/45D), *N. australiensis* (NK11) and *N. gruberi* (CCAP 1518/1e) were obtained from Dr. Rolf Michel, Ernst Rodenwaldt Institute, Koblenz, Germany. Except for *N. gruberi,* all *Naegleria* strains were cultured at 37°C in SCGYEM. *N. gruberi* was maintained at 27°C in Balamuth medium.

Morphological Studies. Trophozoites and cysts, both from axenic and bacterized cultures, were examined under a phase contrast microscope. The trophozoites were further stained with trichome and haematoxylin for further studies. The cysts were stained with PATAG r for identification and classification. Trophozoites, cysts and flagellate stages were also processed for electron microscopic examination.

Lectin Sensitivity Studies. For lectin agglutination, fluorescence tests and electron microscopic visualization of receptors, 72-hour axenic cultures were harvested and washed free of the culture medium. Cells suspended in PBS were made to react with different lectins. The degree of agglutination was scored from weak (+) to strong (+ + +). To determine the specificity of the lectin reaction, a 0.2 M solution of an appropriate inhibitory sugar was added to the lectin solution one hour before incubating it with the cells. The degree of agglutination was scored as above.

Amoebae cultured on coverslips were fixed with 1% gluteraldehyde and incubated with the appropriate lectin-FITC conjugate for one hour. The unbound conjugates were washed off and the stained cells were observed under the fluorescense microscope. Gluteraldehyde- fixed cells were also incubated with lectin-gold conjugates, embedded in Lowicryl and processed for electron microscopic studies. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

Naegleria philippinensis soluble and insoluble protein extracts were subjected to Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE). The separated protein bands were transferred to nitrocellulose membranes according to the method of Kyhse-Andersen (1988). The membranes were reacted with the appropriate lectin-peroxidase conjugate. The bound lectin was visualized using diaminobenzidine (DAB) as a substrate.

RESULTS AND DISCUSSION

Naegleria trophozoites can easily be recognized by the characteristic lobose monopseudopodium and a very prominent nucleus with a centrally located nucleolus. A section of the trophozoite under the electron microscope shows a clear separation of the pseudopodial hyaloplasm and the granular cytoplasm which in turn contains all the membrane-bound organelles such as the elongated dumb-bell shaped mitochondria, numerous vacuoles of varying sizes, rough endoplasmic reticulum, golgi and nucleus. Ribosomes may be observed free in the cytoplasm or attached to the endoplasmic reticulum. The nucleus contains a large, prominent centrally located nucleolus.

Naegleria trophozoites, when trasferred from culture dish to distilled water or saline solution, readily form pairs of flagella originating from the tip of a pear-shaped cell body. At the electron microscope level, the flagellate stage can easily be recognized when the flagellar apparatus is found in the plane of section. This consists of the flagella and their supporting structures, basal bodies and associated fibrils that run parallel to each other, as well as the rhizoplasts. Both flagella exhibit the typical $9+2$ arrangement of microtubules and are enclosed in a membrane which is continuous with the cell surface plasma membrane.

Numerous cysts can be observed in agar plates that have been kept for more than three days. Phase contrast microscopic studies show ovoid to oblong cysts with varying numbers of pores. A new cyst within an old cyst can occasionally be observed. The *Naegleria* cyst wall is made up of two layers: a fragile loosely-associated ectocyst and a very thick endocyst. The cytoplasm inside the cyst contains several mitochondria and membranous vesicles. As in the trophozoite, the nucleus of the cyst contains a prominent nucleolus and condensed chromatin material.

The reactions of the different *Naegleria* species to lectins with various sugar specificities were studied. *N. philippinensis,* as well as *N. lovaniensis,* were readily agglutinated by the lectins used, whereas *N. fowleri* did not react with any of the lectins. With the exception of *N. fowleri,* all species were agglutinated by Con A and *Triticum vulgare* lectin. *N. philippinensis, N. lovaniensis* and *N. australiensis* were strongly agglutinated by *Helix pomatia* but weakly agglutinated by *Lathyrus odoratus* lectin. *N.*

philippinensis showed weak agglutination with *Bauhinia purpurea, Bandeirea simplicifolia* and *Sambucus nigra* lectins. Furthermore, *N. philippinensis* did not agglutinate with *Glycine max* and *Arachis hypogea* before pronase treatment, but weak agglutination was observed thereafter.

To visualize the bound lectins on the surface membrane, lectin-FITC conjugates were employed. The Con A-FITC conjugate was found to be uniformly distributed throughout the surface of the trophozoites. This was also true for *Helix pomatia, Triticum vulgare* and *Ulex europaeus I.* Patching, capping was also observed when live trophozoites were stained with *Helix pomatia*-FITC. No difference was observed with the appearance of the fluorescence patterns among the different *Naegleria* species.

The surface membrane of the trophozoites incubated with *Helix pomatia*-gold conjugate was uniformly covered with colloidal gold particles. No difference was observed in the distribution of gold particles between regions of contact and free areas or areas immediately adjacent to contact points. On the other hand, the gold conjugate was randomly distributed and discrete clusters were observed on the surface membrane of the flagellate stage. No gold conjugate was found in the cytoplasm of the trophozoites or flagellates.

Cysts stained with PATAG r revealed a carbohydrate-rich cyst wall, as well as a collar surrounding the cyst pore. Mannose-specific Con A-FITC reacted with the cyst wall and the collar surrounding the cyst pores. However, galNAc-specific *Helix pomatia*-FITC did not stain the cyst wall as intensely as Con A, though the collar surrounding the pores was clearly detected.

Soluble surface membrane lectin receptors were further characterized on the basis of their molecular weights. Con A receptors separated by SDS-PAGE were found to be within the 20-60 kd range, whereas *Helix pomatia* lectin receptors were within the 30-120 kd range. The reaction was completely inhibited both by a-methyl-mannopyranoside and N-acetyl-galactosamine, respectively, indicating specificity of binding. Insoluble Con A receptors were further detected within a similar molecular weight range as that of the soluble Con A receptor. No insoluble *Helix pomatia* lectin receptor was detected.

N. philippinensis reacted with galNAc and mannose-binding lectins suggesting that these two sugars are predominantly found to be on the surface membrane. The difference in the distribution pattern of these receptors in the cyst, trophozoite and flagellate

stages indicates that modifications in membrane structure occur during transformation. The flagellate stage is usually rigid, has a smaller surface area, does not undergo endocytosis and has a completely different mode of locomotion. On the other hand, the trophozoite has a very fluid surface membrane as evidenced by its ability to patch and cap and internalize surface structures. These carbohydrate moieties may function in relation to cell recognition or cell-substrate adhesion. Other mechanisms of cell recognition may be employed by the flagellate forms.

Table 1. Rections of the various *Naegleria* species to the different Lectins used

LECTIN	SUGAR	phil isolate	N. lovan	N. austrl.	N. gruberi	N. fowleri
Concanavalin A	α–D-methyl manno-pyranoside	++	+	++	++	0
Triticum vulgare	gluNAc	+	++	++	++	0
Helix pomatia	galNAc	+++	+++	+++	0	0
Lathyrus odoratus	α-D-Mannose	+	+	+	0	0
Ulex europaeus I		+	0	+	0	0
Limulus plyphemus	NeuNAc	0	++	+	0	0
Bauhinia purpurea	galNAc	+	++	0	0	0
Bandeira simplicifolia	galNAc	+	++	0	0	0
Sambucus nigra	gluNAc	+	++	0	0	0
Mycoplasma gallisepticum		+	+	0	0	0
Glycine max	galNAc	0	+	0	0	0
Glycine max + pronase		+	+	0	0	0
Arachis hypogea	galNAc	0	++	0	0	0
Arachis hypogea + pronase		+	0	0	0	
tragonolobus purpureus	fucose	0	+	0	0	0
Anti- A		0	+++	0	0	0

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