Cytopathic Effects of a Pathogenic *Naegleria fowleri* and an Anti-Nfal Antibody on Rat Microglial Cells

 아주 대학교 대학원

 의학과

 오영환
Cytopathic Effects of a Pathogenic *Naegleria fowleri* and an Anti-Nfa1 Antibody on Rat Microglial Cells

by

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*Naegleria fowleri*, a free-living amoeba, causes fatal primary amoebic meningoencephalitis (PAME) in experimental animals and humans. On the pathogenic mechanisms of *N. fowleri* concerned with host tissue invasion, the adherence of amoeba to host cells is most important. The *nfa1* gene (360 bp) was previously cloned from a cDNA library of pathogenic *N. fowleri* by immunoscreening, and produced a 13.1 kDa recombinant protein (rNfa1) that showed the pseudopodia specific localization by immunocytochemistry. On the basis of an idea that the pseudopodia-specific Nfa1 protein seems to be involved in the pathogenicity of *N. fowleri*, the cytopathic activity of *N. fowleri* trophozoites co-cultured with rat microglial cells was observed, and effects of an anti-Nfa1 antibody as treating on a co-culture system was elucidated. By a light, scanning and transmission electron microscope, *N. fowleri* trophozoites contacted with microglial cells produced vigorous pseudopodia and food-cups structure. Microglial cells were destroyed by *N. fowleri* trophozoites as showing the necrotic and apoptotic cell death in a time-dependent manner. As the results of $^{51}$Cr release assay, *N. fowleri* showed 17.8, 24.9, 54.6 and 98.0% cytotoxicity against microglial cells at 3, 6, 12 and 24 hr post-incubation, respectively. On contrary, *N. fowleri* treated with an anti-Nfa1 antibody (1:100 dilution) showed 15.5, 20.3, 50.7 and 66.9% cytotoxicity, respectively. And then, microglial cells co-cultured with *N.
*Naegleria fowleri* trophozoites secreted cytokines, TNF-α, IL-1β and IL-6, as the protective immune response.

Key words: *Naegleria fowleri*, PAME, *nfa1* gene, microglial cell, anti-Nfa1 antibody, cytotoxicity
TABLE OF CONTENTS

ABSTRACT i
TABLE OF CONTENTS iii
LIST OF FIGURES v
LIST OF TABLES vi

I. INTRODUCTION 1
II. MATERIALS AND METHODS 8
   A. Amoeba 8
   B. Preparation of an anti-Nfa1 polyclonal antibody 8
   C. Microglial cell culture 9
   D. Light microscope 9
   E. Scanning electron microscope 10
   F. Transmission electron microscope 10
   G. Chromium release assay 11

H. Cytokine measurement 12

III. RESULTS 13
   A. Microscopic findings of microglial cells 13
   B. Morphology of N. fowleri characterized by pseudopodia
      and food-cups structure
   C. Cytopathic effects of microglial cells co-cultured
      with N. fowleri trophozoites 16
      1. Morphologic changes of microglial cells co-cultured
         with N. fowleri trophozoites 16
      2. In vitro cytotoxicity of N. fowleri trophozoites
         on microglial cells 20

D. Cytokines release from microglial cells co-cultured
with *N. fowleri* trophozoites 22

IV. DISCUSSION  26

V. CONCLUSION  30

BIBLIOGRAPHY  31

국문요약  37
LIST OF FIGURES

Fig. 1. Life cycle of *Naegleria fowleri* 2

Fig. 2. Light microscopic findings of primary cultured rat microglial cells 14

Fig. 3. Electron microscopic findings of microglial cells 14

Fig. 4. Electron microscopic findings of *Naegleria fowleri* trophozoites 15

Fig. 5. Light microscopic findings of microglial cells co-cultured with *Naegleria fowleri*, or with an anti-Nfa1 antibody for 6 hr 17

Fig. 6. SEM findings of microglial cell co-cultured with only *Naegleria fowleri* trophozoite, or with an anti-Nfa1 antibody 18

Fig. 7. TEM findings of microglial cell co-cultured with *Naegleria fowleri* trophozoite 19

Fig. 8. Cytotoxicity of *Naegleria fowleri* trophozoites co-cultured with microglial cells, or with an anti Nfa1 antibody 21

Fig. 9. Amounts of TNF-α secreted from microglial cell with *Naegleria fowleri* trophozoites 23
Fig. 10. Amounts of IL-1β secreted from microglial cells with *Naegleria fowleri* trophozoites 24

Fig. 11. Amounts of IL-6 secreted from microglial cells with *Naegleria fowleri* trophozoites 25
LIST OF TABLES

Table 1. The results of the in vitro cytotoxicity of *Naegleria fowleri* trophozoites on microglial cells 21

Table 2. Amounts of TNF-α secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites 23

Table 3. Amounts of IL-1β secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites 24

Table 4. Amounts of IL-6 secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites 25
I. INTRODUCTION

*Naegleria fowleri*

*N. fowleri* a free-living amoeba found in widespread areas in moist soil, water and sediment, exists as a virulent pathogen causing fatal primary amoeba meningoencephalitis (PAME) in experimental animal and humans (Visvesvara and Chang, 1974; Brown, 1979; Zubiaur, 1983). The infection results from introduction of water containing amoebae into the nasal cavity and subsequent passage of the organisms to the central nervous system (CNS) via the olfactory apparatus (Carter, 1968, 1970, 1972). Most human infections with *N. fowleri* have been associated with swimming in warm water, but other reports of infection include tap water (Warhurst and Mann, 1980) and hot baths (De Jonchkeere, 1982).

*N. fowleri* is thermophilic, thriving best in temperatures from 35°C to 46°C tolerating temperature of 40 – 45°C, while *N. gruberi* is nonpathogenic having an optimal growth temperature of 22 – 35°C. In its life cycle, *N. fowleri* has three stages; trophozoite, cyst and a temporary flagellate stage (Fig. 1) (John, 1982; Martinez, 1985; Marciano-Cabral, 1988). Alternatively, trophozoites can occur in two forms, amoeboid and flagellate. The amoeboid form is elongated with a broad anterior end tapered posterior end. The trophozoite, measuring 10 – 20 μm, is the infective and reproductive form and characterized by a nucleus with a large karyosome surrounded by a halo and a dense, prominent central nucleus.
Fig. 1. The life cycle of *Naegleria fowleri*. *N. fowleri* changes its shape according to the environmental conditions. Three stages are amoeboïd, flagellate, and cyst.
It moves with broadly rounded, granule-free processes that erupt from the surface; granular cytoplasm flows into them. Frequently, protrusions are formed in quick succession at different points on the surface of the cell so that its shape is constantly changing. This pseudopodial granular cytoplasm may have ingested erythrocytes. A contractile vacuole, which ruptures by emptying itself and reforms within a few seconds, is visible in the cytoplasm and serves as a valuable aid in diagnosing and recognizing the presence of amoebic trophozoites and also in avoiding the misidentification.

*N. fowleri* trophozoites are found in cerebrospinal fluid (CSF) and tissue, and flagella forms are found in CSF. Cysts have a single nucleus almost identical to that seen in the trophozoite. They are generally round, measuring from 7 to 10 μm, and there is a thick double wall. The flagellate form is pear-shaped, with two flagella at the broad end. These flagellate forms do not divide, but when the flagella are lost, the amoeboid forms resume reduction (Ma et al., 1990).

PAME occurs most commonly in healthy, young adults, nonimmunocompromised children and associated with swimming or bathing in contaminated warm waters. The incubation period of this disease produced by *N. fowleri* may vary from 2 to 3 days to as long as 7 to 15 days. It has an acute onset with severe headache, anorexia, nausea, vomiting, fever (from 38°C - 40°C), ataxia, sign of meningeal irritation, and encephalitis (Ma et al., 1990).

The olfactory neuroepithelium is the anatomic site of the primary lesion in PAME due to *N. fowleri*. Subtentacular cells of the olfactory neuroepithelium are capable of active phagocytosis, which appear to be the
mechanism by which *N. fowleri* penetrates CNS. During it’s invasion and migration into the CNS, *N. fowleri* follows the mesaxonal spaces of the unmyelinated olfactory nerve. The olfactory nerve terminates in the olfactory bulb, which is located in the in the subarachnoid space bated by the CSF. The olfactory bulbs are lined by the pia-arachnoid membranes and are in close contact with the subarachnoid space. This space in richly vascularized and constitutes the route of dissemination of the amoeba to other areas of the CNS (Martinez, 1985).

The final diagnosis of PAME is based on the isolation and culture of free-living amoeba from CSF or the demonstration of amoebic trophozoites in biopsied brain tissue. Antibodies may be detected in serum; however, serologic tests usually are of no value in the diagnosis of infection with free-living amoeba (John, 1982). Amphotericin B reportedly cured one case of PAME (Ma et al., 1990).

**Pathogenicity**

To date, the factors which determine susceptibility to *Naegleria* infection and subsequent development of PAME have not been definitively established. There is no evidence for hereditary immunologic deficiencies. Several investigators have suggested that *N. fowleri* releases cytolytic substance which account for invasiveness and tissue damage in vivo and for cytopathogenicity in vitro (Culbertson, 1971; Brown, 1979; Chang, 1979; Zubiaur, 1983). Indeed, electron microscopy of brain from mice infected with *N. fowleri* revealed areas of extensive demyelinization containing trophozoites surrounded by a clear halo (Carter, 1968; Culbertson, 1971; Chang, 1974). It has been proposed that the release of phospholipolytic enzymes by *N. fowleri*
results in the rapid destruction of brain tissue (Chang, 1979). Acid phosphatase activity of *N. fowleri* amoeba in CNS lesions has been detected (Feldman, 1977). Acid phosphatase has been detected also, in vivo along membranes of the host-parasite interface.

In same free living amoeba, *Acanthamoeba*, cytopathogenic effects of *Acanthamoeba* on host cells require adhesion of amoeba to host cell (Yang et al., 1997; Shin et al., 2001), phagocytosis, amoebic proteolytic enzymes include serine proteases (Hadas and Mazur, 1991; Mitra et al., 1994, 1995), contact-dependent metalloproteases (Khan et al., 2000), elastases (Ferrante and Bates, 1988), cysteine proteases (Hadas, 1993), and cytotoxic proteinases, induced by mannose-mediated adhesion (Leher et al., 1998). It has been shown that adhesion is one of the crucial steps for the pathogenicity of amoeba (Yang, 1997) as non-pathogenic amoeba exhibit significant decreased binding to host cells. In addition, Khan et al. (2000) reported that pathogenicity would be a complex process which involves both contact-dependent and contact-independent pathways in order to kill host cells quickly and to reduce the degree to which defense can be induced.

**Microglial cells involved in host defense within the CNS**

Microglia, the resident macrophages of the CNS (Perri and Gordon, 1989), are generally considered to have a mesodermal origin and to be derived from the macrophage precursors originating in the bone marrow, that infiltrate the brain during fetal development to established a resident population (Cuadros and Navascues, 1989). Resting microglia form a neuron-glial network with potential macrophage-like functions (Nakajima and Kohsaka, 1993), including expression of major histocompatibility complex class
II (MHC class II) (Sedgwick et al., 1993), intercellular adhesion molecule I (ICAM-I) (Zielasek et al., 1993), phagocytosis of microorganisms (Chao et al., 1992; Nakajima and Kohsaka, 1993), and free radicals (i.e. nitric oxide (NO) and reactive oxygen intermediates (RO)) (Chao et al., 1993, 1996; Hu et al., 1995). On the other hand, microglia represent a sensor for pathological events in the CNS (Kreutzberg, 1996). During the development of inflammatory processes, microglia migrate, differentiate, proliferate, and display amoeboid shape, and phagocytose degenerating cells at sites of inflammation. This type of cell, also named activated microglia, plays an important role in immune host defense (Chao et al., 1993, 1994). These cells may also contribute to neuronal injury (Hu et al., 1995; Chao et al., 1996).

Microglial cells occur in three morphological forms following cell differentiation, i.e., an amoeboid form during embryogenesis, a ramified shape in the mature normal brain, and a rod shaped morphology around inflammatory lesions in the CNS (Giulian and Baker, 1986; Suzumura et al., 1991). Moreover, they function as phagocytotic cells and produce cytokines such as interleukin-1 (IL-1), IL-6, and tumor necrosis factor alpha (TNF-α) (Chao et al., 1992; Suzumura et al., 1993). Thus, it has been suggested that microglial cells play an important role as inflammatory cells or immunoregulatory cells in the protective immune system of the CNS (Suzumura et al., 1993).
In the previous study, an antigenic gene, *nfa1*, was cloned from a cDNA library of pathogenic *N. fowleri* by immunoscreening using the immune and the infected sera, and produced a recombinant 13.1 kDa protein. In addition, the immunolocalization study of the Nfa1 protein revealed that this protein was abundant in pseudopodia and around food vacuoles of *N. fowleri* trophozoites (Shin et al., 2001; Cho et al., 2003). It was suggested that the *nfa1* gene might be related with a mechanism of pathogenicity of *N. fowleri* (Cho et al., 2003; Jeong et al., 2004). Any attempt to study the cytopathic effects of pathogenic *N. fowleri* against actual target cells of PAME, that is, rat microglial cells, has not yet been reported. On the basis of an idea that above pseudopodia–specific Nfa1 protein seems to be involved in the pathogenicity of *N. fowleri* concerned with the host–tissue contact, this study was performed. To determine whether a pathogenic *N. fowleri* showed the cytopathic effects against primary cultured rat microglial cells, the morphological changes of microglial cells co-cultured with *N. fowleri* trophozoites was observed by a light, scanning and transmission electron microscope. And then, the in vitro cytotoxicity of *N. fowleri* against rat microglial cells were also observed. By adding an anti-Nfa1 antibody in the co-culture system, the effects of an anti-Nfa1 antibody on the cytopathology and in vitro cytotoxicity of *N. fowleri* against microglial cells was elucidated. In addition, the cytokine release from microglial cells in co-culture system was estimated.
II. MATERIALS AND METHODS

A. Amoeba

The trophozoites of *N. fowleri* (Cater NF69 strain, ATCC NO. 30215) were axenically cultured at 37°C in Nelson’s medium (Willaert, 1971).

B. Preparation of an anti-Nfa1 polyclonal antibody

For the preparation of the sera to a recombinant Nfa1 protein (rNfa1), the expression of a *nfa1* gene were performed by the method mentioned in the previous paper (Jeong et al., 2004). An 8-week-old female BALB/C mouse (Korea Institute of Science and Technology, Daejeon, Korea) was intraperitoneally injected with a mixture of the rNfa1 protein (50 µg) and the same volume of complete Freund’s adjuvant (Sigma). The mouse was boosted biweekly with the rNfa1 protein (25 µg) and a same volume of incomplete Freund’s adjuvant (Sigma). After third boosting, rNfa1 protein (50 µg) was injected intravenously without the adjuvant. Four days later, an anti-Nfa1 polyclonal serum was collected from the mouse blood by centrifuging at 2,500 × g for 30 min at 4°C. ELISA was performed with a purified rNfa1 protein (5 µg/ml) with rabbit anti-mouse (1:10,000 dilution) whole immunoglobulin conjugated with alkaline phosphate (Sigma). Western blotting for a rNfa1 protein was confirmed by the method in a previously paper (Jeong et al., 2004).
C. Microglial cell culture

Microglial cells were prepared by the method of Giulian and Baker (Giulian and Baker, 1991) with some modifications. Mainly, the cortex of the brain were obtained from newborn rats (Sprague-Dawley, purchased from KIST in Daejeon, Korea) and homogenized by pumping with 21-gauge syringe. The mixture was centrifuged at 300 × g for 5 min and resuspended in Dulbecco’s modified eagle’s medium (DMEM; Sigma, St. Louis, MO) with 10% heat-inactivated fetal bovine serum and antibiotics. The suspension was put into 75-cm² tissue culture flasks, and the flasks were incubated for 14 days at 37℃ in an incubator with a humidified atmosphere consisting of 5% CO₂. On 14 days of culture, microglial cells were harvested by vigorously shaking each culture flask, and then filtered with nylon wool to remove any remaining astrocytes and centrifuged at 300 × g for 5 min. The pellet was resuspended in DMEM with 10% FBS, and the mixture was incubated at 37℃ for 2 hr. The attached microglial cells were harvested and subjected to subsequent experiments. The purity of microglial cells was nearly 95% by indirect immunofluorescence staining with a FITC-conjugated mouse anti-rat CD68 (ED 1 antibody) (Serotec, Bicester, United Kingdom), as shown in figure 2.

D. Light microscope

Microglial cells (2×10⁵) were co-cultured with N. fowleri trophozoites (2×10⁵) or lysate (0.1, 0.5 and 1 mg/ml) in 24-well culture plate for 6 hr and 12 hr. After co-incubation, samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer and post-fixed with 1% osmium tetroxide - 1.5% potassium for 1 hr and then were examined with a light microscope.
E. Scanning electron microscope (SEM)

Microglial cells \(2 \times 10^5\) were seeded onto the lab-tek II chamber slide system (Nunc A/S, Roskilde, Belgium) and trophozoites of *N. fowleri \(2 \times 10^5\) were added to the monolayers. Microglial cells and *N. fowleri* trophozoites were incubated for 12 hr. After co-incubation, samples were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), dehydrated with increasing concentrations of ethanol. Samples were vacuum-dried and coated by ultra-thin layer (300 Å) of gold/pt in an ion sputter (E-1010, Hitachi, Tokyo, Japan). An image analyzer program (Escan 4000, Bumi-Mi Universe Co., Ltd., Ansan, Korea) was used to capture the images of cells and modified surfaces. Samples were characterized by scanning electron microscope (S-800, Hitachi, Tokyo, Japan).

F. Transmission electron microscope (TEM)

*N. fowleri* trophozoites were incubated with microglial cells in 24-well culture plate at ratio of 1:1 for 3, 6 and 12 hr, and were fixed in modified Karnovsky’s fixative solution in cacodylate buffer (pH 7.4) and postfixed in 1% osmium tetroxide - 1.5% potassium ferrocyanide. The cells were stained en bloc in 0.5% uranyl acetate, dehydrated through a graded ethanol series, and embedded in resin (Polyscience, Warrington, Pa.). Then, the blocks were sectioned with Ultrostain 1H and 2 (Leica, Vienna, Austria). Specimens were observed and photographed with a Zeiss EM 902 A electron microscope (Leo, Oberkohen, Germany).
G. Chromium release assay

A $^{51}$Cr (chromium) release assay was performed to determine the cytopathic effects of alive *N. fowleri* trophozoites by the methods of a previously study (Moore et al., 1991), with some modifications. Target microglial cells were labeled with 100 $\mu$Ci [Na]$_2$$^{51}$CrO$_4$ per $10^5$ cells for 60 min at 37°C. The cells were washed to remove any unbound radioisotope. Labeled microglial cells ($5 \times 10^4$) were added to each well of a 96-well culture plate and co-incubated with effector *N. fowleri* trophozoites ($5 \times 10^4$) in the absence or presence of an anti-Nfal polyclonal antibody in 5% CO$_2$ for 3, 6, 12 and 24 hr. In addition, labeled microglial cells ($5 \times 10^4$) co-cultured with lysates (0.1, 0.5 and 1 mg/ml) of *N. fowleri* as above-mentioned procedures. Spontaneous release from labeled microglial cells was determined by acquiring the counts per min (cpm) in the supernatant fluid in the absence of trophozoites. All assay were performed in triplicate. At the end of the experimental incubation period, plates were centrifuged at $300 \times g$ for 3 min and the supernatant fluid from each wells was harvested. Each of these for maximal release was lysed with 5% (vol/vol) Triton X-100 and harvested. Following 3, 6, 12 and 24 hr, supernatant fluid and lysed cells were counted separately in a gamma counter. The percentage of the radioisotope released from target microglial cells was determined as the index of lysis using the following formula:

$$\text{Cytotoxicity} (\%) = \frac{\text{experiments release} - \text{spontaneous release}}{\text{maximum release} - \text{spontaneous release}} \times 100$$
H. Cytokine measurement

The amounts of tumor necrosis factor (TNF)-α, Interleukin (IL)-1β and IL-6 from microglial cells in a co-culture system was measured by enzyme-linked immunosorbent assay (ELISA) kits (BioSource International, California, USA). The detection limits for these kits were about 10 pg/ml, 10 pg/ml and 50 pg/ml, respectively. The amounts of cytokine produced by microglial cells were estimated by generating a standard curve according to the manufactures instructions.
III. RESULTS

A. Microscopic findings of microglial cells

Microglial cells were separated from the brain cortex of SD newborn rats and cultured with DMEM medium. Two weeks later, microglial cells with three morphological types were observed by a light microscope (Fig. 2A). The purified microglial cells were identified by the immunostaining of a cell surface marker, CD68. (Fig. 2B). For micro-organic observations, microglial cells were observed by a SEM and TEM (Fig. 3). Amoeboid form of microglial cell have long cytoplasmic projection (Fig. 3A, B) compared with them of round form (Fig. 3C). In particular, microglial cells had short blunted spinous projection from cell surface (Fig. 3A, B). Nucleus was ovoid or contained clumped chromatin and occasional nucleoli (Fig. 3C). Rough endoplasmic reticulum was abundant and tended to form elongated cisternae. Golgi apparatus was moderately developed (Fig. 3C).
In panel A, microglial cells showed amoeboid form (a), rod form (r), and ramified form (ra). (x200). In panel B, microglial cells were immunostained with a FITC-conjugated mouse anti-rat CD68. (x400).

**Fig 3.** Electron microscopic findings of microglial cells. Numerous cytoplasmic projections were showed in microglial cell by a SEM (A, B). The nucleus (N) containing scattered chromatin materials was observed by a TEM (C).
B. Morphology of *N. fowleri* characterized by pseudopodia and food-cups structure

*N. fowleri* trophozoites were observed by a light, SEM and TEM (Fig. 4). Food-cups structure and pseudopodia were observed during the cultivation (Fig. 4A, B). The trophozoite of *N. fowleri*, measuring 10-20 μm, was characterized by a nucleus with a large karyosome surrounded by a halo (Fig. 4C).

![Fig 4. Electron microscopic findings of *Naegleria fowleri* trophozoites.](image)

Food-cups structure was shown by a SEM (4A, B). Obvious karyosome in nucleus was observed by a TEM (4C). N, nucleus; V, vacuole; fc, food-cup structure.
C. Cytopathic changes of microglial cells co-cultured with *N. fowleri* trophozoites

1. Morphologic changes of microglial cells co-cultured with *N. fowleri* trophozoites

By a light microscope, microglial cells co-cultured with *N. fowleri* trophozoites for 6 hr showed severe morphological destruction and decreasing number (Fig. 5C). In comparison, microglial cells co-cultured with *N. fowleri* trophozoites and an anti-Nfa1 antibody showed less destruction than above experimental group (Fig. 5D). Many alive microglial cells remained to be attached on the bottom.

By SEM findings, microglial cells co-cultured with *N. fowleri* trophozoites was attacked and destroyed by *N. fowleri* trophozoites. *N. fowleri* showed broad and sticky attachment onto microglial cells (Fig. 6A, B). When an anti-Nfa1 antibody was treated on *N. fowleri* co-cultured with microglial cells, the morphology of *N. fowleri* was changed into a round form and less sticky attachment than above experiment group (Fig. 6C, D).

By TEM findings, microglial cells co-cultured with *N. fowleri* trophozoites were destroyed in a time-dependent manner (Fig. 7). *N. fowleri* attached to a microglial cell by a vigorous pseudopodium was observed at 3 hr (Fig. 7B). As the incubating time was continued, the food-cups structure of *N. fowleri* trophozoite was observed in the contact site of a microglial cell, and simultaneously a amoeba had dead particles of microglial cells in vacuoles (Fig. 7C). A microglial cell was dead by the necrotic process characterized by membrane swelling and burst of the nucleus membrane (Fig. 7D).
Fig. 5. Light microscopic findings of microglial cells co-cultured with *Naegleria fowleri* trophozoites (C), or with an anti-Nfal antibody (1:100 dilution) (D) for 6 hr. Arrows and arrowheads indicated microglial cells and *N. fowleri* trophozoites, respectively. A, microglial cells only; B, *N. fowleri* trophozoite only. (× 200).
Fig. 6. SEM findings of microglial cell co-cultured only with *Naegleria fowleri* trophozoite (A, B), and an anti-Nfa1 antibody (C, D). In panel A and B, *N. fowleri* showed broad and sticky attachment onto microglial cells. In panel C and D, *N. fowleri* showed weaker attachment and round morphology than those of panel A and B. m, microglia; nf, *N. fowleri*. 
Fig. 7. TEM findings of microglial cells co-cultured with *Naegleria fowleri* trophozoites. Microglia (M) co-cultured with *N. fowleri* (Nf) trophozoite for 0, 3, 6 and 12 hr (A, B, C and D), respectively. In panel B, *N. fowleri* attached to a microglial cell with a vigorous pseudopodia. In panel C, the food-cups structure of *N. fowleri* was shown in a contact region of a microglial cell (Arrow). In panel D, a microglial cell was more destroyed via necrotic process. M, microglia; Nf, *N. fowleri*; N, nucleus; V, vacuole.
2. In vitro cytotoxicity of *N. fowleri* trophozoites on microglial cells

To determine whether *N. fowleri* trophozoites show the cytotoxicity on microglial cells, $^{51}$Cr release assay was carried out. When microglial cells were co-cultured with *N. fowleri* trophozoites, the cytotoxicity of amoeba on microglial cells was increased from 17.8 to 100 % in a time-dependent manner (Table 1, Fig. 8). *N. fowleri* co-cultured with microglial cells and an anti-Nfa1 antibody showed 15.5 ~ 66.9 % cytotoxicity which was more decreased than above experimental groups without antibody (T-test, $P<0.05$) (Table 1, Fig. 8).
### Cytotoxicity of *Naegleria fowleri* trophozoites on microglial cells

<table>
<thead>
<tr>
<th>Groups</th>
<th>Times of cultivation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>3 h</td>
</tr>
<tr>
<td>Microglial cells + trophozoites</td>
<td>17.8 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Microglial cells</td>
<td>15.5 ± 0.9</td>
</tr>
<tr>
<td>+ trophozoites</td>
<td></td>
</tr>
<tr>
<td>+ anti-Nfa1 antibody (1:100)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> % cytotoxicity calculated by the <sup>51</sup>Cr amount released from radiolabelled microglial cells (mean ± standard variation)

<sup>b</sup> Dilution ratio of an anti-Nfa1 antibody

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**Fig. 8.** Cytotoxicity of *Naegleria fowleri* trophozoites co-cultured with microglial cells, or with an anti-Nfa1 antibody. M, microglial cells; A, *N. fowleri* trophozoites; Ab, anti-Nfa1 antibody.
D. Cytokines release from microglial cells co-cultured with *N. fowleri* trophozoites

To determine whether microglial cells show any change in cytokine release as a result of the protective activity induced by pathogenic *N. fowleri* trophozoites, assays for cytokines such as TNF-α, IL-1β and IL-6 were measured with enzyme–linked immunosorbent assay kits. In microglial cells co-cultured with *N. fowleri* trophozoites, the amount of TNF-α was increased at 3 hr and peaked at 6 hr post-incubation, and when an anti-Nfa1 antibody was treated, the increasing was inhibited (Table 2, Fig. 9). The amount of IL-1β was slightly increased at 12 hr post-incubation. No effect of an anti-Nfa1 antibody was shown (Table 3, Fig. 10). The amount of IL-6 was increased from 3 hr to 12 hr post-incubation. No effect of an anti-Nfa1 antibody was shown (Table 4, Fig. 11).
Table 2. Amounts of TNF-α secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Amp (pg/ml) secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Microglial cells</td>
<td>26.9</td>
</tr>
<tr>
<td>+ <em>N. fowleri</em> trophozoites &lt;sup&gt;b&lt;/sup&gt;</td>
<td>26.8</td>
</tr>
<tr>
<td>+ <em>N. fowleri</em> trophozoites + Ab &lt;sup&gt;c&lt;/sup&gt;</td>
<td>28.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values are means ± standard deviations

<sup>b</sup> The ratio (1:1) is the number of microglial cells to the number of trophozoites

<sup>c</sup> Ab, dilution ratio of an anti-Nfal antibody is 1:100

Fig. 9. Amounts of TNF-α secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites. M, microglia; A, *N. fowleri* trophozoites; Ab, anti-Nfal antibody.
Table 3. Amounts of IL-1β secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Amp (pg/ml) secreted</th>
<th>0 h</th>
<th>3 h</th>
<th>6 h</th>
<th>12 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microglial cells with:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ <em>N. fowleri</em> trophozoites</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ <em>N. fowleri</em> trophozoites + Ab</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ a \] Values are means ± standard deviations

\[ b \] The ratio (1:1) is the number of microglial cells to the number of trophozoites

\[ c \] Ab, dilution ratio of an anti-Nfa1 antibody is 1:100

Fig. 10. Amounts of IL-1β secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites. M, microglia; A, *N. fowleri* trophozoites; Ab, anti-Nfa1 antibody.
Table 4. Amounts of IL–6 secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites

<table>
<thead>
<tr>
<th>Culture conditions</th>
<th>Amp (pg/ml) secreted</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Microglial cells with:</td>
<td>37.5</td>
</tr>
<tr>
<td>+ <em>N. fowleri</em> trophozoites (^b)</td>
<td>37.5</td>
</tr>
<tr>
<td>+ <em>N. fowleri</em> trophozoites + Ab (^c)</td>
<td>37.0</td>
</tr>
</tbody>
</table>

\( ^a \) Values are means ± standard deviations

\( ^b \) The ratio (1:1) is the number of microglial cells to the number of trophozoites

\( ^c \) Ab, dilution ratio of an anti–Nfa1 antibody is 1:100

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Fig. 11. Amounts of IL–6 secreted from microglial cells co-cultured with *Naegleria fowleri* trophozoites. M, microglia; A, *N. fowleri* phozoites; Ab, anti–Nfa1 antibody.
IV. DISCUSSION

A prominent feature of fatal human and animal cases of infection by *N. fowleri* is invasion of the CNS and development of PAME. As the major route of invasion for the *N. fowleri* infection, amoeba reach the nasal cavity and attach to then, and invade the nasal mucosa and olfactory nerve. Concerned with the host–tissue invasion, the adherence of the amoeba to host cells is the most important step in the mechanism of pathogenicity of *N. fowleri*, and a specific pseudopodia projection, called an amoebastome, is formed (Marciano-Cabral, 1988). Invasive amoeba able to enter the nervous system digest neuronal tissue and other mammalian cells by usually effective cytolysis and phagocytosis as observed in culture or in sections of infected brain tissue (Marciano-Cabral, 1988). Other cytotoxic toxins and cytolytic proteinases have been proposed that amoeba destroy target cells (John, 1982; Ma et al., 1990).

Microglial cells are resident macrophages within the CNS and exhibit phenotypic and functional features of macrophages localized at peripheral non–neural sites. Microglial cells migrate to sites of injury and infection, Phagocytose dead cells and invading organisms, and produce a variety of factors including cytokines such as TNF-α, IL-1β and IL-6. Because microglial cells appear to possess characteristics of extraneural tissue macrophages, the may be a major cell type involved in host defense against *N. fowleri* in the brain (Suzumura et al., 1993). However, the mechanisms whereby these cells participate in defense against this infection are not understood.

In our study, microglial cells cultured from rat newborn brain were used as target cells, to determine whether *N. fowleri* show the cytopathic effects
against primary cultured rat microglial cells, the morphological change of microglial cells co-cultured with *N. fowleri* trophozoites observed by a light, SEM, TEM. In addition, the in vitro cytotoxicity of *N. fowleri* against microglial cells observed. By adding an anti-Nfa1 antibody in the co-culture system, the efforts an anti-Nfa1 antibody on the cytopathology and in vitro cytotoxicity of *N. fowleri* against microglial cells was elucidated.

In previous report, trophozoites of *N. fowleri* in cultivating system and in mouse brain tissue infected experimentally with *N. fowleri* were well immunostained, as results of immunohistochemistry of the Nfa1 protein (Cho et al., 2003). The immunolocalization study of the Nfa1 protein revealed that this protein was abundant in pseudopodia and around food vacuoles of *N. fowleri* trophozoites (Cho et al., 2003). This suggests that the Nfa1 protein is required for food ingestion and amoeba movement. On the basis of pseudopodia-specific Nfa1 protein involved in the pathogenicity of *N. fowleri* concerned with the host–tissue invasion, cytotoxicity of *N. fowleri* against microglial cells were observed by adding an anti-Nfa1 antibody in the co-culture system to elucidate the antibody effect.

In this study, it was shown that microglial cells co-cultured with *N. fowleri* trophozoites and an anti-Nfa1 antibody showed less destruction than groups without anti-Nfa1 antibody by a light microscope. By a SEM, it was also shown that an anti-Nfa1 antibody was treated on *N. fowleri* trophozoites co-cultured with microglial cells, the morphology of *N. fowleri* was changed into a round form and less sticky attachment than groups without anti-Nfa1 antibody. However, by a TEM, microglial cells co-cultured with *N. fowleri* trophozoites were destroyed in a time-dependent manner. This observations suggested that effector *N. fowleri* trophozoites contacted target microglial, microglial cells were dead by necrotic process accompanied by membrane
swelling and burst in the nucleus. Therefore, the lysis of target microglial cells or ingestion of the target cells via food cups and their subsequent channeling into intracytoplasmic food vacuoles. In particular, Brown (Brown, 1979; Zubiaur and Alonoso, 1983) concluded that N. fowleri injured target cells by repeated nibbling, a process he termed ‘trogocytosis’. In our study, it was confirmed the observation of Brown that N. fowleri can destroy target cells with early stages for piecemeal ingestion. In addition, when microglial cells co-cultured with N. fowleri trophozoites, the high cytotoxicity of amoeba on microglial cells in the 51Cr release assay was increased in a time-dependent manner, but when an anti-Nfa1 antibody was added, the cytotoxicity was decreased than groups without antibody. Moreover, in the previous study, the proliferation and the cytotoxicity of N. fowleri against CHO target cells were observed by adding an anti-Nfa1 antibody in the co-culture system to elucidate the antibody effect. It was shown that anti-Nfa1 antibody inhibited the proliferation of N. fowleri trophozoites in a dose-dependent manner (Jeong et al., 2004). In addition, less CPE has been shown to occur in the presence of an anti-Nfa1 antibody which immobilizes or agglutinates N. fowleri trophozoites.

In previous report about Acathamoeba culbertsoni to induce granulomatous amoeba encephalitis, pathogenic A. culbertsoni induced the cytopathic effects in primary cultured rat microglial cells, with the effects characterized by necrosis and apoptosis of microglial cells (Shin et al., 2000). Necrosis is marked by swelling and bursting of cellular membranes and organelles following the effector cell-target cell contact event. In contrast, apoptosis is characterized by shrinkage in which the target cell undergoes sequential condensation of chromatin, membrane blebbing, loss of the nuclear envelope, cellular fragmentation into apoptotic bodies, and uptake by
phagocytic cells. In this study, necrosis via pore-forming lytic molecules and apoptosis, both of which disrupt cell membrane integrity, were found to be two fundamental mechanisms in the cytolysis of target microglial cells by *N. fowleri*.

To determine whether microglial cells show changes in cytokines release as a result of a CPE induced by *N. fowleri*, the assay for cytokines such as TNF-α, IL-1β and IL-6 was performed with culture supernatants. We observed that TNF-α and IL-6 peaked early and then decreased at 24 hr, whereas IL-1β peaked at 12 hr and continued to accumulate in the medium through 24 hr (data not shown). In addition, when microglial cells were co-cultured with *N. fowleri* and an anti-Nfal antibody for 3 and 6 hr, the amount of TNF-α secreted from microglial cells was inhibited about 20.3%, 14.1%, respectively, but the amount of IL-1β and IL-6 was not decreased. It suggested that cytokine regulations were different modes. It is well known that microglial cells produce proinflammatory cytokines, such as TNF-α, IL-1β, IL-3, IL-6, IL-8, IL-12p40 and IL-15, as well as anti-inflammatory cytokine, such as IL-10, TGF-β, for defense against parasites and brain injury (Marciano-cabral et al., 2000; Benedetto et al., 2001). More extensive studies on the cytokine responses of microglial cells due to *N. fowleri* are necessary.

The results of this study could be considered as the cytopathic effects induced by pathogenic *N. fowleri* against microglial cells and may be more directly prominent to understanding the mechanisms of pathogenic *N. fowleri*. Moreover, further studies need to determine whether the Nfal protein is related to in vivo amoebic pathogenicity.
V. CONCLUSION

Whether *N. fowleri* could induce the cytopathogenicity to primary cultured rat newborn microglial cells as actual target cells of PAME, and an anti-Nfa1 antibody reduce the cytopathogenicity, microglial cells in co-culture system were observed by electron microscopies and quantitative method. *N. fowleri* trophozoites produced pseudopodia to contact microglial cells followed by inducing a necrotic and apoptotic process. Moreover, there were dead particles at larger vacuoles and obvious food-cups structure in *N. fowleri*. Finally, pathogenic *N. fowleri* trophozoites showed the cytopathic effects on rat microglial cells, showing the morphological changes and strong in vitro cytotoxicity which was inhibited with an anti-Nfa1 antibody. And then, microglial cells secreted cytokines, TNF-α, IL-1β and IL-6, as the protective immune response.
BIBLIOGRAPHY


5. Carter RF: Description of a Naegleria species isolated from two cases of primary amoebic meningoencephalitis and the experimental pathological change induced by it. *J Pathol* 100:217–44, 1970


파울러자유아메바 (Naegleria fowleri)는 토양과 담수에서 자유생활을 하는 아메바로써 자연환경에서는 세균을 포식하며, 인체와 실험동물에서 원발성 아메 바성 수막뇌염 (priamry amoebic meningoencephalitis)을 일으키는 것으로 알려 져 있다. 숙주 세포로의 침입과 연관해서 파울러 자유아메바의 병원성 기전에서 표적 세포와의 접촉은 아주 중요하다. 본 연구실에서는 파울러 자유아메바에 대한 감염한 혈청 및 면역혈청을 생산하여 cDNA로부터 immunoscreening을 통해 병원 성 및 항원성과 관련이 있을 것으로 사료하며, 특히 위족(pseudopodia)에 특이성을 갖는 유전자 nfa1을 클로닝 해놓았다. 그리고 클로닝 된 nfa1유전자로부터 13.1 kDa의 재조합 단백질인 Nfa1을 얻었다.

본 연구의 목적은 위쪽 특이성을 갖는 nfa1 유전자가 파울러자유아메바의 병원성과 관련성이 있는지를 입증하기 위해서, 일차 배양한 쥐의 microglia와 함께 파울러자유아메바를 혼합 배양함으로써 아메바 영양형들의 병변효과 및 항 Nfa1 항체의 투여효과를 광학, 주사, 및 투과 전자현미경을 이용해서 관찰하였다. 파울러자유아메바 영양형들은 활발한 위족과 food-cups 구조를 형성하여 microglia에 접촉하는 것을 확인하였다. 혼합 배양 3 시간 후, 소수의 microglia들은 자유아메바들에 의해서 파괴가 되었으며, 배양 시간이 길어지면 따라 microglia들은 심하게 파괴되었다. 생화학적 방법인 ⁵¹Cr 분비 실험결과, microglial 세포들에 대한 자유아메바의 세포독성은 배양 후 3, 6, 12 그리고 24
시간에 각각 17.8, 24.9, 54.6 그리고 98.0% 이었으며, 항 Nfa1 항체(1:100)를 투여 하였을 경우, microglia들에 대한 세포독성이 각각 15.5, 20.3, 50.7 그리고 66.9% 로 감소하였다. 한편, 파울러자유아메바와 혼합 배양한 microglia들은 방어적인 면역반응으로 TNF-α, IL-1β 그리고 IL-6등의 사이토카인(cytokine)들을 분비한 다.

핵심이 되는 말: 파울러자유아메바, 원발성 아메바성 수막뇌염, nfa1 유전자, microglia, 항 Nfa1 항체, 세포독성