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Comparative Analysis on the Cytotoxicity of *Naegleria fowleri* and *N. gruberi* to Macrophages by the Addition of Saccharides

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To elucidate the invasion mechanism of pathogenic *Naegleria fowleri*, especially a receptor-ligand recognition, we investigated the in vitro cytotoxicity of pathogenic *N. fowleri* and nonpathogenic *N. gruberi* to murine macrophages, RAW 264.7, by adding four kinds of saccharides, α-fucose, β-galactose, α-D-mannopyranoside (α-mannose) and xylose. There was not enough of a difference in the cytotoxicity of *N. fowleri* treated with 10 mM of each saccharide. In particular, the cytotoxicity of *N. fowleri* was highly inhibited by 100 mM α-mannose, which was 62.3% inhibition calculated by the analysis of lactate dehydrogenase (LDH) release assay. Although murine macrophages were not significantly destroyed by nonpathogenic *N. gruberi* under hematoxylin staining, the cytotoxicity of *N. gruberi* was inhibited from 31.5% to 14.5% ($P<0.01$) by 100 mM α-mannose treatment. The binding of *N. fowleri* to macrophages was inhibited from 33% to 50% by 100 mM α-mannose. Furthermore, as results of the adhesion assays which were performed to determine whether binding of *Naegleria* is mediated by saccharides-binding protein, the binding ability of *N. fowleri* as well as *N. gruberi* was inhibited by 100 mM α-mannose.

Key Words: *Naegleria fowleri*, *N. gruberi*, Saccharides, LDH, Cytotoxicity

INTRODUCTION

Naegleria fowleri is a free-living amoeba found in widespread areas in moist soil, water and sediment, and exists as a virulent pathogen causing fatal primary amoebic meningoencephalitis (PAME) in experimental animals and humans. Amoebae are invasive, able to enter the nervous system through the olfactory nerve, and digest neuronal tissues by unusually effective cytolysis and phagocytosis as observed in culture or in sections of infected brain tissue (Jung et al., 2008b; Shin et al., 2004). *N. gruberi*, a nonpathogenic species, was the first member of the genus to be described and has been used extensively to define the molecular biology of cellular differentiation (Fulton, 1977).

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The adhesion of parasites to the host cells is clearly a critical first step in the pathogenesis of infection. According to the same-free living amoeba, *Acanthamoeba*, subsequent to adhesion, the parasite produces a potent cytopathic effect leading to target cell death (De Jonckheere, 1980; van Klink et al, 1992). That the *Acanthamoeba* may adhere to host cells via a carbohydrate-binding protein has been suggested by studies demonstrating that: (i) the adhesion of *Acanthamoeba* to corneal epithelial cells in culture as well as to the surface of the corneal buttons can be inhibited by free methyl-α-mannopyranoside but not by a number of other sugars (Cao et al., 1998; Panjwani et al., 1997; Yang et al., 1997), (ii) *Acanthamoebae* bind to a neoglycoprotein, mannosylated-bovine serum albumin but not to galactose-bovine serum albumin (Cao et al., 1998), (iii) mannose-related saccharides that inhibit amoeba binding to corneal epithelial cells are also potent inhibitors of the amoeba-induced cytopathic effect (Cao et al., 1998). Although there are many reports about the adhesion of *Acanthamoeba* spp., there are few about the adhesion of *Naegleria* spp. Recently,

it was reported that an integrin-like protein and protein kinase C in *N. fowleri* adhesion to fibronectin and amoebic cytotoxicity was involved (Han et al., 2004). The mechanism by which *N. fowleri* produces PAME has not been fully elucidated.

In this study, we examined the comparative analyses on the cytotoxicity of pathogenic *N. fowleri* and nonpathogenic *N. gruberi* to murine macrophages by the addition of several saccharides. In particular, the macrophage is applied as a target cell to the amoeba in a mouse brain which contains a similar microglial cell. Furthermore, it was described what saccharides used in this study showed better effects to the binding ability of *Naegleria* to target cells.

MATERIALS AND METHODS

Amoeba and murine macrophage culture

Trophozoites of *N. fowleri* (Carter NF69; ATCC No. 30215) were axenically cultured at 37℃ in Nelson's medium (Willaert, 1971). Nonpathogenic *N. gruberi* (ATCC No. 30960) was cultured at 27℃ in modified PYNFH medium (De Jonckheere, 1993). Adherent murine macrophage RAW 264.7 (ATCC No. TIB-71) was routinely cultured at 37℃ in Dulbecco's modified eagle's medium (DMEM; GibcoBRL) with 4 mM L-glutamine adjusted to contain 1.5 g/L sodium bicarbonate and 4.5 g/L glucose (Raschke et al., 1978). For the in vitro cytotoxcity and adhesion assay, 3×10^5 cells/ml of macrophages was grown in 24-well culture plates. Confluent monolayers were formed within 24 h and used for the in vitro cytotoxicity and adhesion assay.

Hematoxylin staining and in vitro cytotoxicity assay

To examine the effect of saccharides in the interaction of *Naegleria* spp. with murine macrophage, the cytotoxicity assay was performed by the methods of previos paper (Jeong et al., 2004). After murine macrophages were cultured in 24 well plates, *N. fowleri* or *N. gruberi* (9×10^5 amoebae/well) preincubated with four kinds of saccharides [α-fucose, β-galactose, α-D-mannopyranoside (α-mannose) and xylose] at 10, 50, or 100 mM final concentration in 500 μl of DMEM for 40 min was added. For controls, amoebae were incubated with 3% bovine serum albumin (BSA). Interaction of *Naegleria* spp. with murine macrophage was observed using an inverted microscope at culture intervals. At the end of this observation period, the cytotoxicity was assessed visually after hematoxylin staining. In addition, supernatants were collected, and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) released from macrophages with a CytoTox96 Non-radioactive Cytotoxicity Assay kit (Promega, Madison, WI). For LDH assay, 50 μl of reacted supernatant in each well was transferred to 96 well assay plate (Nunc A/S, Roskilde, Denmark). After 50 μl of the reconstituted assay buffer was added, the plate was incubated 30 min at room temperature, and then 50 μl of stop solution was added. The reactants were read at 490 nm with ELISA reader. The formula of cytotoxicity was as follows: sample value-control value/ total LDH release - control value×100 = % cytotoxicity. Control values were obtained from macrophages incubated alone. Total LDH release was determined from macrophages treated with lysis buffer.

Adhesion assays

To examine the inhibition of adherence of *Naegleria* spp. to murine macrophage by saccharides treatment, the adhesion assay was performed (Jung et al., 2008a). *Naegleria* spp. were metabolically labeled by culturing amoebae (5×10^6/ml) in each culture medium containing 100 μCi of ^{35}S-methionine (Pharmacia Biotech, England, UK) at respective culture temperature. Following incubation, flasks were chilled on ice, and radio-labeled amoebae were collected by centrifugation at 1,000 ×g for 5 min and resuspended in 20 ml of phosphate buffer saline (PBS) containing 0.1 mM $CaCl_2$. This process was repeated three times to remove unincorporated ^{35}S-methionine. Finally, amoebae (9×10^5/ well) in 500 μl suspension of DMEM were incubated with murine macrophages for 48 h at respective optimal temperature. The unbound amoebae were removed gently by washing three times using PBS before the addition of 0.4 ml of 2% sodium dodesyl sulfate in PBS to solubilize the murine macrophages and bound amoebae. The specific radioactivity was counted by a scintillation counter (Packard, Illinois, USA). To examine the effect of saccharides, four

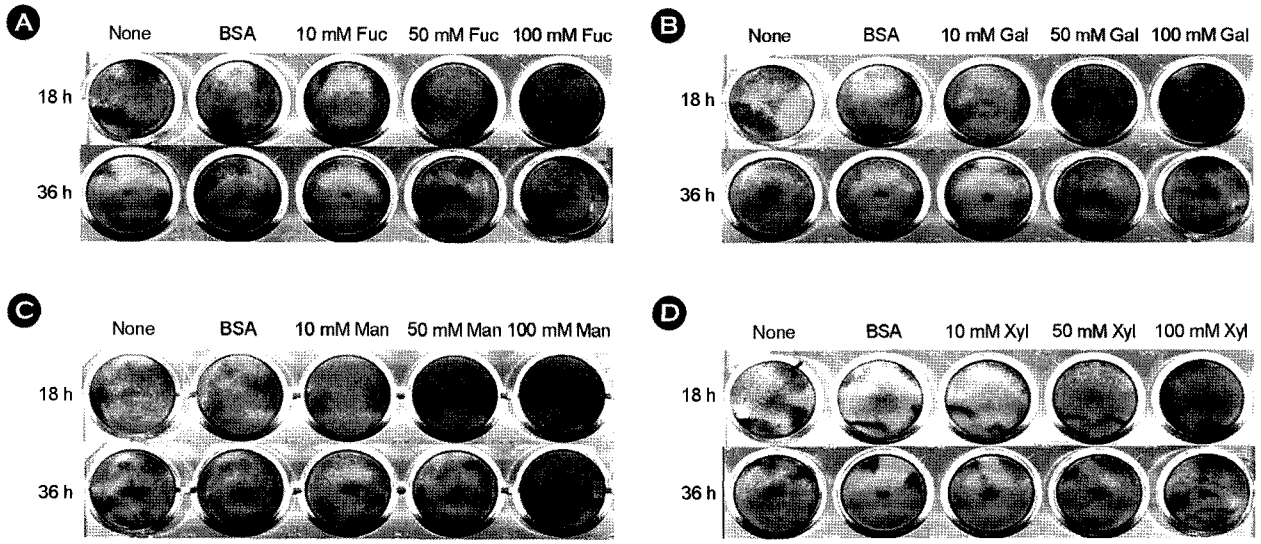

Fig. 1. Hematoxylin staining of murine macrophages co-cultured with *N. fowleri* trophozoites pretreated with α-fucose (Fuc) (A), β-galactose (Gal) (B), α-mannose (Man) (C) and xylose (Xyl) (D). *N. fowleri* (9×10^5) were added to macrophages (3×10^5) in 24 well plates and incubated in 5% CO_2 incubator at 37℃ for 18~36 h. After this incubation, cytotoxicity was determined visually by hematoxylin staining. (A) α-fucose (B) β-galactose (C) α-mannose (D) xylose.

kinds of saccharide were incubated with *Naegleria* spp. as mentioned above.

RESULTS

In vitro cytotoxicity of pathogenic *N. fowleri* by the addition of saccharides

Pathogenic *N. fowleri* was treated with each saccharide under various concentrations, and co-cultured with murine macrophages (RAW 264.7) used as a target cell to observe the effect of saccharides. Overall, according to the hematoxylin staining, macrophages were more severely destroyed by *N. fowleri* trophozoites untreated with saccharides (Fig. 1) in comparison to those treated with saccharides. As the culture time was continued, the extensive loss of macrophage monolayers by *N. fowleri* trophozoites untreated with saccharides was occurred as shown in a hematoxylin staining (Fig. 1). As the concentration of saccharides was increased, the cytotoxic effect of *N. fowleri* preincubated with saccharides was inhibited (Fig. 1). Although the inhibition on the cytotoxicity of *N. fowleri* was not significantly different by kinds of saccharides, it was shown to be maximal at 18 h in α-mannose treatment (Fig. 1). There were not enough differences to be increased in cytotoxicity of *N. fowleri* trophozoites at 10 mM, but it was shown to be significantly inhibited at 50 mM and 100 mM saccharides treatments. In particular, the cytotoxicity of *N. fowleri* was highly inhibited by 100 mM α-mannose among four kinds of saccharides (Fig. 1C). To further examine whether the cytotoxicity is due to the lysis of macrophages or simply disruption of the cell monolayers, LDH release was performed (Fig. 2). The results of LDH release assay were similar to those of hematoxylin staining (Fig. 2). In addition, 100 mM α-mannose treatment for 18 h induced highly the inhibition of cytotoxicity of *N. fowleri* about 62.3% ($P< 0.01$) (Fig. 2C). There were not significant differences by kinds of saccharides at 36 h post incubation.

In vitro cytotoxicity of nonpathogenic *N. gruberi* by saccharides

The inhibition on the cytotoxicity of nonpathogenic *N. gruberi* by saccharides was examined by comparing the effect of saccharides to pathogenic *N. fowleri*. The concentration of saccharides to *N. gruberi* was determined with 100 mM shown the highest effect to *N. fowleri*. By the hematoxylin staining, the differences of inhibiting effect of saccharides treatment were not obscure, but slightly different on α-mannose treatment in comparison with controls (Fig. 3). At 18 h post incubation, the cytotoxicity of *N. gruberi* was highly inhibited by the addition of 100 mM α-mannose

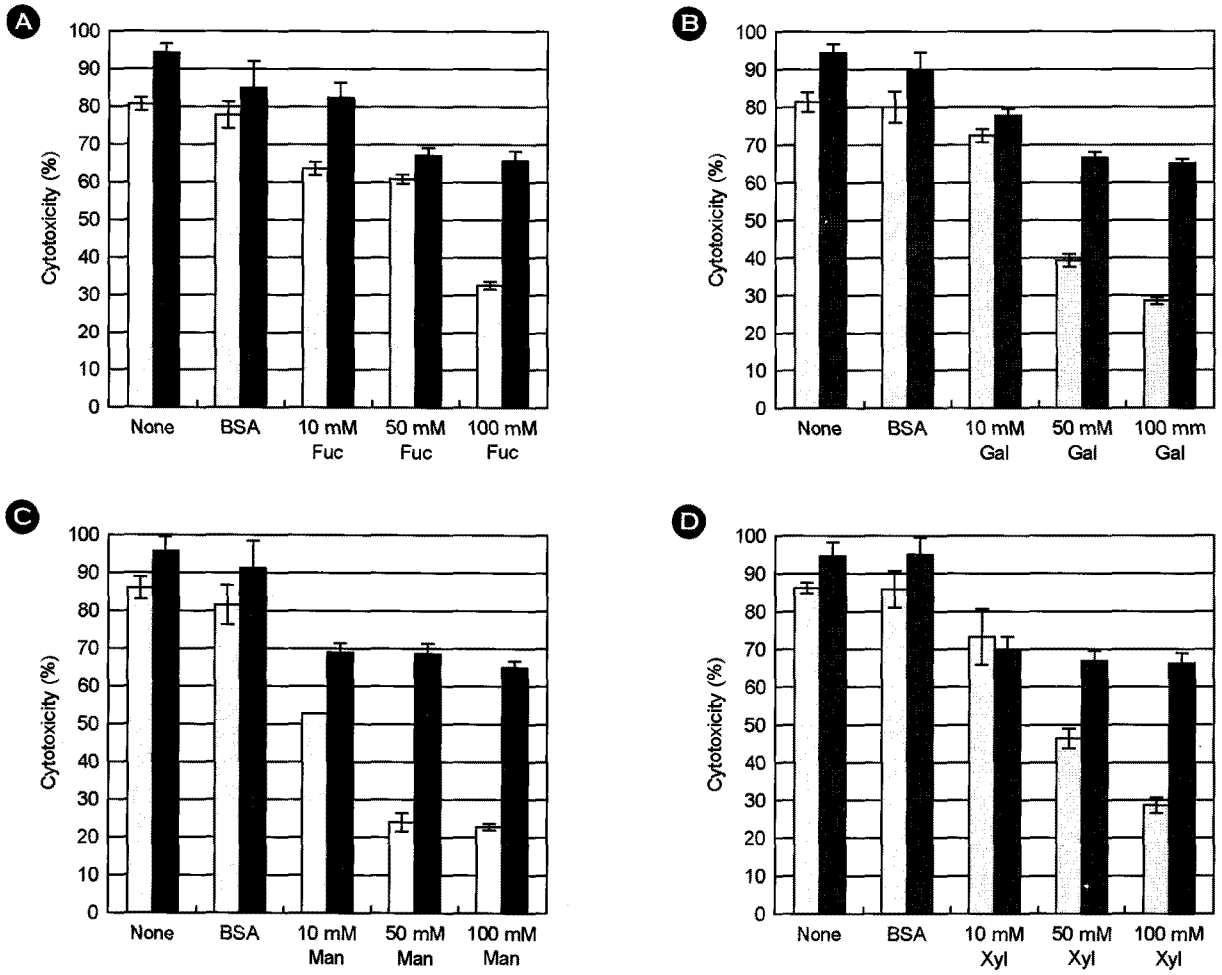

Fig. 2. Analysis of LDH released from murine macrophages by *N. fowleri*. It was performed with culture supernatants of macrophages co-cultured with *N. fowleri* trophozoites pretreated with α-fucose (Fuc), β-galactose (Gal), α-mannose (Man) and xylose (Xyl). The bars of graphs represent standard deviation value. LDH data was calculated with % cytotoxicity, and results represent mean of three independent experiments performed in triplicates for 18 h (white color)-36 h (gray color).

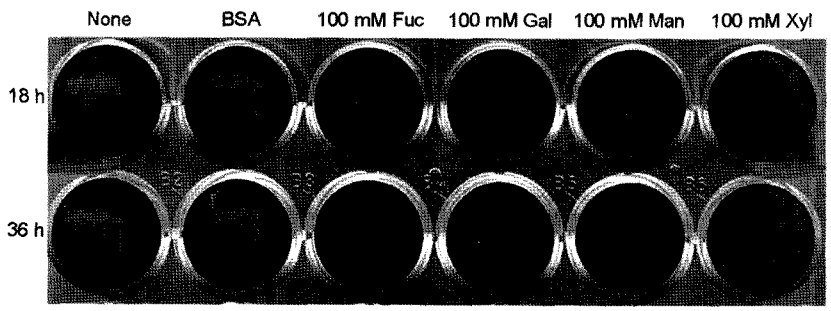

Fig. 3. Hematoxylin staining of murine macrophages co-cultured with *N. gruberi* trophozoites pretreated with α-fucose (Fuc), β-galactose (Gal), α-mannose (Man) and xylose (Xyl). The concentration of saccharides was used with 100 mM which was shown highest effect to *N. fowleri*. *N. gruberi* (9×10^5) were added to macrophages (3×10^5) in 24 well plates and incubated in 5% CO_2 incubator at 37℃ for 18~36 h. After this incubation, cytotoxicity was determined visually by hematoxylin staining.

(Fig. 3C). In results of LDH release assay, although murine macrophages were not significantly destroyed by non-pathogenic *N. gruberi*, they were destroyed about 31.5% by 100 mM β-galactose ($P<0.01$) (Fig. 4). The cytotoxicity of *N. gruberi* was inhibited with about 14.5% by 100 mM α-mannose ($P<0.01$) (Fig. 4). Although there were differences of the inhibition of cytotoxicity in *N. fowleri* and *N. gruberi*, the cytotoxicity was highly inhibited by the addition of 100 mM α-mannose.

Comparison of the binding ability of *N. fowleri* vs. *N. gruberi* by saccharides treatment

To test whether binding of *N. fowleri* vs. *N. gruberi* was highly inhibited by a saccharide-binding protein, adhesion assay was performed in the presence of four kinds of saccharides, α-fucose, β-galacotse, α-mannose, and xylose (Fig. 5). At 24 h post incubation, the binding of radio-labeled *N. fowleri* to macrophages was inhibited from 33% to 50% by saccharides treatment of which 100 mM α-mannose showed highest effect about 33% (Fig. 5A). Although the adhesion ability of nonpathogenic *N. gruberi* was lower than pathogenic *N. fowleri*, it was diminished a little by saccharides treatment of which 100 mM α-mannose showed highest effect about 18% (Fig. 5B). There was different specificity of saccharides against *N. fowleri* or *N. gruberi*, but the binding ability of *N. fowleri* as well as *N. gruberi* was significantly inhibited by 100 mM α-mannose used in this study.

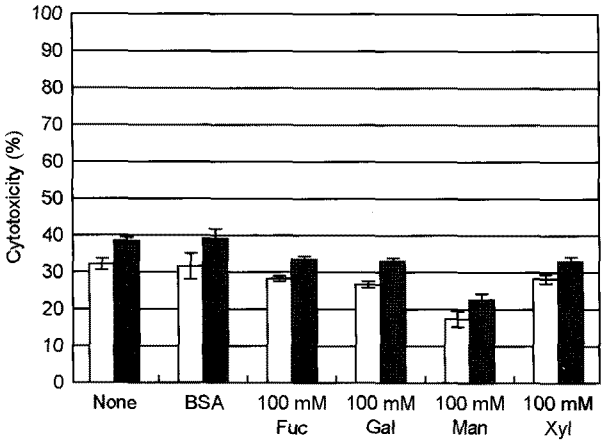

Fig. 4. Analysis of LDH released from murine macrophages by *N. gruberi*. The concentration of saccharides was used with 100 mM. It was performed with culture supernatant of macrophages co-cultured with *N. gruberi* trophozoites pretreated with α-fucose (Fuc), β-galactose (Gal), α-mannose (Man) and xylose (Xyl). The bars of graphs represent standard deviation value. LDH data was calculated with % cytotoxicity, and results represent mean of three independent experiments performed in triplicates for 18 h (white color)-36 h (gray color).

DISCUSSION

PAME is a rapidly progressive and fatal infection caused by a free-living amoeba, especially *N. fowleri*, however, the pathogenesis and pathophysiology of this disease are not fully elucidated. In same free-living amoeba, genus *Acanthamoeba*, the cytopathogenic effects of *Acanthamoeba* on host cells require (i) adhesion of the amoeba to host cell (Khan, 2001), (ii) secretion of proteases (Khan et al., 2000), and (iii) phagocytosis (Marciano-Cabral et al., 2004).

It has been shown that adhesion is one of the crucial steps for the pathogenicity of amoeba, as non-pathogenic amoeba exhibit significant decreased binding to host cells (Khan et al., 2000). It was also reported that the first step in the pathogenesis of *Acanthamoeba* infection involve attachment of the parasite to the mannose residues of the plasma

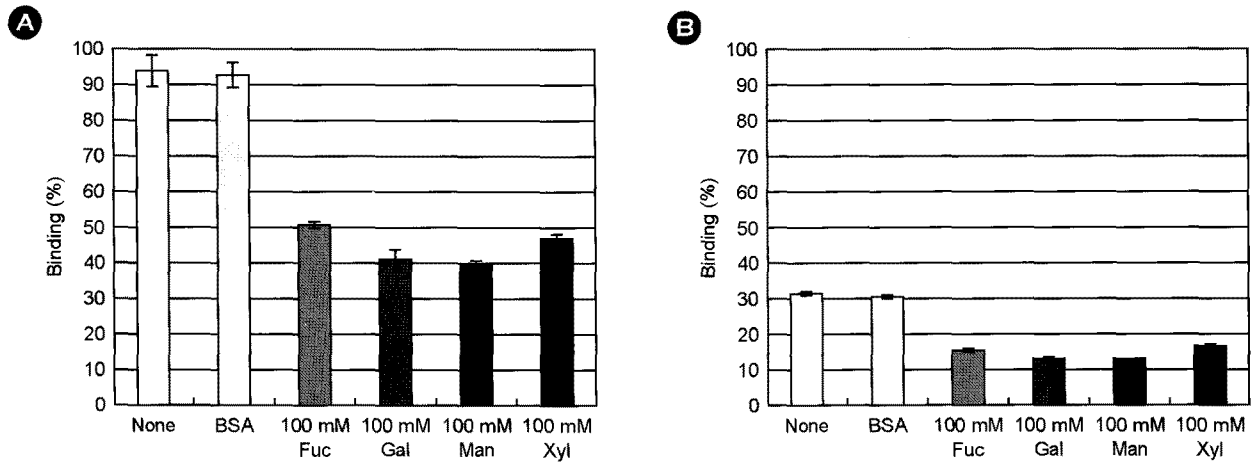

Fig. 5. Adhesion assay of *N. fowleri* (A) and *N. gruberi* (B) to murine macrophages. Radio-labeled *N. fowleri* and *N. gruberi* trophozoites were incubated with murine macrophages for 24 h in the presence or absence of various saccharides, α-fucose (Fuc), β-galactose (Gal), α-mannose (Man) and xylose (Xyl). The radioactivity of bound parasites was counted using scintillation counter. Results represent mean of three independent experiments performed in triplicates. The bars of graphs represent standard deviation value.

membrane glycoproteins of host cells (Yang et al., 1997). It was demonstrated that *Acanthamoeba* express a ~400 kDa mannose-binding protein that is constituted of multiple 130 kDa subunits (Garate et al., 2004). According to Khan (2001), the binding of *Acanthamoeba* to corneal epithelial cells was acanthopodia-dependent as no binding of non-pathogenic amoeba (which exhibit significant acanthopodia) with epithelial cells was observed. Also, he 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 (Khan, 2001).

In the case of *Trypanosoma cruzi*, participation of sugar-binding proteins in host-cell recognition has been suggested (Araujo Jorge et al., 1984; Piras et al., 1987). In particular, galactose residues from the host cell surface glycoconjugates have been shown to be relevant in the parasite attachment and invasion (Araujo Jorge et al., 1986; Ajaujo Jorge et al.,; Villalta et al., 1984).

The purpose of this study was to determine the correlation between saccharides and amoebic pathogenicity. The experiments of cytotoxicity by LDH release and adhesion assay by ^{35}S-methione release were done simultaneously and in triplicates. The results showed that pathogenic *N. fowleri* could destroy macrophages more than nonpathogenic *N. gruberi*. When four kinds of saccharides were treated with *N. fowleri* or *N. gruberi*, the amoebic cytotoxicity to macrophages was significantly decreased. In particular, α-mannose used showed the highest inhibiting effect, and the cytotoxicity of *N. fowleri* or *N. gruberi* was inhibited by saccharides in a time- and concentration-dependent manner. When 100 mM saccharides chosen in the cytotoxicity experiment were used in the adhesion ability test, the adhesion ability of *N. fowleri* was significantly decreased about 54.2% by α-mannose treatment. Although the effect on the inhibition of cytotoxicity and adhesion was not significantly differently from kinds of saccharides, it was supported that α-mannose especially play a important role in *Naegleria* interactions with murine macrophages.

Previously, it was reported that the reasons why the binding of *Acanthamoeba* of T7 genotype with brain microvascular endothelial cells was not significantly inhibited by α-mannose were (i) binding of T7 isolate was minimal to observe any differences in the presence or absence of α-mannose, (ii) mannose-binding protein is absent in T7 isolate tested, or (iii) mannose-binding protein is structurally different in T7 isolate tested (Alsam et al., 2003). In comparison with *Acanthamoeba* mentioned above, this present study suggested that more mannose-binding proteins than other saccharides-binding proteins were present in *N. fowleri* than in *N. gruberi*, and there were no structural differences of mannose- or other saccharides-binding proteins. Previously, galactose-specific lectin was identified in *Entamoeba histolytica* (Ravdin, 1989). This lectin, although equally present in pathogenic and nonpathogenic strains, appears to be antigenically different in non-invasive strains (Petri et al., 1990). When *A. castellanii* is stimulated with methyl-α-D-mannopyranoside, the amoebae secrete a new 133 kDa protein (MIP-133) that is highly cytolytic to both human and hamster corneal epithelial cells in vitro. The production of the MIP-133 protein correlates with an amoeba's ability to cause disease (Hurt et al., 2003; Leher et al., 1998).

Related with the reports mentioned above, the saccharide-binding proteins in *Naegleria* will be studied to be related with the amoebic pathogenicity for further analysis. We described the significant differences between pathogenic *N. fowleri* and nonpathogenic *N. gruberi* in their ability to bind macrophages as well as to produce in vitro cytotoxicity for the first time. It also may be helpful for providing the important informations on pathogenic mechanisms in *Naegleria* infection.

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