2019

The Infectivity of Naegleria fowleri Cysts in vivo and in vitro, and Mediation of Encystment by cAMP

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The Infectivity of *Naegleria fowleri* cysts *in vivo* and *in vitro,* and Mediation of Encystment by cAMP

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By

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Richmond, Virginia

May, 2019

Acknowledgment

The author would like to thank several people who helped along the development of this thesis. I would like to thank my advisor and P.I., Dr. Francine Marciano-Cabral. My graduate school experience became fantastic and inspiring when I entered your lab. I am so grateful for all of your help, patience, guidance, and kindness in teaching.

I'd like to thank my brother, Alex Evdokiou, for never hesitating to be there for me, and for being the example of accomplishment that I strive to achieve myself. I would also like to thank my parents, Leah and Kostas Evdokiou, for the unending support and unconditional love that has been my stepping stone to success throughout my entire life. I'd like to thank Tyler Poe for his friendship throughout this entire program. Lastly, I'd like to thank Zach Frazier for being my flashcard guy and for always supporting me whenever I needed it.

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List of Abbreviations

LDH= Lactate dehydrogenase

PAM= Primary amoebic meningoencephalitis

CDC= Centers for Disease Control and Prevention

GAE= Granulomatous amoebic encephalitis

CNS= Central nervous system

TNF= Tumor necrosis factor

MAC= membrane attack complex

cAMP= cyclic adenosine monophosphate

ABSTRACT

Evdokiou, Anna. M.S. Department of Microbiology and Immunology, Virginia Commonwealth University, 2019. The Infectivity of *Naegleria fowleri* Cysts *in vivo* and *in vitro*, and Mediation of Encystment by cAMP.

The free-living amoeba and causative agent of Primary Amoebic Meningoencephalitis, *Naegleria fowleri*, has three life stages: the trophozoite, the flagellate, and the cyst. This study examined the ability of the cyst to attach to, excyst upon, and destroy cell cultures grown to confluent monolayers, and to cause Primary Amoebic Meningoencephalitis in a murine animal model. The co-culture of cysts with P388D.1, CHME3, Vero, human nasal epithelial, and rat primary mixed glial cells resulted in destruction of the monolayer of all cell types once the cysts attached and excysted. One day post exposure to cysts, the mixed glial cells exhibited a two-fold increase in lactate dehydrogenase (LDH) release compared to cells without cysts, and on day eight post exposure, showed a nearly four-fold increase in LDH. In this study, the cysts of *N. fowleri* were shown not to be infective *in vivo* in a murine model using $B_6C_3F_1$ male mice. The mediation of the encystment process by the intracellular concentration of the secondary messenger cAMP, as described in other closely related genera and species of amoeba, was also investigated. Encystment of *N. fowleri* was shown to be mediated at least in part by the secondary messenger cAMP by treating cultures of the trophozoite with 100 uM dipyridamole, an inhibitor of cAMP-specific phosphodiesterases. Dipyridamole (100 μM) increased the rate of encystment by nearly two-fold compared to 0.1% DMSO by the end of a five day period of observation. This suggests that cAMP is an essential mediator of the encystment process within *Naegleria fowleri.*

Vita

Anna Lynn Evdokiou was born on November 8th, 1994 in Porter County, Indiana, and is an American citizen. She graduated from William Mason High School, Mason, Ohio in 2013. She received her Bachelor of Science in Biology with an emphasis on Molecular and Cellular Biology from University of the Cumberlands, Williamsburg, Kentucky in 2017, where she completed an undergraduate research study developing a noninvasive detection assay for skin cancer under Dr. Joan Hembree.

INTRODUCTION

Naegleria fowleri: Brain-eating Amoeba

Naegleria fowleri is a free-living amoeba that causes the disease Primary Amoebic Meningoencephalitis (PAM) in the host (John et al., 1982, Siddiqui et al., 2016). Most often, diagnosis and cause of death is only identified when an autopsy is performed, due to the quick onset of symptoms and rapid deterioration of the host (Jamerson et al., 2017, da Rocha-Azevedo et al., 2009). PAM involves a swift deterioration of brain tissue due to cell lysis by the amoeba through secreted cytolytic factors, such as phospholipase A, phospholipase B, neuraminidase, elastase, and a pore forming protein similar to perforin, called naegleriapore (Vivesvara et al., 2007, Maricano-Cabral, 1988, Herbst et al., 2002, Eisen et al., 1987). Mechanical consumption of living cells by the amoeba also destroys host brain tissue (Vivesvara et al., 2007, Maricano-Cabral, 1988, Sohn et al., 2010, John, 1982). Symptoms of PAM can include headache, stiff neck, fever, and nausea, and these are typically indistinguishable from bacterial meningitis (da Rocha-Azevedo 2009, CDC). However, about three to ten days after initial exposure, the host will die without immediate, early treatment (Centers for Disease Control and Prevention (CDC), 2017, Maricano-Cabral, 1988).

PAM begins with entry of amoeba into the nasal passages, usually carried by contaminated freshwater, and then attachment to the nasal mucosa (John, 1982, Marciano-Cabral, 1988, CDC, 2017). Subsequent migration up olfactory nerves, and through the cribriform plate, leads to replication within the frontal lobe (Jarolim et al., 2000, Marciano-Cabral, 1988, CDC, 2017, Siddiqui et al., 2016). Once replication begins in the brain, it triggers a strong innate immune response involving macrophage, neutrophil, and eosinophil recruitment (Jamerson et al., 2017, Marciano-Cabral et al., 2007, Marciano-Cabral, 1988). *N. fowleri* secretes its cytolytic factors and

proteases that lyse the cells (Jamerson et al., 2017), leading to hemorrhagic necrosis of central nervous system (CNS) tissue. The amoeba uses its food-cup structure to phagocytose lysed brain tissue material in the surrounding environment (Marciano-Cabral, 1988, John, 1984). The food-cup, or amoebastome, is an extension of cytoplasmic appendages called pseudopodia (Brown, 1979). Actin is highly localized in the food-cup to allow cytoskeleton rearrangement during trogocytosis, which is when the amoeba takes small portions out of its target cell's membrane and cytoplasm, and phagocytizes it (Zysset-Burri et al., 2015, Vivesvara et al., 2007). The high expression of actin is considered a virulence factor in *Naegleria fowleri* strains due to its essential function within the food-cup (Sohn et al., 2010). The amoebastome is a highly specialized organelle that facilitates attachment to host tissue (Sohn et al., 2010) and both trogocytosis and phagocytosis of lysed cells (Sohn et al., 2010, Zysset-Burri et al., 2015, Marciano-Cabral, 1988, John, 1984).

PAM was first diagnosed in southern Australia in 1965 (Fowler & Carter, 1965), and was eventually dubbed "primary amebic meningoencephalitis" in order to differentiate between another amebic brain infection by *Entamoeba histolytica* that is rare, and secondary to an intestinal infection (Butt, 1966, Carter et al., 1968). Around that time, reports of PAM started coming forward from all around the world and in the United States, including states such as North Carolina, South Carolina, Florida, Virginia, Missouri, Arkansas, Georgia, Nevada, Oklahoma, Louisiana, Mississippi, New Mexico, New York, Texas, and California (John, 1982, Yoder et al., 2010, Vyas et al., 2015, Dos Santos et al., 1970). Usually, cases of PAM come from victims swimming in contaminated warm water sources like lakes, ponds, and streams. Children are more likely to contract PAM, and this is possibly due to behavioral predisposition to exposure by swimming vigorously and diving in natural water sources during warm weather (Martinez et al., 1997).

However, sometimes pool water or water from pipelines can contain the amoeba and cause infection by intranasal inoculation (Miller et al., 1982, Marciano-Cabral et al., 2003). There has been a case of tap water in the United States being the source of a fatal infection of *Naegleria fowleri* resulting in PAM (Marciano-Cabral et al., 2003, Cope et al., 2013). *Naegleria fowleri* was shown to be the causative agent of this fatal case of PAM by collection of the tap water in question and subsequent culture of viable *Naegleria fowleri* from these samples (Cope et al., 2003). There are two other species within the *Naegleria* genus that cause disease in animal models, *Naegleria australiensis* and *Naegleria italica*. However, these have not been shown to cause disease in humans (De Jonckheere et al., 2004). *Naegleria lovaniensis* is the most closely related genetically to *Naegleria fowleri* (Liechti et al., 2018), however it is not pathogenic in humans or animal models (Jamerson et al., 2012). There are also other genera of free-living amoebae, such as *Acanthamoeba* and *Balamuthia mandrilaris*, that can cause a fatal CNS infection, called granulomatous amebic encephalitis (GAE), but usually only affects immune compromised patients (Marciano-Cabral et al., 2003, Vivesvara et al., 2007, Martinez et al., 1997). PAM can manifest in immune competent individuals, making it less of an opportunistic pathogen than other free-living amoebae (Marciano-Cabral et al., 2007). *Naegleria fowleri* has also been shown to be almost ubiquitous in nature, as it can survive in water or soil and remain a viable pathogen to animals and humans alike (Marciano Cabral et al., 2003, Page, 1974, Vivesvara et al., 2007).

Infection with *Naegleria fowleri* causes PAM, however it is not fully understood why some people contract an active infection and develop PAM, while others who had been participating in the same activity do not. The existence of different strains of the amoeba could explain any variances in disease manifestation among patients. There have only been 4 documented survivors of active *Naeglera fowleri* infection out of 143 U.S. cases (CDC, 2017). The survivors were

diagnosed early, almost immediately after displaying symptoms, and started on treatments. Strains isolated from human victims of PAM vary widely in virulence (John, 1982), so the survival of these patients was attributed to infection with a less virulent strain of *Naegleria foweri*. It was cultured and shown to cause cytolytic damage to cells slower than other strains. The early diagnosis and treatment of these patients was also a crucial factor in their survival (CDC, 2017).

Treatments for PAM

Once *Naegleria fowleri* establishes an infection, treatment includes a combination of amphotericin B, rifampin, miltefosine, fluconazole, azithromycin, and steroids to help with brain swelling (Grace et al., 2015, Schuster et al., 2006). Due to the amoeba's intolerance of colder temperatures, hypothermia can also be used to slow its progress (Marciano-Cabral, 1988), however, even with these treatments available, mortality for *N. fowleri* infection is 95% (CDC, 2017). The drug of choice for treatment of *Naegleria fowleri* infection is Amphotercin B. It is most effective at preventing proliferation at 0.1 ug/mL and is entirely amoebicidal at 0.78 ug/mL (Grace et al., 2015, Kim et al., 2008). According to the CDC, increasing intravenous dosages for treatment over at least a ten day period from 0.25 to 1.5 mg/kg/day should be administered for adults, and 0.5 to 0.7 mg/kg/day for children (CDC, 2017). There are significant toxicity issues associated with higher doses of amphotericin B, including kidney failure (Stevens et al., 1981). Rifampin is another popular therapy for PAM, although the efficiency of its CNS penetration has been thrown into question (Grace et al., 2015). It has been purported that the physiological concentration of Rifampin in the brain after the recommended dose is administered is not high enough for efficacy against *Naegleria fowleri* (Grace et al., 2015). Miltefosine is a drug of potential, as it has been shown to be useful *in vitro* for killing *Naegleria fowleri* (Schuster et al., 2006), and is thought to have some limited CNS penetration (Grace et al., 2015). Fluconazole is an antifungal agent that is

sometimes added to treatment with Amphotercin B to increase efficacy due to its ability to pass through the blood-brain barrier. The CDC recommends an intravenous dosage of fluconazole of 10 mg/kg/day for 28 days (CDC, 2017). Azithromycin is an antibiotic that reportedly suppresses growth of *Naegleria fowleri* during *in vitro* experiments at 10-100 mg/mL. *In vivo* experiments, however, show that for mice, a dosage of 75 mg/kg/day prevents death by PAM (Grace et al., 2015). The CDC suggests 10 mg/kg/day for 28 days (CDC, 2017).

Life Cycle of *Naegleria fowleri*

Naegleria fowleri is a free-living amoeba that thrives in freshwater sources such as: lakes, ponds, and streams in warm climates (Marciano-Cabral, 1988). Depending on environmental conditions, it can exist in three phenotypically distinct stages: trophozoite, flagellate, and the cyst (Siddiqui et al., 2016). The transformation to each stage is dependent upon changes in environmental conditions. The trophozoite is the amoeboid form associated with the onset of PAM, as it is the feeding stage of the organism. It is the most prevalent form of *N. fowleri* in nutrient rich environments and is the feeding stage of the amoeba. The trophozoite is easily recognizable and displays a distinct, large nucleus and nucleolus, along with visible vacuoles and organelles. The amoeba has most of the typical eukaryotic membrane-bound organelles including: mitochondria, endoplasmic reticulum, ribosomes, and a contractile vacuole (Siddiqui et al., 2016, Vivesvara et al., 2007, Marciano-Cabral, 1988)

When in a nutrient-depleted aqueous environment, the trophozoite transforms into a flagellate (Dingle et al., 1966, Maricano-Cabral, 1988) The pear-shaped flagellate features two flagella with some, but not all, of the afore mentioned organelles, such as a nucleus and nucleolus in the anterior region, mitochondria, rough endoplasmic reticulum, and vacuoles. In order to move, the flagella are powered by kinetosomes, rhizoplasts, and thin-walled fibrils. Interestingly, only

the flagellates contain kinetosomes or rhizoplasts (Dingle et al., 1966, Marciano-Cabral, 1988). This suggests that synthesis of these organelles begins once the flagellate form is initiated. Tubulin is also expressed in the flagellate (Jamerson et al., 2017), and it uses its motility to escape an air-water interface, or nutrient deplete environments. (Siddiqui et al., 2016, Marciano-Cabral, 1988, Vivesvara et al., 2007).

The cysts of *Naegleria* species vary in conformation among different species and, based upon observed differences in behavior within certain conditions, possibly strains as well (Fouque et al., 2012). The cysts of other free-living amoebae, such as *Acanthamoeba* species and *Naegleria gruberi* have been better characterized. The cysts of *Acanthamoeba* are double-walled, with an endocyst and an ectocyst, and contain acid-insoluble fibers and cellulose. *N. gruberi* also forms a double-walled cyst, with a bumpy ectocyst. The ectocyst is thinner than the endocyst and is about 25 nm thick. The endocyst is markedly thicker, usually between 200-400 nm (Fouque et al., 2012). These two layers are joined at pores called ostioles, plugged by mucus-like opercula, through which it has been proposed that the trophozoite excysts (Fouque et al., 2012). The cyst itself is not destroyed during excystment (Marciano-Cabral, 1988). Reports on the morphology of *Naegleria fowleri* cyst walls were conflicted, and this could come from the configuration of its wall. Originally, it was thought that *Naegleria fowleri* had a double-walled cyst, just like *Naegleria gruberi* (Macriano-Cabral, 1988). However, it has been shown that unlike *Naegleria gruberi*, the wall is a single endocyst-like, very thick, fibrous layer with ostioles, rather than separate layers (Fouque et al., 2012, Marciano-Cabral, 1988). *Naegleria.* cyst walls are all thought to contain some carbohydrate similar to cellulose (if not cellulose), and chitin, analogous to *Entamoeba* cysts. During cyst formation of *Naegleria fowleri*, it has been observed that enolases are induced and

likely located at the cyst wall during formation, suggesting some role in encystment (Fouque et al., 2012).

While *N. fowleri* has three distinct life stages, including the trophozoite amoeba, the flagellate, and the cyst, most studies focus upon on the trophozoite, which is known to be infective. However, limited research has been performed upon the protective cyst stage of *N. fowleri.*

Naegleria fowleri and Immunity

The reaction of the human immune system to an infection of *Naegleria fowleri* can be described as ineffective against all of the immune evasion mechanisms the amoeba has acquired. Humans make antibodies against *N. fowleri* (Rivera et al., 2001, Dubray et al., 1987, Marciano-Cabral et al., 2007), but these have been shown to offer no protection against PAM (Marciano-Cabral, 1988). Additionally, activation of complement is not protective (Jamerson et al., 2017). The amoeba has been shown to possess resistance to tumor necrosis factor α (TNF- α), IL-1, and membrane attack complexes as well (Marciano-Cabral et al., 2007). *N. fowleri* can avoid complement-mediated lysis by membrane vesiculation, and subsequent shedding or degradation, of any membrane attack components on its surface (Toney et al., 1992, Marciano Cabral et al.,1994). It also has a CD59-like protein that is thought to disrupt the membrane attack complex (MAC) and other complement-mediated components of cell lysis (Fritzinger et al., 2006). Serine/threonine and tyrosine kinases are associated with amoebae that have higher resistance to complement-mediated lysis (Chu et al., 2000).

Within the brain, the reaction of the immune system is comprised mainly of innate cells such as: neutrophils, eosinophils, and macrophages (Marciano-Cabral et al., 2007, Marciano-Cabral, 1988). In both humans and mice, neutrophils are the dominant cell type during early

infection (Martinez, 1985, Marciano-Cabral et al., 2007). *In vitro*, neutrophils have been shown to be able to kill *Naegleria* when activated by lymphokines such as TNF- α. (Marciano-Cabral et al., 2007). TNF-α therefore may play a large role in controlling early infection, with its ability to augment neutrophil number and activation. Mice that have had TNF-α depleted show a greater susceptibility and more rapid mortality when infected with *Naegleria fowleri* (Ferrante et al., 1988). TNF-α can also activate macrophages and lead to killing of *Naegleria fowleri*, by secreting cytolytic factors and phagocytosis (Marciano-Cabral, 1988, Siddiqui et al., 2016). Soluble factors collected from activated macrophages are capable of killing the amoebae (Fischer-Stenger et al., 1992, Marciano-Cabral et al., 2007). When highly pathogenic *Naegleria fowleri* is co-cultured with microglia, the resident macrophage population of the CNS, there is strong expression of pro-inflammatory cytokines including IL-1α, IL-1β, IL-6, and TNF-α (Marciano-Cabral et al., 2007). Interestingly, the pathogenicity of the strain seems to correspond with the level or intensity of activation of pro-inflammatory pathways and cytokines (Marciano-Cabral et al., 2001). However, *Naegleria fowleri* was able to destroy and ingest most cell cultures under normal conditions.

Adaptive immunity seems to play a minimal role. However, according to a survey of serum from people hospitalized for various illnesses or injuries not related to infection with *Naegleria fowleri*, the majority of humans appear to have antibodies for *Naegleria*, and other free-living amoebae, suggesting introduction to the amoebae and subsequent blockade of infection (Dubray et al., 1987). In this study of randomized serum samples from patients in the U.S, antibodies to *Naegleria fowleri* were found in 101 out of 115 of them (Dubray et al., 1987, Marciano-Cabral et al., 2007). It has been suggested that IgA and IgM are most important in nasal mucosal secretions, possibly to help prevent the initial attachment of amoebae to nasal epithelium (Rivera et al., 2001,). Upon challenge with *N. fowleri* by nasal inoculation, mice develop an IgG response as well

(Vivesvara et al., 2007), however it has also been suggested that low initial serum IgA could be the deciding factor in fatality of infection (Cursons et al., 1979). However, the amoeba has the ability to internalize and degrade antibody, and passive immune therapy has been shown to be ineffective against active infections of *Naegleria fowleri* in rabbits (Marciano-Cabral et al., 2007). Therefore, the efficacy of humoral immunity is debatable, but minimal in the clinical sense.

Mediation of Encystment of Amoebae

As proposed by Du et al (2014), enzymes called phosphodiesterases are the regulating factor of amoebic encystment. Specifically RegA, which is responsible in *Acanthamoeba* for changing cyclic AMP to linear AMP. They propose that the intracellular level of cAMP is the main factor in whether the organism will remain a trophozoite or begin the transformation into its protective cyst form. Higher levels of intracellular cAMP, thus less active RegA, or other cAMP-specific phosphodiesterases, would signal encystment. Lower levels of intracellular cAMP, thus more active RegA or other cAMP specific phosphodiesterases, would allow continued feeding in the trophozoite stage. This acts as a signal to the cell about the availability of nutritional sources and the rate of ATP production and usage within the cell.

This notion is supported in other species of amoeba by Wessels et al (2000), where once again it is reported that RegA, the cAMP-specific phosphodiesterase, is responsible for the regulation of movement and chemotaxis of the social amoeba *Dictyostelium.* RegA's phosphodiesterase activity is determined when its sensor histidine kinases and phosphatases act upon its intrinsic response regulator domain (Du et al., 2014). Inactive RegA causes the amoebae to aggregate into small clumps, and affects the coordinated action of myosin when forming pseudopods. Starving amoebae will coordinate their movements in order to form a fruiting body, which is a rigid stalk that contains protective cyst-like spores ready to disperse throughout the

surrounding environment. Extracellular cAMP acts upon G-protein coupled receptors to initiate sporulation. It is also known that the intracellular level of cAMP and its effect on protein kinase A (PKA) is important for sporulation (Wessels et al., 2000). Thus without RegA, the colony loses all sense of direction and ability to coordinate their movement, and subsequently to regulate spore formation in *Dictyostelium*. Here, RegA is shown to be an important regulator in other amoebae of movement and life cycle management. The cAMP phosphodiesterases seem to be most important in life cycle determination and coordination of movement in amoebic species. Specifically, RegA continues to be mentioned in the literature.

Through studies of many inhibitors of phosphodiesterases, Du et al. (2014) discovered that the cAMP-specific phosphodiesterase inhibitors, namely a compound called dipyridamole, was most effective in inducing early and rapid encystment of amoebae. On the other hand, W-7 HCl, an inhibitor of non-cAMP-specific phosphodiesterases, did not affect the speed of encystment of amoebae, and was used as a control to ensure specificity of the experiment in regards to which phosphodiesterases are responsible for controlling encystment. Granted, the species they used were not of the genus *Naegleria*. They used *Acanthamoeba castellanii* amoebae. However, based upon current literature, it can be hypothesized that the mechanism is highly similar, if not the same, in both social and free-living amoebae alike. I hypothesize that if the cAMP-specific phosphodiesterase inhibitor, dipyridamole, is placed upon a culture of amoebae, then they will encyst more rapidly when compared to other methods of encystment, and that W-7 HCl will not affect the speed of encystment. This would support the argument that cAMP-specific phosphodiesterases, such as RegA, are responsible for mediating encystment in *Naegleria fowleri*, as they do in other species of amoebae.

Aims of the Study

Overall the aims of this study can be summarized and categorized into three sections; *in vitro* studies, *in vitro* studies, and investigating the role of cAMP and phosphodiesterases in the encystment process. The first aim would include studies of the cyst concerning *in vitro* infection of cell cultures, and questions that should be answered by these *in vitro* experiments include: Does cell type matter in regards to infection of a cell culture and destruction of the monolayer by the amoebae that excyst? Will cysts attach readily to epithelial cells? Will cysts attach readily to immune cells? Will immune cells affect the ability of the cysts to excyst? Will using a mixture of cell types, rather than a single cell line, improve cell survival in an infection model? How long do cysts take to become trophozoites in culture on a cell monolayer, and long to destroy it after excystment?

Regarding the second aim, which includes *in vivo* studies in a murine animal model, questions that should be answered are as follows: Are cysts of *Naegleria fowleri* infective? Does inoculation with a pure culture of cysts lead to PAM in a murine model? How many cysts are needed for disease? Does the age of the mouse affect disease development?

The last aim of this study includes the investigation of cAMP and phosphodiesterases as mediators of the encystment process in *Naegleria fowleri*. Questions for this aim that should be answered are: Will inhibition of phosphodiesterases lead to the rapid encystment of trophozoites of *Naegleria fowleri*? Does intracellular cAMP concentration specifically mediate encystment in *N. fowleri* as it does in other species of amoeba?

MATERIALS AND METHODS

Amoebae

Naegleria fowleri (ATCC 30894) were obtained from the American Type Culture Collection. ATCC 30894 was originally cultured from a human patient with PAM. Amoebae were cultured in a 37°C incubator using Oxoid medium for 24-48 hours and subcultured at least every five days. Oxoid medium was prepared and consists of 0.55 % liver digest, 0.30 % glucose, 0.50 % proteose peptone, 0.25 % yeast extract, 1 % fetal calf serum (FCS) and 1 µg hemin/ml in Page's saline. The mixture was autoclaved before addition of FCS and hemin. *N. fowleri* was passaged through male $B_6C_3F_1$ mice once a month to maintain pathogenicity and virulence of the strain.

Mammalian Cell Culture.

P388.D1 murine macrophage phenotype lymphoma cells (ATCC-CCL-46), Human Nasal Epithelial cells (ATCC-CCL-30), Mixed Primary Rat Glial cells, Vero cells, and CHME3 human immortalized microglia (ATCC-CRL-3304) cells were seeded into T-25 and T-75 vented flasks and cultured at 37°C in 5% (v/v) CO2. Vero and Human Nasal Epithelial cells were cultured in Dulbecco's minimal essential medium (DMEM) purchased from ThermoFisher (Waltham, MA). The DMEM was supplemented with 10% fetal calf serum, 1% L-glutamine, 1% non-essential amino acids, 1% HEPES buffer, and 1% DMEM vitamins. P388.D1 cells, CHME3 cells, and Mixed Primary Rat Glial cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 medium purchased from ThermoFisher (Waltham, MA) with 10% fetal calf serum, 1% HEPES buffer, and 1% L-glutamine.

Cyst Culture

Cysts of *N. fowleri* were obtained utilizing a novel combination of Nelson's medium containing 50 mM $MgCl_2$. Cysts were cultured in 100 mm x 15 mm petri dishes, enclosed in larger 150 mm x 15 mm petri dishes for ease of handling and protection from contamination. Both petri dish sizes were purchased from ThermoFisher (Waltham, MA). 13 mL of Nelson's medium containing 50 mM $MgCl_2$ was added to each petri dish once confluency of 90-100% of amoebae had been reached after 24-48 hours of growth. After 7-10 days, the petri dishes were scraped using a sterile cell scraper (Southern Labware (Cumming, GA)) to harvest cysts for counting and then immediate use in *in vitro* experiments or intranasal inoculation of mice.

Cell Counts

A small volume of the cell suspension, about 200 μL, was taken and placed into a sterile microcentrifuge tube. The tube was vortexed to ensure distribution of cells. 20 uL of the cell suspension was taken and placed onto a hemocytometer with a coverslip. Four quadrants of sixteen squares were counted for cells on the hemocytometer, and this was repeated four times, twice for each side of the hemocytometer. The average number of counted cysts per quadrant was taken and multiplied by 10,000 to get the number of cells/mL of cell suspension.

Co-Culture of Mammalian Cells and Cysts of *N. fowleri*

Once removed from the petri dish, cyst cultures were placed into sterile 50 mL centrifuge tubes and spun at 1250 rpm for 8 minutes. The supernatant was removed and the pellet was washed with completed RPMI or DMEM, and then spun again at 1250 rpm for 8 minutes. Supernatant was decanted off of the pellet, and cysts were resuspended in either completed RPMI or DMEM, depending on suitability for the cell type in question. The co-culture was allowed to incubate at

37°C and 5% (v/v) CO_2. The cells with cysts were monitored and photographed using an Olympus inverted microscope for several days until complete cellular destruction, monolayer detachment, and growth of trophozoites. Media (300 µL) from the Mixed Primary Rat Glial cell co-culture were removed every 24 hours and placed into a sterile microcentrifuge tube. It was then centrifuged at 1250 rpm for 5 minutes, and the supernatant was removed to be placed in a new sterile microcentrifuge tube. It was then stored for future analysis at -80°C.

Lactate Dehydrogenase (LDH) Cytotoxicity Assay

A Pierce LDH Cytotoxicity Assay kit (Thermo Fisher, Waltham, MA) was used to measure the release of LDH from damaged cells. Mixed glial cells (p1) in a T-75 flask were trypsinized with 0.25% trypsin once 100% confluent and subcultured into two new T-75 flasks. Once both flasks were 90-100% confluent, 14 mL of a 5.6 x 10^5 cysts/mL solution in RPMI media were placed onto one flask of mixed glial cells, while 14 mL of RPMI without cysts were placed onto the other flask of mixed glial cells. Culture supernatant (200µL) was removed from each flask consecutively for eight days. Once removed, the supernatant was centrifuged for 10 minutes at 1250 rpm and stored at -80˚C until use in the LDH cytotoxicity assay. In a CorningStar flat-bottom 96 well plate, a positive LDH control was prepared by placing 50 µL of 1x Positive LDH Control in duplicate wells. 50 µL each of the treated group (the supernatant from mixed glial cells with cysts) and the untreated group (the supernatant from mixed glial cells without cysts) were placed in duplicate wells. 50 µL of fresh RPMI media were placed in duplicate to account for base LDH activity. To each well, 50 µL of previously prepared Reaction Mixture was added. The plate was allowed to incubate at room temperature for 30 minutes with minimal light exposure. Then, 50 µL of Stop Solution was added to each well, and the

absorbance was measured at 490 nm and 680 nm. The absorbance at 680 nm was subtracted from the absorbance at 490 nm to account for the background signal of the instrument.

Trypan Blue Exclusion Assay

Equal parts (1:1) 0.4% Trypan Blue solution was added to co-cultures of cells and amoebae after several days of observation to the point of monolayer disruption and destruction. The mixture was incubated at room temperature for one minute before being pipetted into both sides of a hemocytometer and observed under 40x magnification. Cells that were colored blue were considered to be non-viable.

Co-cultures For Scanning Electron Microscopy

P388D1 and Human Nasal Epithelial cells were cultured on coverslips placed in 6-well plates. Once cells reached confluency of the monolayer, 2 mL of pure cyst cultures of 5.5×10^5 cysts/mL in completed DMEM were placed on each well of the Human Nasal Epithelial cells, and 7.5×10^5 cysts/mL in completed RPMI were placed on each well of the P388D1 cells. Coverslips of each cell-type with cysts were placed in a 4% solution of glutaraldehyde for fixing at 24 hours, 48 hours, and 72 hours after inoculation with the cyst cultures. Scanning electron microscopy photos were taken by Judy Williamson.

In vivo Inoculation of Mice

Male $B_6C_3F_1$ (3-8 weeks) were inoculated intranasally with cysts ranging from 1×10^4 cysts to 4×10^5 cysts. The mice were observed daily for symptoms of Primary Amoebic Meningoencephalitis, including bowed head and loss of appetite. The mice were sacrificed no sooner than ten days to allow ample time for disease manifestation. Brain tissue from sacrificed

mice was removed and placed in an incubator at 37C with Oxoid medium to culture for trophozoites of *Naegleria fowleri*.

Inhibitors of Phosphodiesterases

Dipyridamole and W-7 HCl were purchased from ThermoFisher (Waltham, MA). Amoebae were cultured in a 6 well plate, in Oxoid medium, for 24 hours at 37C in the incubator. 2 mL of 100 uM Dipyridamole in 0.1% DMSO and 100 uM W-7 in 0.1% DMSO were placed in separate wells, while 2 mL of Oxoid, 50 mM MgCl2 Nelson's, Molecular Biology grade filtered water, and 0.1% DMSO were placed on the other four wells as controls. The plate was monitored and photographed for several days using an Olympus inverted microscope, using field of view (20x) cyst counts to calculate encystment percentages over time (n=3). Percent encystment= ((# of cysts)/(# of amoeba + # of cysts)) x 100

Inhibitor-Treated Cyst Culture cAMP Competitive ELISA

Cell lysates prepared using a freeze-thaw method were treated with 0.1M HCl to stop endogenous phosphodiesterase activity, and Triton X 100 to ensure lysis of the cysts. To ensure maximum antibody binding, samples were acetylated with 10 μL of a mixture of 1:2 acetic anhydride and triethylamine per 200 μL of sample or standards. Neutralizing Reagent (50 μL) was added to each well except the total activity and the blank wells. HCl (0.1M, 100 μL) was added to non-specific background (NSB) and maximum binding wells. 50 μL additional 0.1M HCl were added to the NSB wells. Then 100 μL of sample or standards were added to the appropriate wells. Blue cAMP-AP conjugate (50 μL) was added to each well, except the total activity, blank, and NSB wells. cAMP antibody (50 μL) was added to each well, except the total activity, blank, and NSB wells. The plate was sealed and incubated at room temperature on a rocker for 2 hours. The

wells were emptied by vigorously tapping on a clean paper towel, and then washed by adding 400 µL of the 1x wash buffer, emptying, and repeating for a total of three times. All liquid was emptied from the wells before 5 µL of the cAMP-AP conjugate was added to the total activity wells. Then, 200 µL of the pNpp substrate solution was added to each well and incubated at room temperature for one hour. Stop solution (50 µL) was added to each well and the plate was read by a microplate reader at 405 nM, making sure to subtract the blank readings from all others. To generate the standard curve and analyze the raw data from the ELISA, the Stats Blue Logarithmic Regression Calculator software at http://stats.blue/Stats_Suite/logarithmic_regression_calculator.html was utilized.

Statistical Analyses

Statistical analyses were performed using a student's t-test. A p-value of <0.01 was considered statistically significant (Microsoft Excel 2016).

RESULTS

Cysts of *Naegleria fowleri* attach to and facilitate the destruction of cell cultures of many different phenotypes.

To determine the infectivity of cysts in an *in vitro* experimental model, cysts were co-cultured with cells of different phenotypes (Figures 2-5). These studies show that the cysts are able to attach to epithelial, immune, and mixed cell cultures (Figure 1, Figure 8, Figure 9). Excystment begins as early as 48 hours post inoculation (Figure 7). Even a fast growing, aggressive cell type with a macrophage phenotype was unable to slow the excystment process (Figure 4). The firm attachment of cysts occurred within 1-1.5 hours post-inoculation when the co-culture was placed in the incubator at 37°C and 5% CO_2 (v/v) (data not shown), and even forceful movement of the flask or plate could not dislodge them. This was true of all cell types investigated in this study. The mixed glial cells were the cell type upon which the cysts excysted the quickest, and subsequently were destroyed by the amoebae most efficiently (Figure 1, Table 2). The CHME3 cells, a cell line of human immortalized microglia, lasted the longest upon a challenge with cysts, on average (Figure 1, Table 2). It took longer for the cysts to excyst and for the monolayer to be destroyed when co-cultured with CHME3 cells.

Cysts May Damage Cells Upon Initial Adhesion To the Monolayer, and Facilitate Further Damage by Trophozoites Upon Excystment.

The course of potential damage by cysts and amoebae was documented by an LDH Cytotoxicity assay performed on the supernatant of a co-culture of glial cells with cysts, and compared to a culture of the same glial cells that remained uninfected. The LDH release of the co-culture by day 8 was significantly increased from the LDH release of the uninfected culture (Figure

6). Day 1 post-inoculation showed a spike in LDH release, as well as day 5. The increase in LDH release on day 5 coincides with the observation of trophozoites having excysted in the culture (data not shown).

Cysts of *Naegleria fowleri* Are Not Infective *in vivo* and Do Not Facilitate the Development of PAM in a Murine Model

Cysts were inoculated intranasally into male $B_6F_3C_1$ mice in four separate trials. Immediately after inocluation, cysts from each inoculum were cultured in Oxoid medium to check for viability, and each batch of cysts utilized were viable (data not shown). After being allowed to develop any symptoms of disease for a suitable amount of time, the mice were sacrificed and their brain tissue was cultured in Oxoid medium for the presence of trophozoites. All trials, regardless of decreasing the age and increasing the number of cysts for inoculation, show the cultures to be negative, and that the cysts do not facilitate PAM in a murine model (Table 1).

Encystment is Partially Mediated By Intraceullular cAMP and cAMP specific Phosphodiesterases

To show that the encystment process is mediated, at least partially, by the intracellular cAMP concentration and by the function of phosphodiesterases, the phosphodiesterase inhibitors dipyridamole and W-7 were placed onto cultures of trophozoites, as well as 0.1% DMSO as a control, and monitored for encystment rates. Dipyridamole-treated cultures exhibited a significant increase in the rate of encystment compared to 0.1% DMSO and W-7 (Figure 10, Figure 11, Table 3). To confirm the efficacy of the inhibitors utilized, the intracellular concentration of cAMP was measured using an ELISA. The intracellular concentration of cAMP was only increased significantly by the dipyridamole (Figure 12) when compared to 0.1% DMSO.

Table 1. *In vivo* Inoculation of Mice Shows No Infectivity of Cysts. Summary of *in vivo* experiments intranasally inoculating male $B_6C_3F_1$ mice with cysts of *Naegleria fowleri.* D.p.i sac'd refers to the number of days post-inoculation with cysts that the mice were sacrificed.

Trial	# of Mice	# of Cysts	D.p.i. sac'd	Result
1	6	1×10^4	10	Negative
2	3	2×10^4	10	Negative
3	3	4×10^4	19	Negative
4	3	1×10^5	22	Negative

In vivo experiments intranasally inoculating male B6C3F1 mice with cysts of *Naegleria fowleri* were performed. The first trial included six 8 week old male mice, and each was given 1×10^4 cysts in a volume of 20 μL of 1x PBS. After 10 days to allow the appearance of symptoms of a PAM-like disease, the mice were sacrificed when none were observed. The brain tissue was removed and placed into Oxoid medium in T-25 flasks to culture any trophozoites present. The brain tissue was negative for *Naegleria fowleri* infection. The second trial included three 3 week old male mice, and each was given 2×10^4 cysts in a volume of 20 μL of 1x PBS. After 10 days to allow the appearance of symptoms of a PAM-like disease, the mice were sacrificed when none were observed. The brain tissue was removed and placed into Oxoid medium in T-25 flasks to culture any trophozoites present. The brain tissue was negative for *Naegleria fowleri* infection. The third trial included three 3 week old male mice, and each was given 4×10^4 cysts in a volume of 20 μL of 1x PBS. After 10 days to allow the appearance of symptoms of a PAM-like disease, the mice were sacrificed when none were observed. The brain tissue was removed and placed into Oxoid medium in T-25 flasks to culture any trophozoites present. The brain tissue was negative for *Naegleria fowleri* infection. The fourth and last trial included three 3 week old male mice, and each was given 1×10^5 cysts in a volume of 20 μL of 1x PBS. After 10 days to allow the appearance of symptoms of a PAM-like disease, the mice were sacrificed when none were observed. The brain tissue was removed, and placed

into Oxoid medium in T-25 flasks to culture any trophozoites present. The brain tissue was negative for *Naegleria fowleri* infection.

Table 2. Co-Cultures of Cells and Cysts Show Infectivity *in vitro*. Averages of days to excyst and days to destroy the monolayer of each cell type (n=3). Each cell type was monitored over the course of destruction by *N. fowleri* after inoculation with cysts ranging from 5 x 10^5 to 7.8 x 10^5 cysts/mL, depending on cell type and trial. The co-cultures were then monitored and photographed each day using an EVOS FL digital inverted microscope (ThermoFisher, Waltham, MA) until complete destruction of the monolayer was observed. The days on which the cysts excysted and on which the monolayer was observed to be destroyed were recorded and cells were removed and were confirmed to be at least 95% dead by trypan blue exclusion assay.

Cell Type:	**VERO**	**CHME3**	**P388D1**	**Mixed Glial**
Avg. Days to Excyst	5.330 ± 1.528	8.000 ± 1.414	5.000 ± 1.732	3.333 ± 0.577
Avg. Days to Destroy Monolayer	11.67 ± 1.699	13.00 ± 1.000	9.000 ± 1.414	8.333 ± 2.054

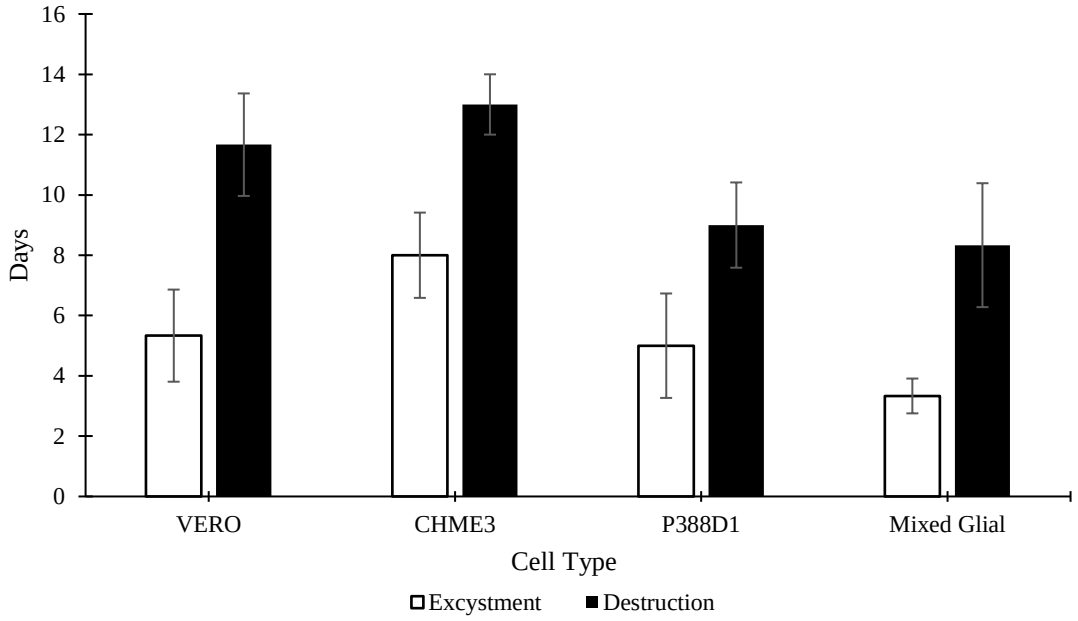

Figure 1. Excystment of *N.fowleri* and Destruction of Cells. Each cell type was monitored over the course of destruction by *N. fowleri* after inoculation with cysts ranging from 5 x 10^5 to 7.8 x 10^5 cysts/mL, depending on cell type and trial. The co-cultures were then monitored and photographed each day using an EVOS FL digital inverted microscope (ThermoFisher, Waltham, MA) until complete destruction of the monolayer was observed. The days on which the cysts excysted and on which the monolayer was observed to be destroyed were recorded and cells were removed and were confirmed to be at least 95% dead by trypan blue exclusion assay.

Vero Cells

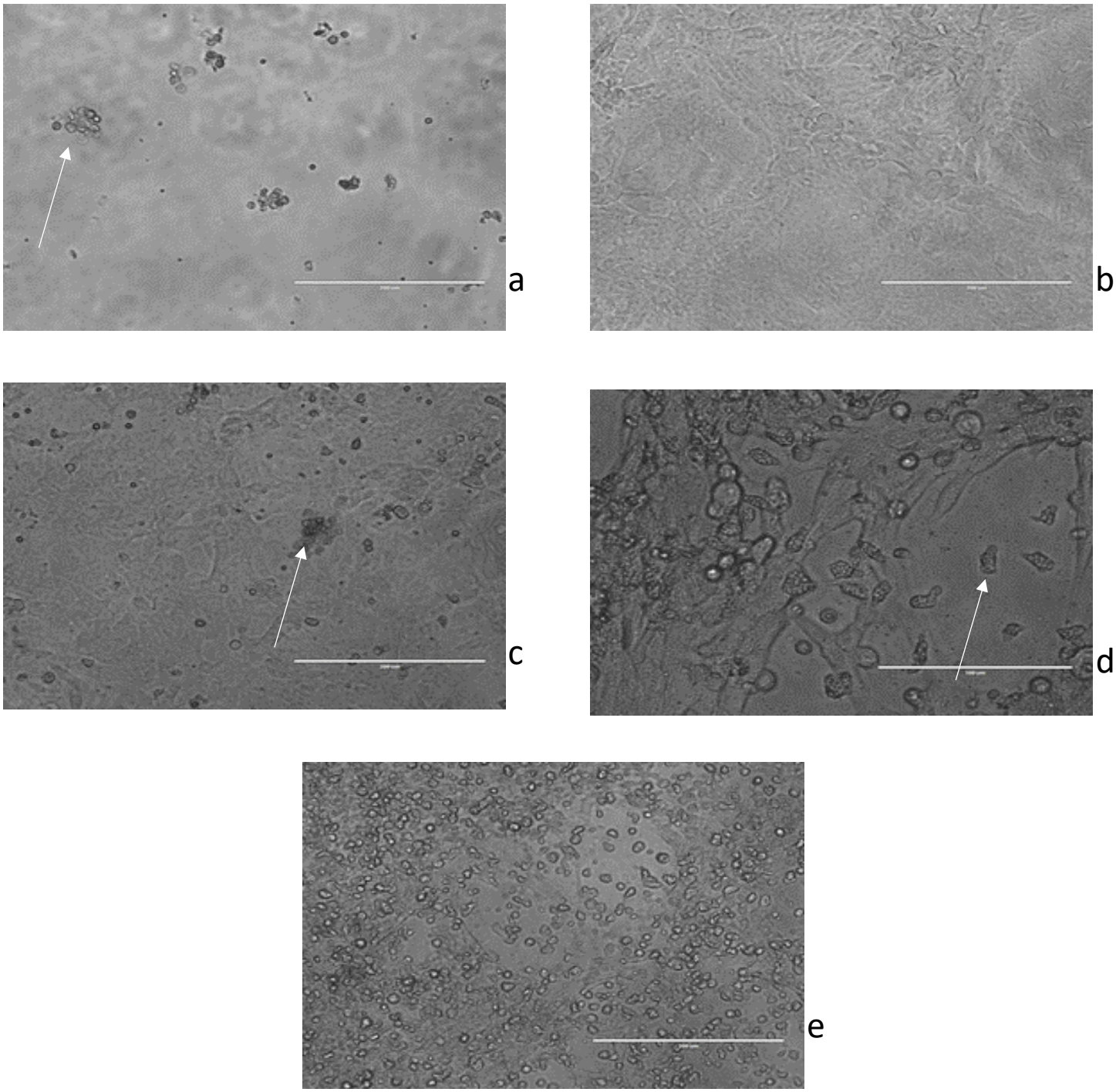

Figure 2. Representative Vero Cell Co-Culture with Cysts. Representative Vero cells inoculated with *N.fowleri* cysts at 20x magnification. Scale bar = 200 uM. a) Cyst inoculum (arrow) of 1.58 x 10^5 cysts/mL,

1.8 mL, 6 well plate, b) Vero cell monolayer, c) inoculation of cysts (arrow) onto monolayer of Vero cells, d) excystment of amoebae (arrow) begins at 7 d.p.i.,, at 40 x magnification e) total destruction of Vero cell monolayer and marked growth of amoebae at 10 d.p.i.

CHME3 Cells

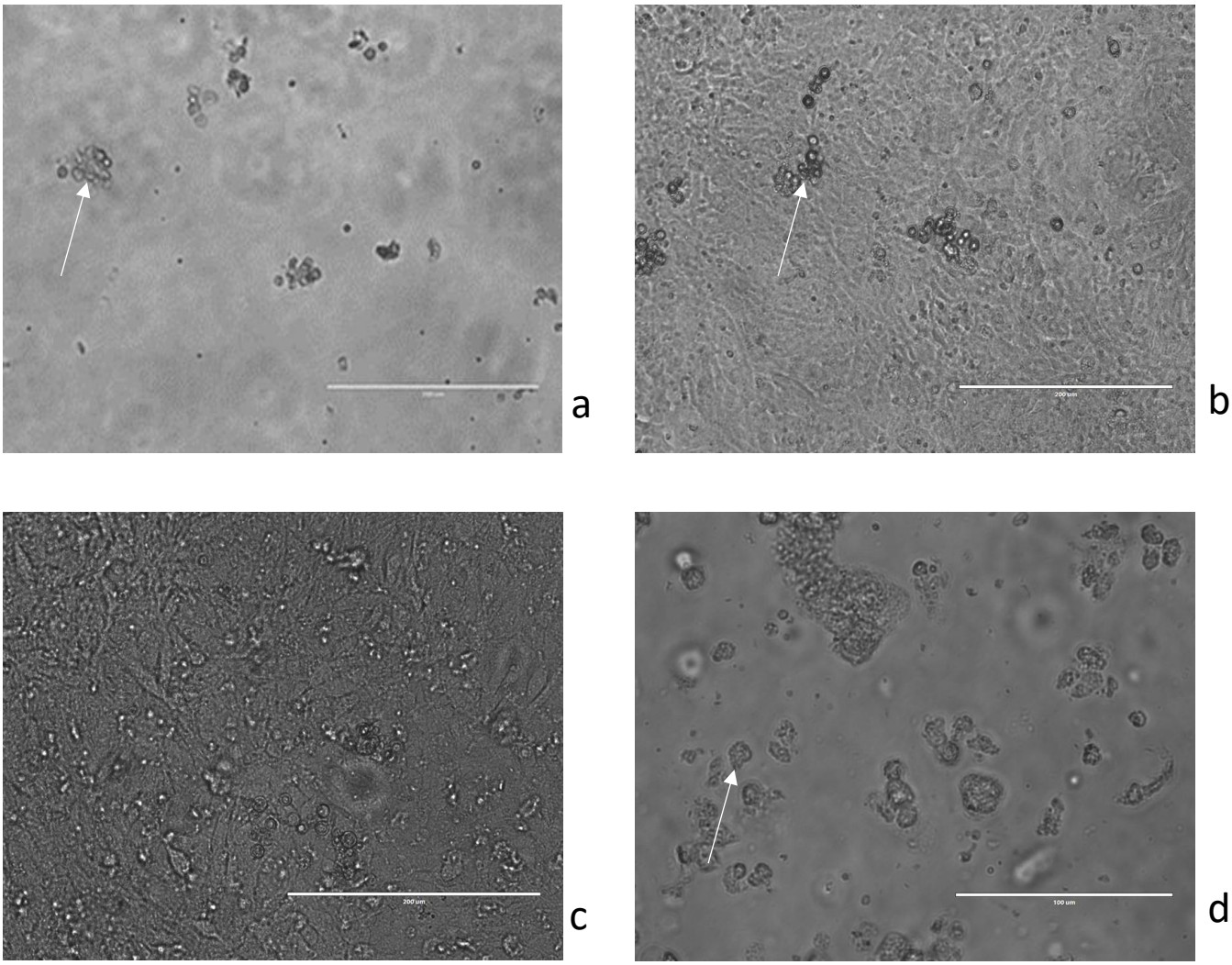

Figure 3. Representative CHME3 Cell Co-Culture with Cysts. Representative CHME3 cells inoculated with cysts of *N.fowleri* at 20x magnification. Scale bar = 200 uM. a) Cyst inoculum (arrow) of 7.55 x 10^5 cysts/mL, 2 mL, 6 well plate, b) Inoculation of cysts (arrow) upon the monolayer of CHME3 cells, c) excystment of *N.fowleri* begins at 8 d.p.i., d) destruction of the CHME3 monolayer at 12 d.p.i. by amoebae (arrow).

P388D1 Cells

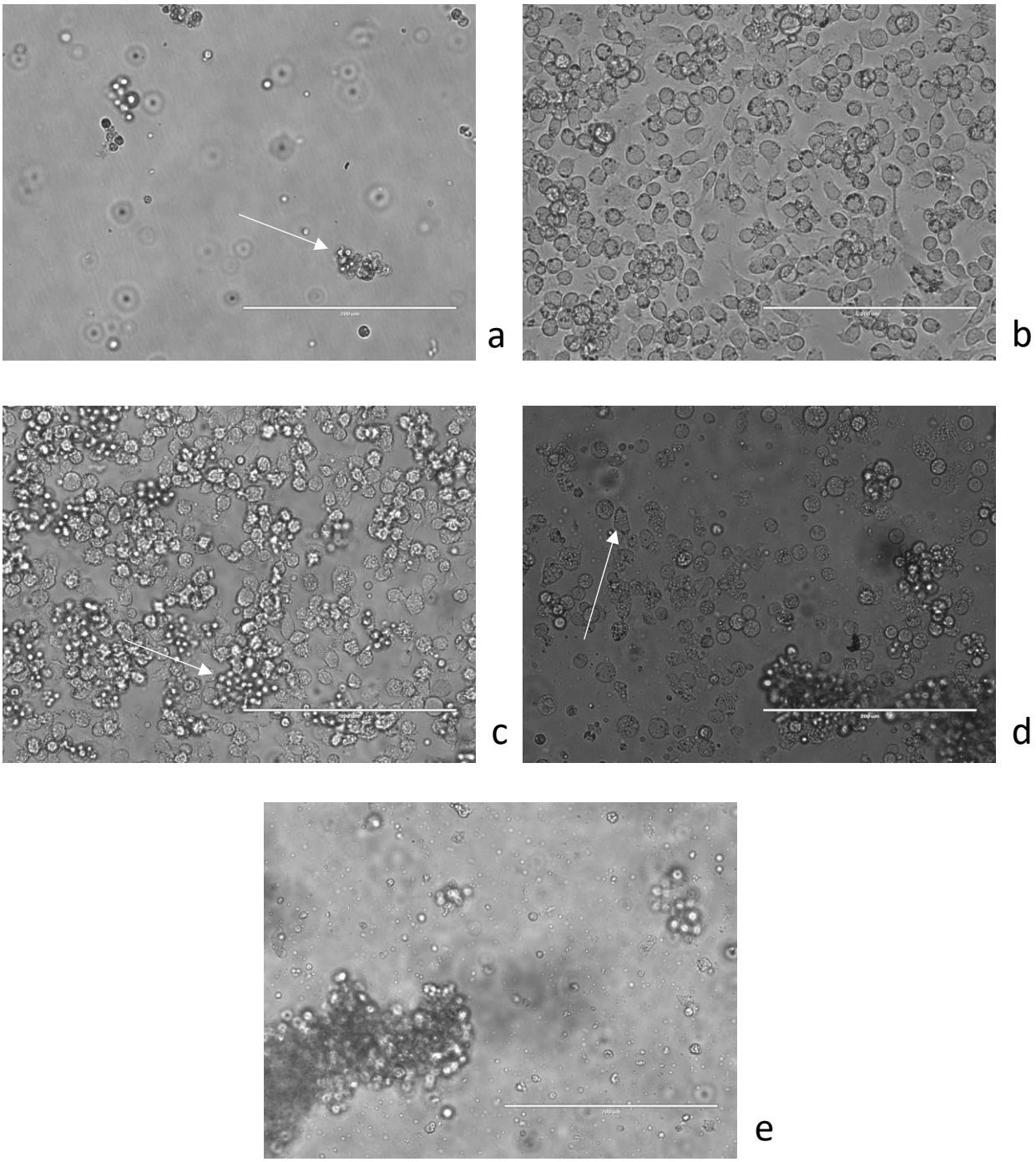

Figure 4. Representative P388.D1 Cell Co-Culture with Cysts. Representative P388D1 cells inoculated with cysts of *N.fowleri* at 20x magnification. Scale bar = 200 uM. a) Cyst inoculum (arrow)

of 7.55 x 10^5 cysts/mL, 2 mL, 6 well plate, b) Monolayer of P388D1 cells, c) Inoculation of cysts (arrow) onto the monolayer of P388D1 cells, d) Excystment of amoebae (arrow) begins at 4 d.p.i., e) Destruction of the monolayer of P388D1 cells by amoebae at 8 d.p.i.

Rat Primary Mixed Glial Cells

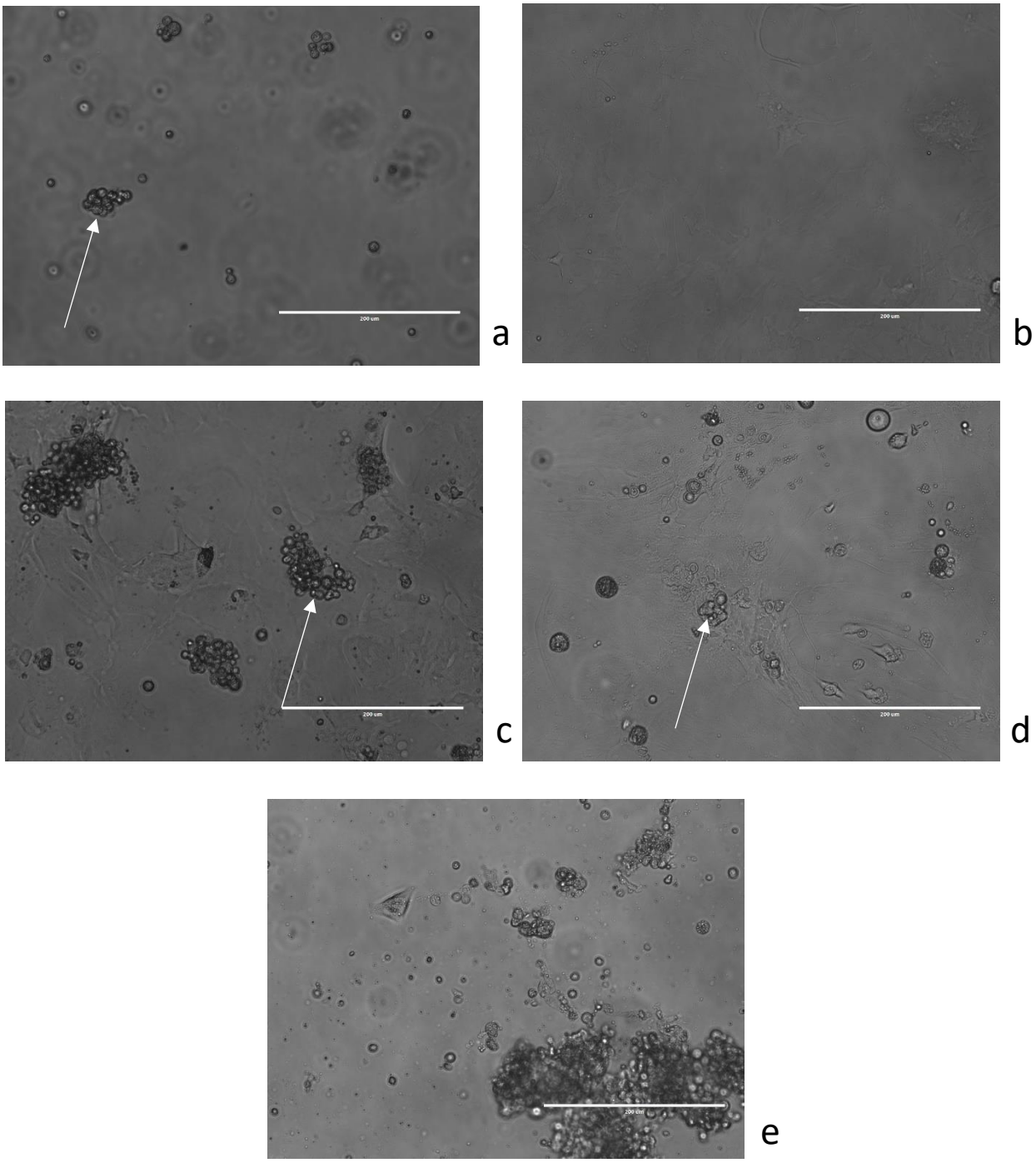

Figure 5. Representative Rat Primary Mixed Glial Cell Co-Culture with Cysts. Representative Rat Primary Mixed Glial cells inoculated with cysts of *N.fowleri* at 20x magnification. Scale bar = 200 um. a)

Cyst inoculum (arrow) of 5.5 x 10^5 cysts/mL, 30 mL, large flask, b) Monolayer of Rat Primary Mixed Glial cells, c) Inoculation of cysts (arrow) onto the monolayer of Rat Primary Mixed Glial cells, d) Excystment of amoebae (arrow) begins at 4 d.p.i., e) Destruction of the monolayer of Rat Primary Mixed Glial cells by amoebae at 11 d.p.i.

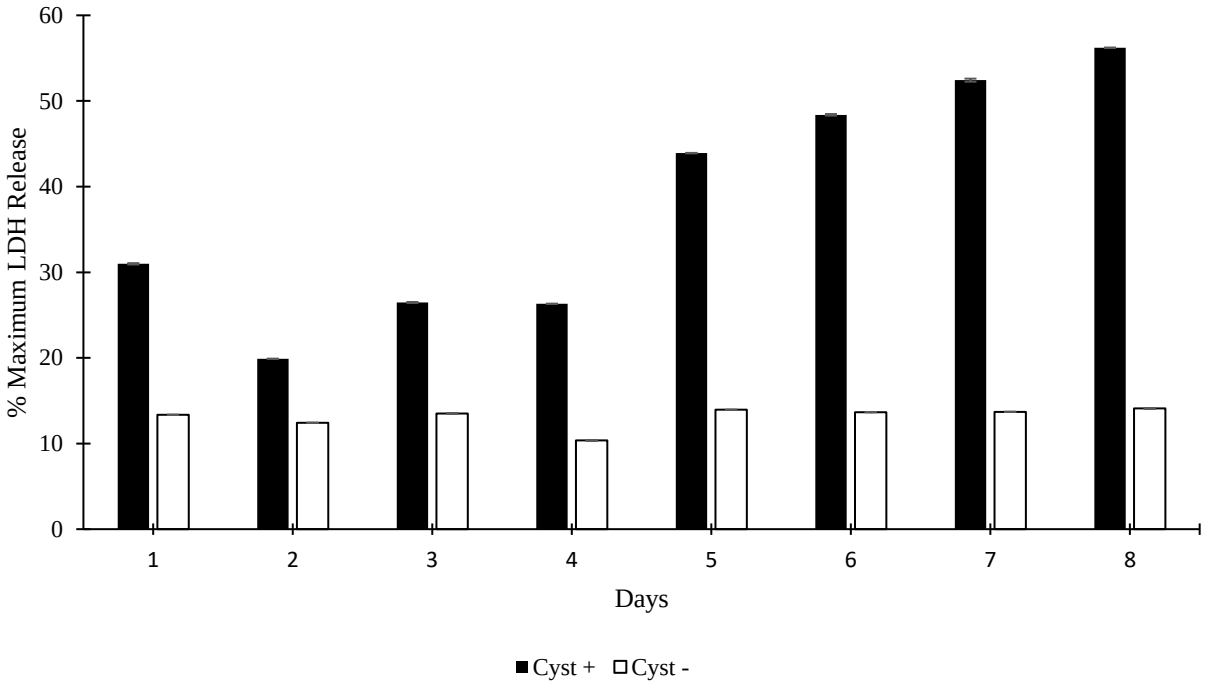

Figure 6. Rat Mixed Glial Cells Released LDH Quickly After Inoculation with Cysts. 14 mL of a 5.6 x 10^5 cysts/mL suspension in RPMI was placed on a 100% confluent T-75 flask of rat mixed glial cells. Each day post-inoculation with cysts, 200 μL of supernatant was removed from of the co-culture and centrifuged in a microcentrifuge tube for 10 minutes at 1250 rpm. The supernatant from placed in a new sterile microcentrifuge tube and stored at -80ºC until analysis. Simultaneously, a culture of the same batch of rat mixed glial cells was grown in a T-25 flask, without cysts, and the same procedure was followed over a period of eight days for both the untreated and cyst treated cultures. An LDH Cytotoxicity Assay was performed in duplicate on the untreated and treated cultures, and each reading was compared to the reading for the concentrated LDH positive control, to yield a percentage of maximum reading, or maximum LDH release.

Scanning Electron Microscopy

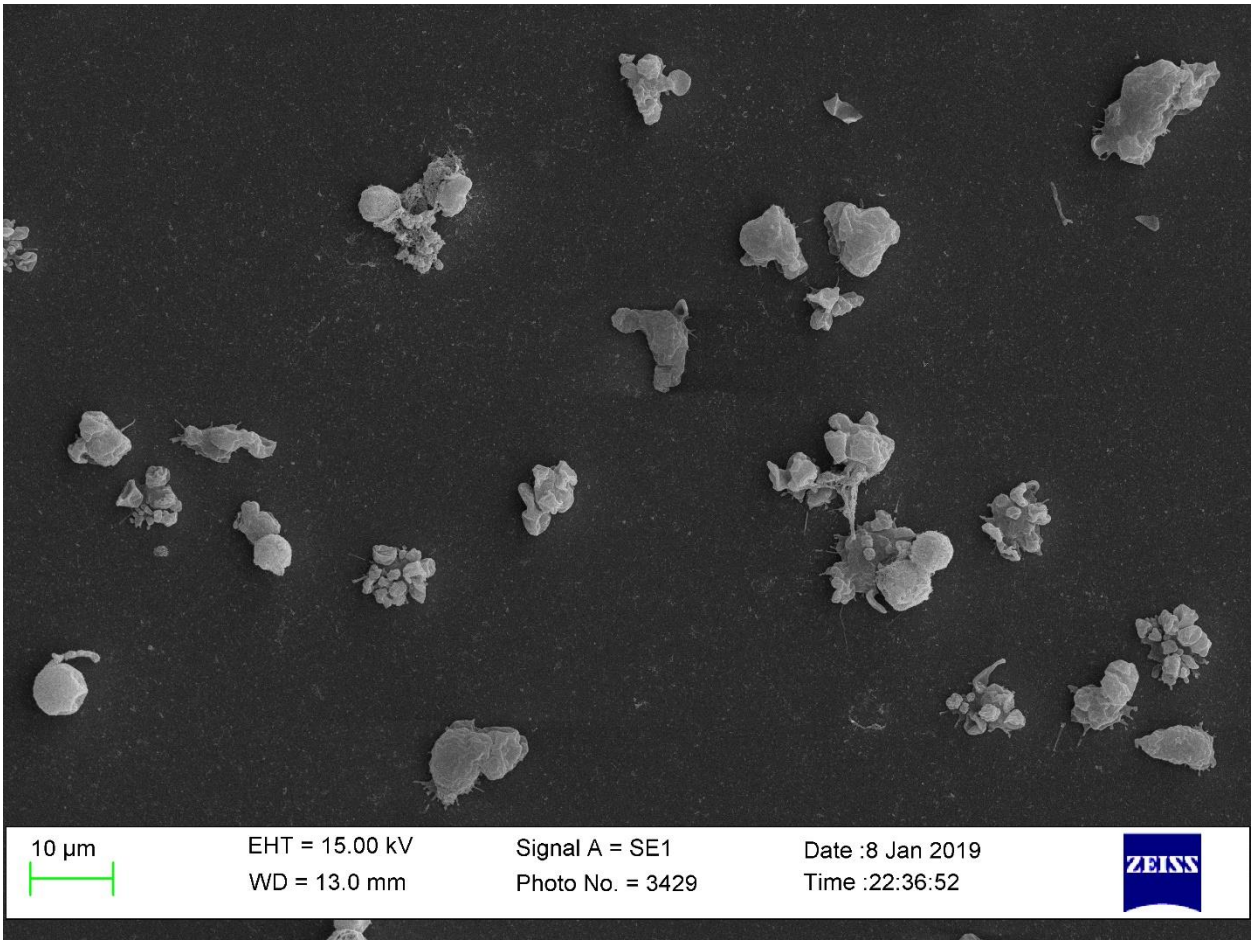

Figure 7. Scanning Electron Micrograph of Excysting Cysts In The Presence of Cells. A scanning electron micrograph of a culture of cysts of *N. fowleri* beginning to excyst. To culture these cysts, a T-75 flask of amoebae was cultured at 37ºC for 48 hours until 100% confluency. The flask was bumped to dislodge the amoebae into suspension. A 100 mm petri dish was seeded with 4 mL of this suspension, and 6 mL of fresh Oxoid media was added. The 100 mm petri dish was placed within a 150 mm petri dish for protection. After 24 hours of growth at 37ºC, the Oxoid was removed and 14 mL of Nelson's Medium containing 50 mM $MgCl_2$ was added. The petri dish was placed onto a rocker to prevent drying of the cysts within the petri dish. Once the culture was 100% encysted, these cysts were placed onto a 50% confluent culture of human nasal epithelia in DMEM. This shows excystment at 48 hours post-inoculation.

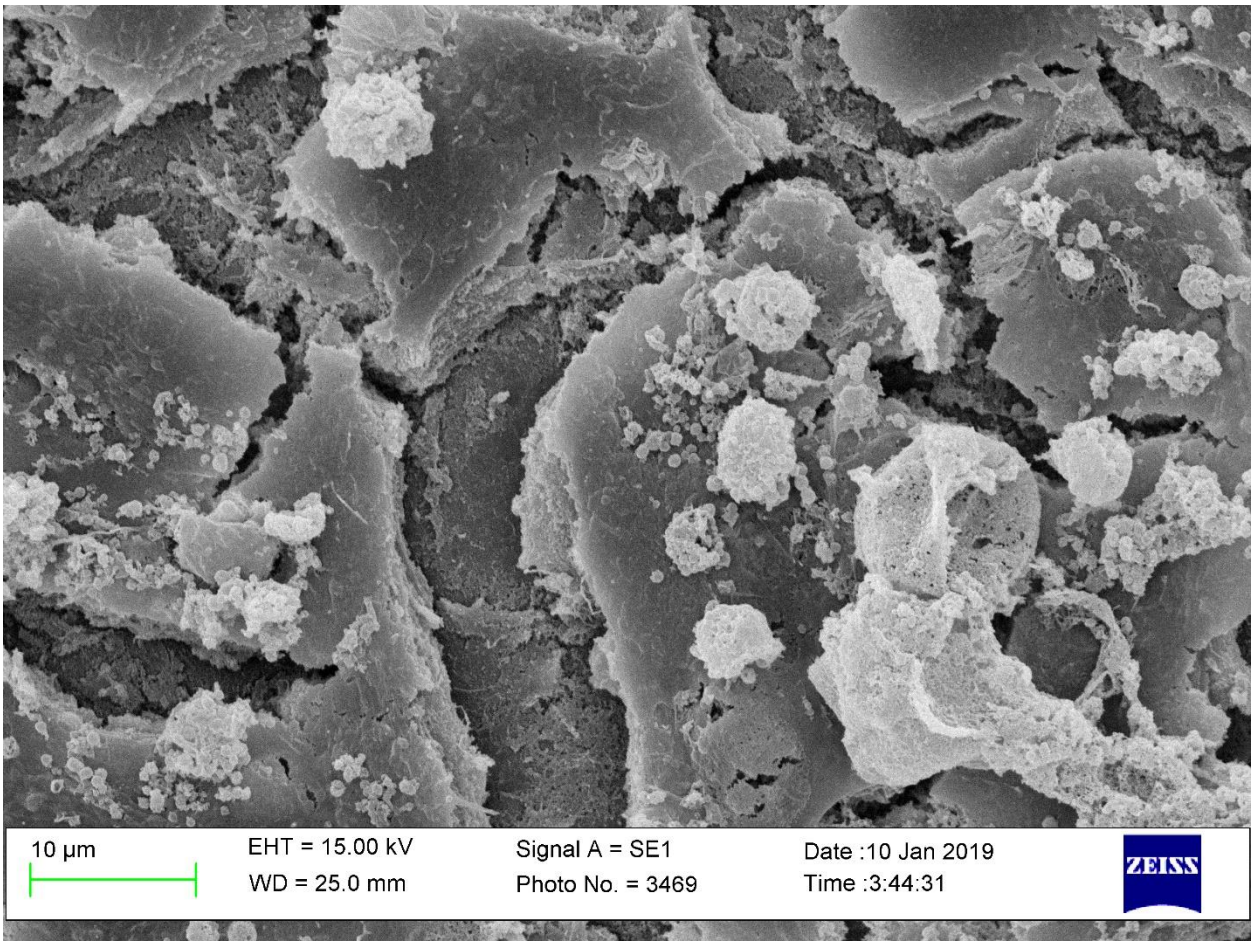

Figure 8. Scanning Electron Micrograph of Cysts on Human Nasal Epithelia. A scanning electron micrograph of a culture of cysts of *N. fowleri* beginning to excyst on human nasal epithelia. This shows firm attachment to the human nasal epithelium 48 hours post-inoculation with cysts. The culture of human nasal epithelium was cultured in a T-25 flask for 5 days before being trypsinized and placed onto coverslips within a 6-well plate, with one coverslip per well. The culture was allowed to grow to 50% confluency before adding 2 mL to each well of a 5.5 x 10^5 cysts/mL solution of DMEM. The co-culture was monitored for 48 hours before adding a 0.4% solution of glutaraldehyde to fix the co-culture for scanning electron microscopy.

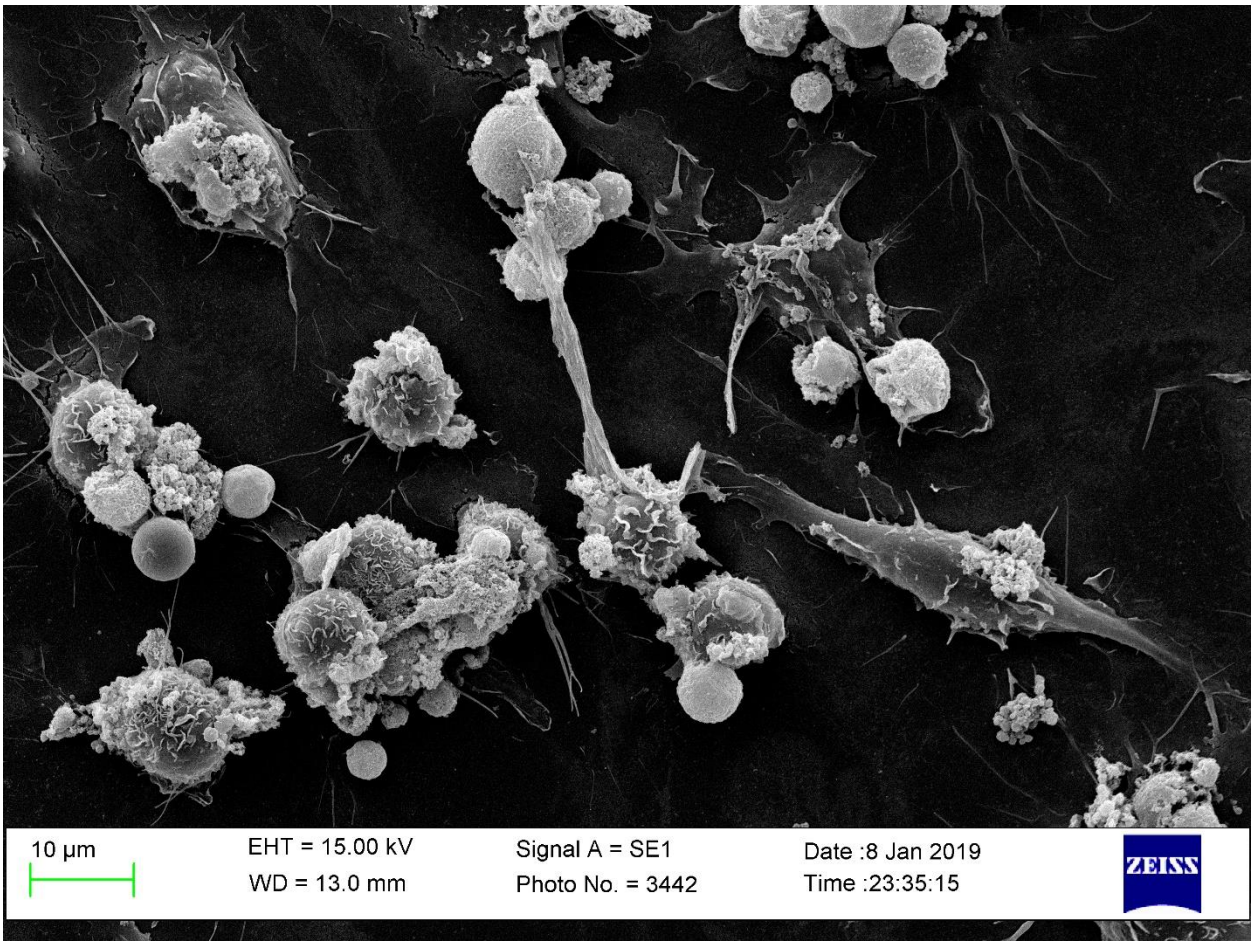

Figure 9. Scanning Electron Micrograph of Cysts on P388.D1 Cells. A scanning electron micrograph of a culture of cysts of *N. fowleri* attaching to and beginning to excyst on P388.D1 cells. This shows firm attachment to the cells 48 hours post-inoculation with cysts. The culture of P388.D1 cells was cultured in a T-75 flask for 5 days before being trypsinized and placed onto coverslips within a 6-well plate, with one coverslip per well. The culture was allowed to grow to 80% confluency before adding 2 mL to each well of a 7.5 x 10^5 cysts/mL solution of RPMI. The co-culture was monitored for 48 hours before adding a 0.4% solution of glutaraldehyde to fix the co-culture for scanning electron microscopy.

Table 3. The Rate of Encystment Was Increased by Dipyridamole. A summary of percentages of encystment of amoebae on each day post-treatment with each solution. Percentages were calculated by average total counts of cysts out of total amoebae in quadrants for each field-of-view, in field-of-view at 20x magnification (n=3).

	100 µM Dipyridamole (%)	100 µM W-7 (%)	200 µM Dipyridamole (%)	200 µM W-7 (%)	50 mM MgCl2 Nelson's Medium (%)	0.1% DMSO (%)	MBG H2O pH 7 (%)
24 hours	8.90	5.94	4.99	N/A (Fatal)	9.50	4.17	3.38
48 hours	37.24	13.20	10.44	N/A (Fatal)	16.88	17.75	21.50
72 hours	43.39	21.93	16.07	N/A (Fatal)	24.61	39.96	42.39
96 hours	51.50	51.61	27.00	N/A (Fatal)	35.50	47.80	67.53
120 hours	81.59	58.40	32.21	N/A (Fatal)	45.97	62.55	58.46

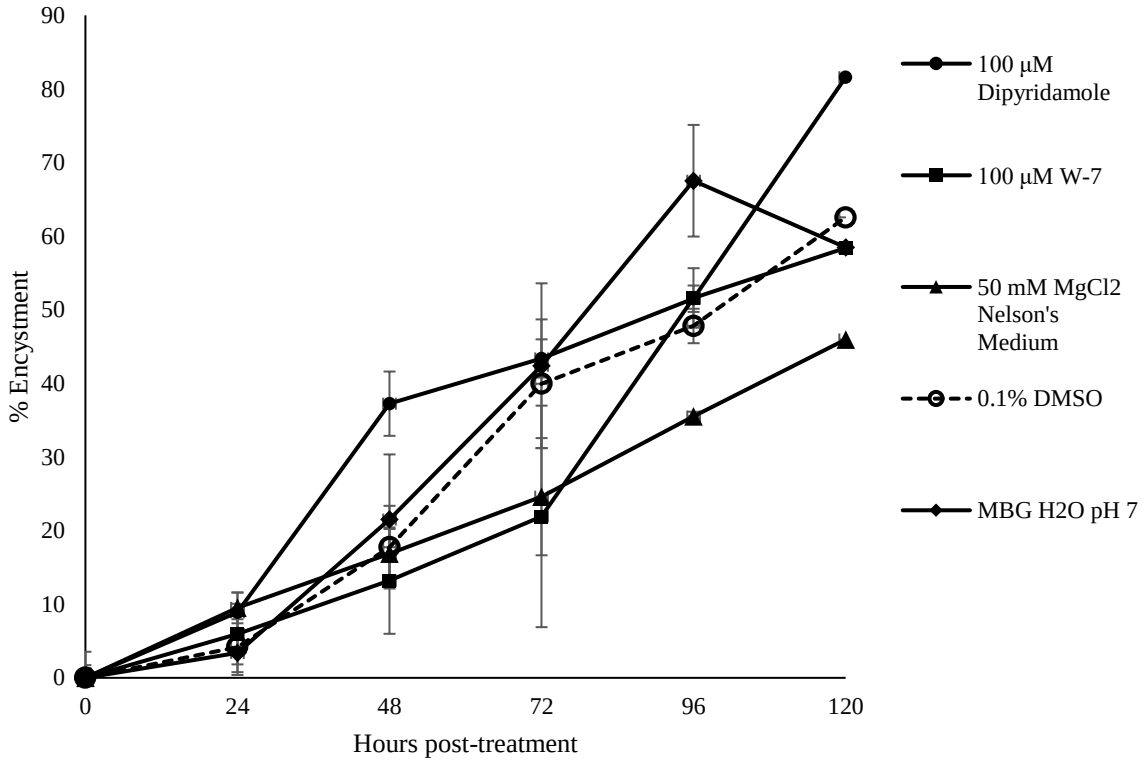

Figure 10. Dipyridamole Induced More Rapid Encystment Than Any Other Treatment. A graph of the data in Table 2 showing the rates of encystment of *N. fowleri* over time upon addition of each indicated solution. Amoebae were cultured in a 6 well plate, in Oxoid medium, for 24 hours at 37C in the incubator. 2 mL of 100 uM Dipyridamole in 0.1% DMSO and 100 uM W-7 in 0.1% DMSO were placed in separate wells, while 2 mL of Oxoid, 50 mM MgCl2 Nelson's, Molecular Biology grade filtered water, and 0.1% DMSO were placed on the other four wells as controls and comparisons. The plate was monitored and photographed using an EVOS FL digital inverted microscope for several days, using field of view (20x) cyst counts to calculate encystment percentages over time (n=3). Percent encystment= ((# of cysts)/(# of amoeba + # of cysts)) x 100

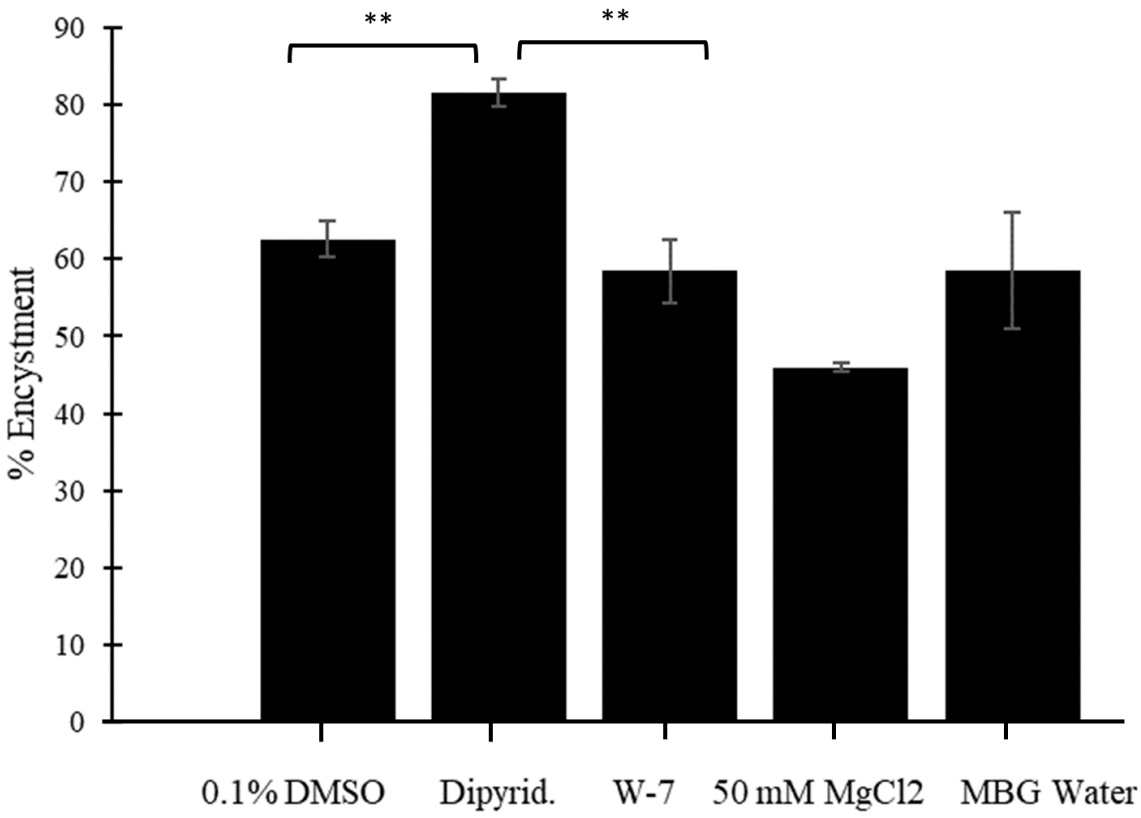

Figure 11. Dipyridamole Significantly Increased Encystment By Day 5. A comparison of the percentages of encystment of amoebae after 5 days of treatment with the indicated solutions. Amoebae were cultured in a 6 well plate, in Oxoid medium, for 24 hours at 37C in the incubator. 2 mL of 100 uM Dipyridamole in 0.1% DMSO and 100 uM W-7 in 0.1% DMSO were placed in separate wells, while 2 mL of Oxoid, 50 mM MgCl2 Nelson's, Molecular Biology grade filtered water, and 0.1% DMSO were placed on the other four wells as controls and comparisons. The plate was monitored and photographed using an EVOS FL digital inverted microscope, using field of view (20x) cyst counts to calculate encystment percentages at day five post-treatment with the above solutions (n=3). Percent encystment= ((# of cysts)/(# of amoeba + # of cysts)) x 100. Significance was determined with a two sample Student's t-test assuming unequal variances ($p<0.01$).

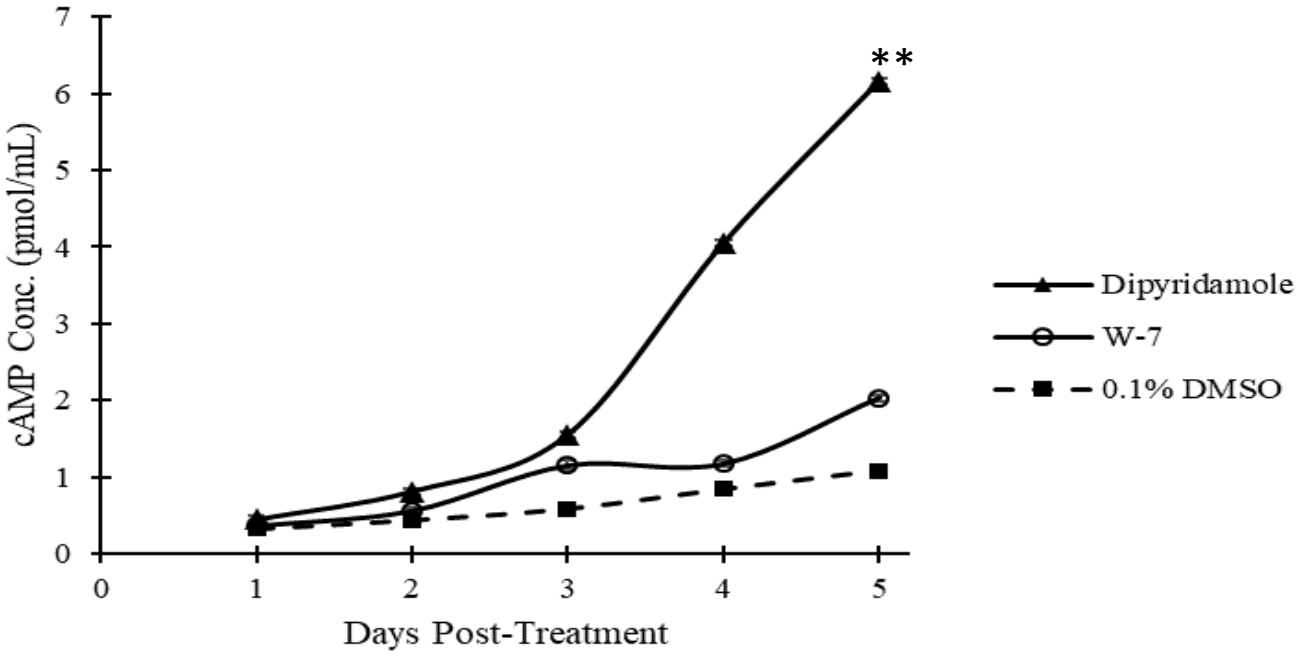

Figure 12. Dipyridamole Significantly Increased Intracellular cAMP. cAMP ELISA data showing the intracellular concentration of cAMP over a period of 5 days. Fifteen T-75 flasks of amoebae were cultured for 48 hours at 37°C in Oxoid medium until 100% confluency. The Oxoid was removed and in five flasks each, 10 mL of each treatment (100 uM dipyridamole, 100 uM W-7, or 0.1% DMSO) was placed on the cultures. On each day (24 hours) post-treatment, one flask from each treatment group was scraped and the amoebae/cyst suspensions were removed from the flask and placed into a 50 mL conical tube (Sigma-Aldrich, St. Louis, MO). The tubes were centrifuged for 20 minutes at 1250 rpm and the pellet was washed with 1 x PBS. The tubes were centrifuged again for 10 minutes and the supernatant was removed. The pellet was resuspended in 700 μL of 0.1M HCl before undergoing the freeze-thaw method of lysate preparation. The samples were aliquoted and stored at -80°C until analysis by cAMP competitive ELISA according to manufacturer's instructions (ThermoFisher, Waltham, MA). Samples were normalized for 1 mg of protein before use. Stars signify a difference between dipyridamole and 0.1% DMSO. Significance was determined with a two sample Student's t-test assuming unequal variances ($p<0.01$).

DISCUSSION

The cysts of the free-living amoeba *Naegleria fowleri* are persistent and hardy, able to withstand harsh environments without nutrients for prolonged periods of time. All of the infectivity and disease manifestation studies to date have been performed on the trophozoite of *Naegleria fowleri.* It was unknown whether or not the cyst form of the species can infect living hosts and manifest disease *in vitro* and *in vivo.* This current study shows that they lose their ability to infect nutrient-rich mammalian hosts when in cyst form. Even increasing numbers of cysts inoculated intranasally into young mice (three weeks old) did not result in manifestation of disease (Table 1). Often times, the age of the animal will affect the ability of a pathogen to establish disease. Indeed, the pattern of infection that fatal cases of PAM has followed in the past includes mostly young male children, rather than adults. In this case, that did not hold true, as neither mice at three weeks or eight weeks of age were positive for infection with *Naegleria fowleri*. Yet, the cysts were able to infect cell cultures *in vitro* and completely disrupt and lyse the monolayers of all cell types used in this study (Table 2). *In vitro* experimental results cannot be translated into *in vivo* experiments. Often times, the complexity of living organisms is lost in *in vitro* experiments, so while the cysts were able to attach to and ultimately destroy cell cultures, there is clearly some reason for why the cysts cannot do the same for living organisms. Perhaps given enough time to attach to the nasal epithelium of the mice, the cysts may have been able to excyst in time to migrate up the olfactory nerves. However, the behavioral tendency of mice to expel the foreign liquid in their nasal area and to scratch at their nose could all be contributing factors to the lack of disease manifestation. The cysts appear to require a stationary scaffold upon which to attach to living tissue, making it impossible to infect a living animal with cysts. This problem does not occur with the trophozoite, as it possesses the machinery necessary to attach firmly with pseudopodia to a living surface,

despite a moving target. Another consideration to take into account is that the inoculation protocol may be able to be improved. Perhaps in order to prevent expulsion of cysts by the mice, the same number of cysts should be inoculated into the animal within a smaller volume. This could help to minimize the effects of behavioral barriers to infection such as expulsion of liquid, through sneezing or scratching of the nose.

Regarding *in vitro* infection of cell cultures, *Naegleria fowleri* has a robust ability to attach to cells, in both cyst and trophozoite form. Across all cell types utilized in this study, the cyst was able to firmly attach to the cell surface. After a certain amount of time, shown in Table 2, the amoebae were able to fully excyst and begin secreting cytolytic factors and pore-forming proteins that ultimately led to the complete disruption of the monolayer, and subsequent lysing of the cells. The hypothesis that immune cells may be able to last longer against a challenge by cysts of *Naegleria fowleri* was moderately supported, as the CHME3 cells and the Rat Primary Mixed Glial cells lasted the longest out of the cell types. Future experiments should include the analysis of cytokines, especially TNF-α, secreted by these immune cells and by the community of cells in the Rat Primary Mixed Glial cells. Considering the implications of the importance of TNF-α in the literature, it would be crucial to examine the effect of crosstalk between the cell types in the Rat Primary Mixed Glial cells.

The basis for examining cAMP's role in mediating the encystment process is firmly supported by literature that utilizes closely related species of free-living and social amoebae. Phosphodiesterases and cAMP are essential for regulating transformation from one life stage to another in genera such as *Acanthamoeba* and *Dictyostelia*. In addition, the close relative *Naegleria gruberi* has been shown to possess a copy of the gene RegA that is known to be a cAMP-specific phosphodiesterase found in *Acanthamoeba* genomes. The hypothesis that the encystment of

Naegleria fowleri is also mediated by a cAMP-specific phosphodiesterase, and thus the level of intracellular cAMP, was supported, as shown in Table 3 and Figures 11 and 12. The dipyridamole, an inhibitor of cAMP-specific phosphodiesterases, caused encystment to occur at a higher rate and to yield the highest percentage of cysts within a culture of amoebae after 5 days ,or 120 hours, of treatment.

There were differences that were noted between the *Acanthamoeba* experiments [Du et al. 2014] and these studies upon *N. fowleri. Acanthamoeba* encysted quicker, with up to 80% encystment being achieved upon day 3 of treatment (Du et al., 2014). In this study, *N. fowleri* encysted up to 81% by day 5, and so the encystment process in relation to incubation with the inhibitor diypridamole is slower by two days. This is not unusual, as the phylogenetic distance between the two genera is enough such that phenotypic differences are to be expected. Yet, the data suggests that the pathway of encystment, utilizing cAMP as a secondary messenger, is highly similar between *Acanthamoeba* and *Naegleria*.

Interestingly, increasing the concentration of the inhibitors changed the results. The encystment percentage for 200 μM dipyridamole decreased significantly (Table 3), while the 200 μM W-7 appears to be fatal to *Naegleria fowleri.* This is particularly fascinating, as W-7 can be used in humans as a drug to treat ribosomal disorders, fibrotic eye disorders, and in combination with other drugs. Perhaps W-7 could be a promising drug for early treatment of PAM if the physiological concentration is relevant. It is not likely that the physiological concentration of W-7 after being administered to a patient will be as high as 200 μM, however, perhaps it may become useful as more is discovered about *Naegleria fowleri*'s interaction with host immunity.

The examination of cytotoxicity through the use of an LDH Cytotoxicty assay showed, as expected, a substantial increase in the release of LDH by mixed glial cells after inoculation with

cysts. By day 8 of co-culturing the mixed glial cells with the cysts, the LDH release was nearly four-fold what the untreated mixed glial cells were releasing (Figure 6). This increase coincides with the trophozoites coming out of the cyst and beginning to digest the cells. However, something unexpected was that the LDH release increased by two-fold within one day of co-culture with the cysts, when compared to the untreated cells. This is unexpected because the cysts are metabolically inactive. They do not release cytolytic factors, and are physically unable to mechanically consume cells. It makes it interesting that there is substantial damage to cells from the cysts alone. Perhaps the act of adhesion itself is damaging to the mixed glial cells by compromising the integrity of the cell membrane. The cysts were observed to adhere to surfaces very firmly when culturing them in petri dishes, as they require a cell scraper to remove, and require longer centrifugation in order to make sure all cysts are in the pellet and not adhered to the walls of the tube. It is also possible that there is a mechanism of attachment that is currently unknown that damages cells while it is in the cyst form. Further study is required to elucidate the source of this finding.

A question that remains unanswered has to do with the outlier for the Rat Primary Mixed Glial cells (data not shown). The first trial of co-culture with cysts of *Naegleria fowleri* and the Rat Primary Mixed Glial cells ended with the cysts dying, and the monolayer surviving. This trial was inoculated at the same time and with the same culture of cysts that the third trial of CHME3 cells were inoculated with, with 2 mL each well in a 6 well plate of 7.55 x 10^5 cysts/mL in completed RPMI medium. CHME3 cells were completely killed by amoebae in 8 days. The glial cells were damaged at first by trophozoites excysting and beginning to lyse them. However, the cells recovered and the cysts were checked for viability. The cysts floating in the media were all nonviable, and the cell culture began recovering the patches that had been cleared by the amoebae on day 41 post-inoculation with cysts. The cysts were clearly viable, since amoebae came out and

began to lyse parts of the monolayer, before disappearing and becoming cysts once more. In hindsight, it would have been most pertinent to have been collecting media supernatant and cell samples off of the co-culture in order to do proper analyses, such as flow cytometry to determine cell type composition. Perhaps the composition of an important cell type for survival was higher in this particular primary culture. In the future, more replicates would need to be done to attempt to repeat and recreate this anomaly. Otherwise, the Mixed Glial cells followed a similar pattern of disruption and destruction as the other co-cultures did. The hypothesis concerning the better ability of a community of cells to survive destruction by the cysts and amoebae was not supported by these results. It shows that both single cell type cultures and community cultures are quickly disrupted and lysed by the amoebae once they excyst. In fact, the mixed glial cells fared the worst compared to the single cell type cultures, as far as excystment rate and destruction of the monolayer (Figure 1).

In conclusion, the aims of this study answered many questions that had never been asked about the cyst form of *Naegleria fowleri*. Concerning *in vivo* studies, it has been shown that the cyst is not capable of infecting a mammalian host under these circumstances. The age of the mouse did not affect disease manifestation, nor did the number of cysts used for inoculation.

For *in vitro* studies, the data demonstrates that the cysts are infective on cell cultures, in that they are able to attach to multiple cell types regardless of phenotype and facilitate destruction through excystment into the trophozoite. The adhesion of the cyst itself may cause damage to the cell membrane itself, as evidenced by day one monitoring the LDH release (Figure 6). All cell types typically have a basal level of LDH activity in culture, due to normal cell turnover. However, this spike in LDH is most likely not attributed simply to the addition of cysts, as another amoeba genera, *Dictyostelium*, was surveyed for enzyme activities, and Lactate Dehydrogenase activity

was found to be significantly lower, almost negligible, compared to other surveyed cell types (Cleland et al. 1968). Therefore, the spike in LDH activity can reasonably be attributed to the adhesion of the cysts on day one, and not simply their presence in the co-cultures.

Concerning the role of cAMP and phosphodiesterases in the mediation of encystment, this study shows that dipyridamole significantly increased both the intracellular concentration of cAMP and the rate of encystment when compared to 0.1% DMSO, and W-7. This indicates that intracellular cAMP specifically plays a role in the encystment process.

Taking all of these findings into account, in future studies utilizing *Naegleria fowleri*, more studies on glial cells should be performed to answer the question: Why did one trial of glial cells survive inoculation with cysts? The gender of the rats from which the cells came from should be noted, and compared between males and females. Cytokine ELISAs can help clarify the immune reaction to inoculation with cysts, and help to show the reaction of the cells immediately upon cyst adhesion. It's likely that they will release pro-inflammatory cytokines, such as TNF-α and IL-1, which would support the claim that cysts may damage cells during the adhesion process. Flow cytometry should be performed upon the glial cells to show what cell subpopulations are present and in what percentages. For example, this could show that a high number of a certain cell type is most important in survival. Without knowing exactly what subsets of cells, and how many of them they are in the culture to begin with, this remains a variable that could be crucial to the understanding of infectivity *in vitro.*

In addition to glial cell studies, it could be important to study how the addition of chitinases and cellulases to a culture of cysts would affect excystment. Since the cyst form is markedly less harmful to cell cultures than the trophozoite form, and knowing that the cyst is composed of chitin and cellulose, perhaps adding inhibitors of enzymes that may be used to excyst could keep the

amoeba in the cyst form longer. This would be useful given that the amoebae eventually die while in the cyst form if kept inside of it long enough. If patients were given enough time to recover, and could keep the amoebae inside the cyst long enough for them to die, this could improve the suvivability of PAM, which is abysmal at present.

Another future direction would be to perform these studies with different strains of *N. fowleri.* There are many different strains with very different virulence patterns, and this could be reflected in different phenotypes regarding infection.

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