IN VITRO CELL-TO-CELL INTERACTION OF THAI *ACANTHAMOEBA* ISOLATED FROM THE ENVIRONMENT

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Abstract. Free-living amebae of the genus *Acanthamoeba* can be opportunistic pathogens when they have to survive in human tissues and utilize host cells as a food source. The present study was performed to investigate the potential virulence of a natural *Acanthamoeba* isolated from an aquatic resource in Buri Ram Province, Thailand. The ameba can grow at 37 ˚C and 45˚C and non-pathogenic to mice by nasal instillation. The trophozoites were co-cultured with human erythrocytes and three carcinoma cells: epidermoid laryngeal carcinoma (HEp-2), cervical carcinoma (HeLa), and oral carcinoma (KB) cells. The amebae made contact first with culture cell lines within three hours and induced cytopathic changes later. They were manifested by its destruction up to a total replacement of the culture cells by amebae. The characters and dynamics of the cell changes of HEp-2, HeLa, and KB cells were similar in interaction with the amebae. No excystation or target cell destruction occurred when the *Acanthamoeba* cysts were inoculated. When co-incubated with erythrocytes, adherence occurred only in the presence of plasma, and most to the trophozoite stage and few attachments to the round up and cyst forms. Some small clumps of erythrocytes were found after 2 days of incubation. The present results show that cytoadherence and cytopathic effects can be produced by either *Acanthamoeba* pathogenic or non-pathogenic strains, which may be natural behaviors to survive or adapt to the constraints of different environments.

INTRODUCTION

Numerous strains of *Acanthamoeba*, free-living amebae, have been isolated from the environment all over the world, including Thailand (John, 1998; Nacapunchai *et al*, 1999, 2001) with increasing importance among human infections (Marciano-Cabral, *et al*, 2000; Sithinamsuwan *et al*, 2001; Wanachiwanawin *et al,* 2001). In the recent years, the interest of investigators in *Acanthamoeba* has increased, as it was proven to be the etiologic agent of a reported case with granulomatous amebic encephalitis. (Sithinamsuwan *et al*, 2001). The following investigations showed the high prevalence of free-living amebae have been isolated from the environment all over the country in Thailand belonged mainly to the genus *Acanthamoeba* (Nacapunchai *et al*, 1999, 2001). The next step of great interest for investigators is to recognize those with potential pathogenicity for human infections. Pathogenic or clinical isolates of *Acanthamoeba* trophozoites have the ability to destroy the various mammalian cell cultures *in vitro*, which is an indirect way to test the pathogenic or virulent potential of such amebae (De Jonckheere, 1980; Shin *et al*, 2001). The purpose of the present study was to investigate and compare the *in vitro* cell-to-cell interaction of the environmental isolated *Acanthamoeba* with four different cell types of human derivation.

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MATERIALS AND METHODS

Ameba cultures

The amebae were isolated from an aquatic resource in Buri Ram Province, in the northeast of Thailand (Nacapunchai *et al,*1999). *Acanthamoeba polyphaga* was identified based on cyst morphology (Page, 1988). The ameba can grow at 37°C and 45° C and is non-pathogenic to mice by nasal instillation. The axenic strain was maintained in PYG medium (Garcia and Bruckner, 1993) containing penicillin G sulfate (400 U/ml) and streptomycin sulfate (400 µg/ml) at 35°C. For preparation of inocula, incubation times varied from 72 to 96 hours for production of trophozoites (>98%) and from 6 to 8 weeks for the development of cysts (70 to 97%). Trophozoites and cysts were harvested and washed with phosphate-buffered saline (PBS) pH 7.4. Washed trophozoites or cysts were suspended in disinfectant solutions to appropriate concentrations (~10^6 cells/ml), as determined from cell counts performed in a hemocytometer counting chamber. All experiments were conducted at least in triplicate.

Human cell types

Three different types of cell lines were used: epidermoid laryngeal carcinoma (HEp-2), cervical carcinoma (HeLa), and oral carcinoma (KB) cells. They were maintained in Eagle's minimal essential medium (MEM) supplemented with 10% heat-inactivated fetal calf serum (FCS), 200U of penicillin G, and 200µg of streptomycin sulfate per ml and grown in 25-cm^2 Corning tissue-culture flasks at 37°C in a humid 5% CO_2 atmosphere. Erythrocytes were obtained from five healthy volunteer donors and plasma was separately collected. The washed erythrocytes were suspended (~10^5 cells/ml) in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum and incubated at the same condition.

The ameba/cell co-culture

A total of 10^5 target cells was suspended in 1 ml of fresh medium (MEM with 5% FCS); and seeded in 24-well tissue culture dishes containing round cover slips 12 mm in diameter. When the cells formed monolayers, 1 ml of ameba suspension was added to confluent monolayers at 1:50 ameba:cell ratios and incubated at the same condition. Cell cultures inoculated with amebae and noninoculated (control) cell cultures were examined after incubation at the selected time intervals of 3, 6, 12, and 24 hours, and observed daily for 2 weeks by inverted microscopy (100×, 200×). The cover slips were removed, fixed in methanol, stained with Giemsa's stain, mounted and examined by light microscope (400×, 1,000×). In these experiments, amebae were counted with a hemocytometer when complete CPE was recorded.

The ameba/erythrocyte co-incubation

One ml of erythrocyte suspension were added to 24-well tissue culture dishes and 1 ml of ameba suspension in RPMI 1640 medium was added at 1:100 ameba: erythrocyte ratio. The co-incubation was incubated and observed at the same condition. The experiment was also done in parallel with the addition of 50µl plasma (diluted 1:10 in PBS). Each test was done in duplicate.

Statistics

Statistical differences between groups were determined by Student's *t* test. Differences were considered significant at $p<0.05$.

RESULTS

The characters and dynamics of all three cell monolayer changes were similar after ameba inoculation. Differences were found only in the intensity and time of appearance of the changes. They presented as detached cells, disruption, formation of cell islands and destruction up to the total replacement of the culture cells by amebae. The common features were that the amebae situated in close contact with the cells produced active pseudopodia (Fig 1). The amebae located at a distance from the cells had normal characteristics. Control cell cultures were normal. Only some insignificant changes of some cells were found: cells with sharp ends; enlarged nuclei; packing of the nuclei; granulation of the nuclear chromatin. Most of the cells (95%) had a normal appearance.

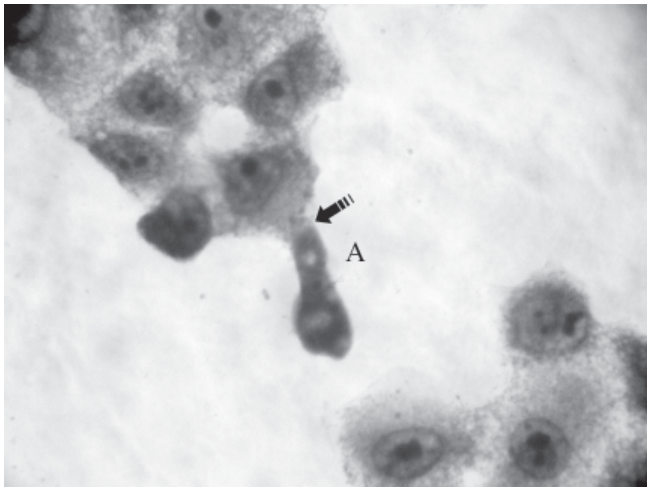

Fig 1- Co-culture of HEp2 monolayer cells with *Acanthamoeba* trophozoites for 24 hours. Note the alteration of monolayer and formation of cell islands, an amoebae (A) attached to the surface of HEp2 cell (at arrowhead) with a vigorous pseudopodium. Giemsa stain, 400×.

Acanthamoeba trophozoites formed pseudopodia that made intimate contact with the target cell membranes (Fig 1). We observed that amebae used the leading acanthapodia to move forward, adhere and utilize from the outer rim of the monolayer or cell island. The times of total replacement by amebae for KB was 4.5 days, and for HEp2 and Hela, 6.0 days were observed. The amebae when complete CPE were increased in number and size compared to the control. Cyst formation occurred 2-3 days thereafter. Inoculation of *Acanthamoeba* cysts did not produce CPE in the cell cultures.

When co-incubated with erythrocytes only in the presence of plasma, the adherence occurred mostly to the amebic trophozoites and few attachments to the rounded-up and cyst forms. The erythrocytes adhered to the parasite surface and could be seen in small or large clumping, which they carried while moving forward (Fig 2). The intensity of attachment was high (large clump) in 1 sample and low (small clump) in 4 samples. Hemolytic activity was not detected and no phagocytosis was seen, but the amounts of erythrocytes decreased compared to those from the control well during cultivation. Adherent erythrocytes could be seen in a small clump and found free-floating in culture fluid after 3 days of incubation. No adherence or hemolysis was found when co-incubated without plasma.

DISCUSSION

Acanthamoeba are natural scavengers of living cells, such as bacteria, algae, and yeasts (Upadhyay *et al,* 1968; Wright *et al,* 1981; Allen *et al*, 1990; Weekers *et al,* 1993). Not only feeding on microorganisms, they also have the ability to utilize mammalian cells as food sources (De Jonckheere, 1980; Taylor *et al*, 1995). As was also found in the present study, the amebae can destroy with complete replacement all types of cell monolayers, but encystation occurred with loss of the target cell. The feeding mechanism started from protrusion of the cytoplasm to form acanthapodia or digipodia, finger-like projections arising from the leading edge which adhered to target cells; thus, the critical first step began, which was the same as the previous study (Cao *et al*, 1998) and in other pathogenic protozoa (Voigt *et al,* 1999; Rosset *et al,* 2002).

The present experiment showed the adherence of erythrocytes to the amebae occurred only in the presence of plasma, and the difference in attachment intensity suggested that the human or host may contribute to this parasite's mechanism, leading to pathogenesis of infection (Cao *et al,* 1988; Na *et al,* 2001). In contrast with the monolayer cells, erythrocytes after adherence to the trophozoites were transported over the cell surface to the posterior end, which could be seen in a large clump. This occurrence was also found in adherent bacteria or yeast to amebae that are often seen to remain in position relative to the substrate as the cell flows beneath, until they reach the uroid where engulfment or phagocytosis take place (Allen *et al*, 1990; Khan, 2001). The decrease in erythrocytes may be due to hemolytic activity, but the mechanism of damage to the cellular membrane of the parasite remains to be elucidated. In addition,

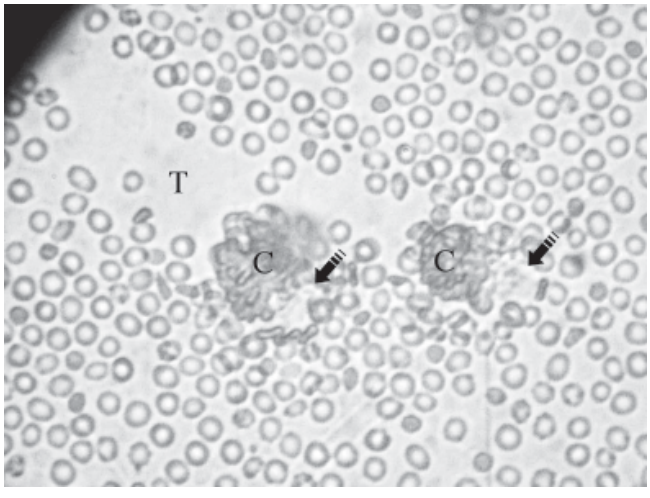

Fig 2- Coincubation of human erythrocytes with *Acanthamoeba* trophozoites for 24 hours. Note the interaction showing the large clumps of erythrocytes (C) attached on the surface of two amoebae which they carried while moving forward by protruding the clear pseudopodium (arrowhead) and left the clear track behind (T). Finding obtained by inverted microscope, 200×.

hemolytic factors may be unsuitable nutrients for the parasite and feedback inducing trophozoite encystment, which occurred in shorter times than by monolayer cells.

On the basis of the present data, this environment isolated *Acanthamoeba* appears to provide an example in which either pathogen- or non-pathogen induced cell-to-cell interaction and resulting inflammation benefit both the pathogens and the hosts. There was a direct indicator of tissue specificity, but an indirect indication for the pathogenic property of the ameba.

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