COMPARISON OF PHYSIOLOGICAL, CYTOPATHOGENIC AND IMMUNOLOGICAL PROPERTIES BETWEEN TWO ENVIRONMENTAL ISOLATES OF *ACANTHAMOEBA* SPP

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Abstract. The aim of this study was to determine whether pathogenic and less-pathogenic isolates of environmental *Acanthamoeba* exhibit differences in adhesion to human erythrocytes. Based on physiological properties of temperature, tolerance, and rapid growth, *Acanthamoeba* were divided into pathogenic and less-pathogenic isolates. *Acanthamoeba* were tested for their ability to produce cytopathic effects (CPE) using two human cell lines, HEp-2 and KB cells. Both ameba isolates caused CPE to both cell lines with the same pattern without significant difference. Human erythrocytes from 20 healthy volunteers were used to study the erythrocyte reactivity of *Acanthamoeba* by co-incubation with trophozoites. The pathogenic *Acanthamoeba* exhibited significantly higher erythrocyte adhesion as compared to the less-pathogens ($p<0.05$). Erythrocyte activity occurred in the presence of plasma in all blood samples, suggesting the role of plasmatic components and contact-dependent mechanisms to produce host cell cytotoxicity. The present results showed correlation between the physiological properties and erythrocyte reactivity of *Acanthamoeba*.

INTRODUCTION

Free-living amebae of the genus *Acanthamoeba* can be opportunistic pathogens for humans, causing granulomatous amebic encephalitis, skin ulcers, granulomatous sinusitis, and chronic keratitis. Although acanthamoebae are widespread in nature, frequent exposure to humans rarely causes clinical disease. (Marciano-Cabral *et al,*2000; Lam *et al*, 2002; Marciano-Cabral and Cabral, 2003). Our previous survey showed that acanthamoebae are commonly isolated from natural sites and most of them belong to morphological group II, which has various temperature tolerance, growth rate and cytopathic effects (Nacapunchai *et al,*1999, 2001, 2004). Morphological group II, the majority of environmental isolates, contain both pathogenic and non-pathogenic strains (Martinez and Visvesvara, 1997) and also belong to sequence type T4 from the twelve 18S rDNA sequence types (Gast *et al,*1996; Stothard *et al,* 1998; Walochnik *et al*, 2000; Khan *et al,* 2002). However, mechanisms of pathogenicity have not yet been well-defined, though several studies have examined links between morphology, physiological features, genotypes, protein profiles and pathogenicity of acanthamoebae (Byoung-Kuk *et al,* 2001; Howe *et al,* 1998; Khan *et al,* 2001, 2002, 2003; Ledee *et al,* 2003;Walochnik *et al,* 2000, 2004). A new perspective in differentiation of pathogenic and non-pathogenic acanthamoebae is their immunobiological properties. Many studies have indicated that host resistance mechanisms operative against *Acanthamoeba* may play an important role in the control of *Acanthamoeba* infections via both innate and acquired immunity (Walochnik *et al,* 2001; Mattana *et al,* 2002). The aim of the present study was to determine the reactivity of environmental *Acanthamoeba* isolates to human erythrocytes and compare to their cytopathic and physiological properties.

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MATERIALS AND METHODS

Ameba isolates

The isolated acanthamoebae were obtained from environmental samples of our previous study (Nacapunchai *et al,* 1999). Two strains of isolated *Acanthamoeba* were collected from soil and water samples in northern Thailand and selected depending on their physical properties. They were identified based on cyst morphology as *Acanthamoeba* gr II (Pussard and Pons, 1977) and axenically maintained in PYG medium at 35°C (Garcia and Buckner, 1993).

Physiological properties

To examine the temperature tolerance, 20 μl of *Acanthamoeba* suspension (10^6 parasites/ml) in PYG medium was dropped onto the center of non-nutrient

agar plates and incubated for 5 days at 37°C and 42°C (n=3). The plates were examined for amebae using an inverted microscope. Growth was indirectly determined by measuring the distance of the amebae moving from the outer rim of the inoculated circle line as the migration rate (mm/d), since amebae replicated as they migrated. A rapid growth rate was equated with rapid growth as evidenced by the high density of cells at the advancing front.

Cytopathic assay

To evaluate the *Acanthamoeba*-induced CPE, the assays were performed by using human epidermoid laryngeal carcinoma (HEp-2) and oral carcinoma (KB) cells as previously described (Nacapunchai *et al,* 2004). Briefly, an aliquot 200 µl of the parasite (>95% trophozoites) suspension (10^6 parasites/ml) was added to each well in 24-well plates of confluent cultures. The plates were then incubated at 37°C in a CO_2 incubator and periodically examined under inverted microscope. Control wells contained the target cells without the parasites.

Erythrocytes

Human heparinized blood smaples were obtained from 20 healthy volunteers. Each fresh blood sample was centrifuged at 250*g* for 10 minutes and the plasma was collected individually. The packed erythrocyte pellet was washed three times with sterile 0.9% NaCl solution to create a suspension containing 10^5 cells/ml in RPMI 1640 medium.

The ameba/erythrocyte co-incubation

The erythrocyte suspension (200 µl) was added to 24-well tissue culture dishes and 100 µl ml of ameba suspension in RPMI 1640 medium was added at 1:100 of ameba: erythrocyte ratio. The co-incubation was incubated at 37°C in a 5%CO_2 atmosphere and observed by inverted microscope. The experiment was also done in parallel with the addition of 50µl autologous plasma (diluted 1:10 in PBS). Each test was done in triplicate including the control wells of erythrocyte, plasma, and ameba. The number of red blood cells was counted after 3 days post-incubation (dpi) and the average value was calculated from 20 fields by 200×.

Statistics

The statistical difference between groups was determined by using Student's *t* test. Differences were considered significant at $p<0.05$.

RESULTS

Physical properties

With respect to temperature tolerance, 45°C and high growth rate (0.65±0.14 mm/h) were the selected properties to be the pathogenic strain of *Acanthamoeba*. Another isolate that can grow only at 37°C and lower migration rate (0.05±0.01mm/h), was selected as the less-pathogenic strain (De Jonckheere, 1980; Walochnik *et al,* 2000; Khan *et al,* 2001, 2002).

Cytopathic effect

The characters and dynamics of target cell changes were similar for both *Acanthamoeba* isolates in producing CPE but slightly different in intensity and time of utilization. The pathogenic isolate produced extensive CPE with complete loss of cell layers of KB and HEp2 in 4.5 and 6.0 dpi, respectively. The less-pathogenic isolate destroyed 50-60% of the target cells on 5 dpi.

Human erythrocyte reactivity

The adhesion of erythrocytes to *Acanthamoeba* trophozoites occurred within 10 minutes and only in the presence of plasma in all blood samples. The erythrocytes adhered to the parasites' membrane in a rosette form and covered all of the surface while the amebae were moving foreword. The pathogenic isolate had a significantly higher intensity of erythrocyte attachment than those of the less-pathogenic isolate ($p<0.05$). After 3 dpi, a number of small erythrocyte clumpings were found which may have been from detachment or autoagglutination. On 5 dpi, the clumpings disappeared but a few erythrocytes remained attached to the round-up and cyst forms of some amebae. Partial hemolysis was found at 6 ± 3 dpi in both isolates but no complete hemolysis and phagocytosis was observed.

In plasma control wells, it was found that six human samples caused the pathogenic ameba isolates to round up and form cysts after 5 dpi. Without plasma, no erythrocyte reactivity and hemolysis was observed, including the change of both ameba strains in all samples.

DISCUSSION

Morphological characteristics, physiological features, virulence in laboratory animals, extracellular proteases, mitochondrial sequences, mtDNA RFLP, and genetic markers have all been used to differentiate putative pathogenic from non-pathogenic strains of *Acanthamoeba* (Howe *et al,*1998; Byoung-Kuk *et al,* 2001; Khan *et al,* 2002, 2003; Ledee *et al,* 2003; Walochnik *et al,* 2000, 2004). Our study showed no correlation between physiological properties and cytopathic effect because the amebae are natural scavengers that can ingest many living cells such as

bacteria, algae and yeast as food sources as well as mammalian cells (De Jonckheere, 1980; Nacapunchai *et al,* 2004). The critical first step in the feeding mechanism of the amebae started with adhesion to target cells, including erythrocytes, which could lead to disease like other pathogenic protozoa such as *Entamoeba histolytica* (Ravdin and Guerront, 1981). However, the red blood cell or its hemolysate may have been unsuitable nutrients for the amebae, thus inducing parasite encystment as occurred in our experiments.

The ability of ameba trophozoites to invade host tissues depends on several pathogenic factors. One of the most important factors is the one that mediates cell surface adherence (Mattana *et al,* 2002). The present study revealed that host factors, especially extracellular matrix in human plasma, contributed to the trophozoite-induced adhesion of erythrocytes and subsequent hemolysis or hemagglutination, leading to pathogenesis. In contrast, some human plasmas of the present study showed that growth inhibition or encystment induction of the trophozoites may be due to some factors or specific antibodies against the ameba (Walochnik *et al,* 2001). However, no result was obtained concerning adherence-inhibiting antibodies in this study.

Erythrophagocytosis is of interest because it is a characteristic property that distinguishes the pathogenic from the non-pathogenic parasites as found in *Trichomonas vaginalis* and *Entamoeba histolytica* (Petri *et al,* 1990; Potamianos *et al,* 1992). No erythrophagocytosis was observed in the present study, which may be the characteristic of this parasite. On the basis of the present data, the environmental *Acanthamoeba* isolates, whether pathogenic or non-pathogenic, can both induce inflammation or pathogenesis via a host-parasite interaction that depends on host response and parasite strain.

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