CYTOPATHOGENICITY OF *ACANTHAMOEBA* ISOLATES ON RAT GLIAL C6 CELL LINE

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Abstract. The pathogenicity of *Acanthamoeba* isolates from keratitis patients (the Hamburg isolate from Germany, H-1 and a Philippine isolate, IB-1-7) as well as an environmental isolate, W4 was assayed *in vitro* using rat glial C6 cell line. Results indicate that both live amebae and cell-free supernatants from H-1 and IB-1-7 clones produced cytopathic effects (CPE) on rat glial C6 cells in a dose-and-time-dependent fashion. A dose of 10^5 cells/ml induced death and moderate areas of destruction of individual cells after 48 hours of incubation. Results of both free zone capillary electrophoresis and sodium dodecyl sulphate polyacrylamide gel electrophoresis suggest the release of amebic products to the culture medium that could at least partially explain the observed cytopathogenicity after 48 hours. Furthermore, results of SDS-PAGE indicate differences between the secretions of the isolates, with bands produced by the two ocular isolates that were not seen with the environmental isolates. That the secretions can produce a cytopathic effect (CPE) has been shown by the cytotoxicity assays using protein concentrations of the secretory products. Protein concentration of 0.30 µg/µl of culture supernatants from H-1 and IB-1-7 clones produced similar effects on the cell monolayers after 2 hours of incubation. This concentration caused the highest % cell death as measured by both trypan blue exclusion (TBE) and 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide (MTT) assays. In contrast, using W4 clone, corresponding concentrations of both trophozoites and culture supernatant did not cause significant cell death and cellular disintegration.

INTRODUCTION

Acanthamoeba species, a widely distributed group of free-living ameba, can infect humans and spread hematogenously after direct interaction with the mucosal surfaces. These organisms have been shown to be the causative agents of life-threatening granulomatous amebic encephalitis and sight-threatening corneal keratitis.

Although *Acanthamoeba* trophozoites have been observed to produce extensive CPE on a variety of target cells, the exact mechanism underlying such cell damage is still poorly understood. Trogocytocis (Diaz *et al,* 1991; Dove Pettit *et al,* 1996), apoptosis (Alizadeh *et al,* 1994) and secretions of cytolytic enzymes such as elastase (Ferrante and Bates, 1988), collagenase (He *et al*, 1990), neuraminidase (Pellegrin *et al*, 1991), and proteinases (Hadas and Mazur, 1993; Mitro *et al*, 1994) have been cited as likely mechanisms to explain host cell destruction.

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This paper compares the ability of ocular isolates H-1 and IB-1-7 with an environmental isolate W4 to produce a CPE on C6 glial cells, with a description of the sequence of events observable by light microscopy and a limited discussion of the possible mechanisms involved. It also compares the ability of the secretory products of the three different isolates to produce a CPE with details of initial preliminary investigations to compare and characterize the said secretions.

MATERIALS AND METHODS

Strains of amebae used

Three *Acanthamoeba* isolates were used in this study. Two are from keratitis patients and one is an environmental isolate. The Hamburg isolate, H-1 was taken from a female patient with keratitis in Krankenhaus, Heidberg, Hamburg (Matias *et al*, 1991). The Philippine isolate, IB-1-7 was obtained in August 1992 from a male patient at the Ophthalmology Department, Philippine General Hospital, University of the Philippines. The third isolate, W4 was collected from a deep-well water source in Quezon City, Philippines.

Axenization and cultivation of *Acanthamoeba* isolates

The cloned isolates were axenized and cultivated in proteose peptone-glucose (PPG) medium (10 g proteose peptone and 18 g glucose dissolved in 1 liter ameba saline) and subsequently harvested after 72 hours as previously described (Matias *et al*, 1991). For the experiments on cytopathic effects, amebae were cultivated in 10-ml petri plates containing PPG medium.

Assay of cytopathic effects (CPE)

To find out the possible mechanisms of virulence *in vitro*, a series of experiments was performed using cocultures of rat glial C6 cell line and amebae. Axenically grown *Acanthamoeba* isolates, W4, H-1, and IB-1-7 were used to infect the C6 cells. These cells were grown to confluence in petri plates with cover slips, containing 10 ml Dulbecco's minimal essential medium (DMEM), supplemented with 5% fetal calf serum (FCS, Gibco) and incubated at 37°C at 5% CO_2. *Acanthamoeba* cultures in log-phase of growth were harvested as described by Matias *et al* (1991). The old medium was removed prior to addition of new medium and amebae. For each *Acanthamoeba* clone, 10^5, 10^4, and 10^3 viable trophozoites in 100 µl PBS were added to each of triplicate wells containing confluent monolayers of target C6 cells. As a control, one group of wells received 100 µl PBS. DMEM with 5% FCS was added and cocultures were incubated at 37°C at 5% CO_2 for 96 hours. Observation of CPE was done every 12 hours. The culture medium was removed and cocultures at 24, 36, 48, and 96 hours were fixed *in situ* with freshly prepared 1.5% glutaraldehyde, stained with Heidenhein's hematoxylin and photographed under the Axiovert 100 (Carl Zeiss) phase contrast microscope. Cytopathic effects were qualitatively assessed by morphological changes such as rounding up and/or disruption/detachment from the culture dish (cytopathic), disintegration and/or lysis (cytolytic), and death (cytotoxic) of the target cells.

Assay of cytotoxicity

The cloned *Acanthamoeba* strains used for cytotoxicity tests were either grown in a 1-liter stirred bioreactor or 250-ml flasks containing PPG medium. The growth curves of the amebae were determined in both bioreactor and flasks. Log-phase cultures of *Acanthamoeba* trophozoites were harvested, counted on a hematocytometer, and centrifuged. Thereafter, the supernatants were collected in 250-ml plastic beakers. Frozen cell-free supernatants were lyophilized using a LABCONCO lyphlock 1. A stock solution of 100 µg protein/100 µl of cell-free supernatant from each *Acanthamoeba* clone was prepared in PBS and filter sterilized. The concentration of protein in the fresh PPG medium and lyophilized conditioned culture media was determined using the Bradford method (Bradford, 1976).

From the stock solution, 0.05, 0.15, and 0.30 µg protein/100 µl were prepared and added to confluent cultures of C6 cells which were grown in 24-well microtiter plates. One hundred microliters fresh PPG medium was added to the control cells. The cultures were incubated at 37°C at 5% CO_2 for 96 hours. Effects of treatments were observed on an inverted Axiovert 100 (Carl Zeiss) phase contrast microscope at 1 hour interval and photographed. After 3 hours of incubation, C6 cells were trypsinized for the TBE assay.

In a separate set-up, cells were cultured in 96-well microtiter plates containing DMEM, supplemented with 5% FCS and incubated with ameba-culture supernatants at 37°C at 5% CO_2. The MTT assay was performed using 20 µl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl-tetrazolium bromide and incubated at 37°C at 5% CO_2. After 4 hours, 100 µl DMSO was added to each well and absorbance values were read at 570 nm using Beckman DU-65 model spectrophotometer. Values were compared with cells supplemented with fresh PPG medium to determine cellular disintegration and cell death.

Free zone capillary electrophoresis

To compare the profiles of the fresh PPG medium with the *Acanthamoeba*-conditioned culture medium, 10 µl each of 10 ng sample of fresh PPG medium and H-1 conditioned media were loaded into a Beckman P/ACE Series 5000 capillary electrophoresis machine. The length of the capillary used was 57 cm and the samples were ran using borate buffer (pH 8.35) at 25,000 volts and at 40.5 mA for 10 minutes. The samples were read at 214 nm and the average of three trials was obtained.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis

To detect the possible presence of excretory and secretory proteins in the conditioned culture media, cell-free supernatants of the *Acanthamoeba* clones and fresh PPG medium were subjected to 12% SDS-PAGE using an LKB 2117 Multiphor Electrophoresis System. The gels were visualized by silver staining.

RESULTS

Cytopathic effects

The disintegration of the monolayer as a result of coculture with the *Acanthamoeba* clones was dose-and-time-dependent (Table 1). Partial destruction of the target cell monolayers occurred at 36 hours with a dose of 10^5 cells of both H-1 and IB-1-7 clones. Marked destruction of the monolayers by H-1 and IB-1-7 clones was observed after 48 hours and 96 hours with a dose of 10^5 cells and 10^4 cells, respectively. On the other hand, 10^5 cells of W4 and 10^3 cells of H-1and IB-1-7 produced contraction of the target cell processes and gaps in the monolayer after 96 hours. In contrast, control cells appeared unchanged over a 96 hours period (Fig 1).

Light microscopic studies of the interaction between axenically grown *Acanthamoeba* and rat glial C6 cells showed varying magnitude of cytopathic effects. The sequence of events in the devel-

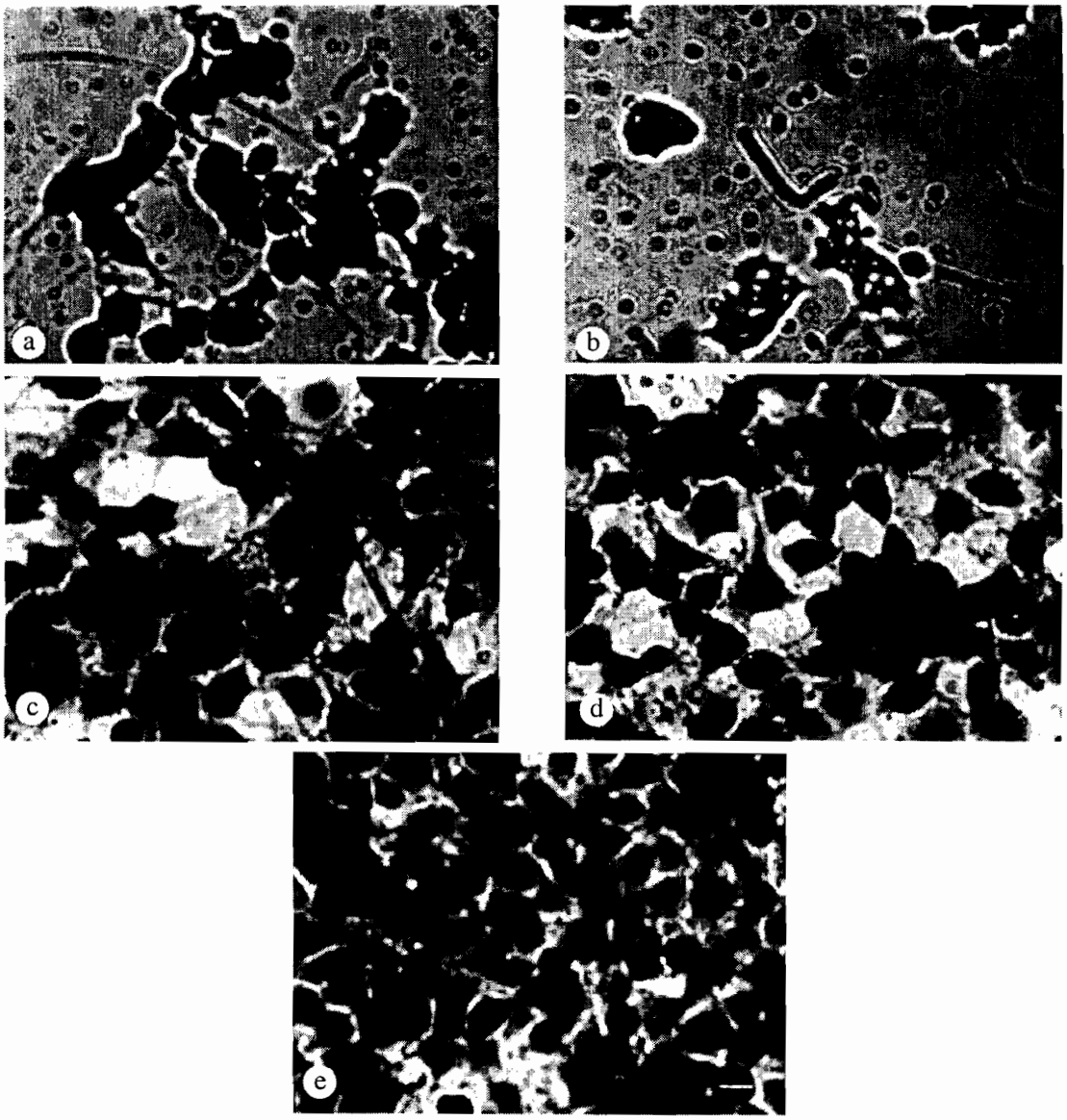

Fig 1–Light micrographs showing the cytopathic effects (CPE) produced by trophozoites of *Acanthamoeba* clones on rat glial C6 cell line after co-cultivation for a) 36 hours, and b) 48 hours with 10^5 cells H-1 and IB-1-7, c) 96 hours with 10^4 cells H-1 and IB-1-7, d) 96 hours with 10^5 cells W4, 10^3 cells H-1 and IB-1-7, e) control (Heidenhein's hematoxylin. Bar = 3 μm).

Table 1
Cytopathic effects of live *Acanthamoeba* clones on rat glial C6 cell line.

Dose level	Clone	Control	Incubation time (Hour)							
			12	24	36	48	60	72	84	96
		PBS	-	-	-	-	-	-	-	-
	W4		-	-	-	-	-	-	-	+
10^5	IB-1-7		-	+	+	++	++	++	++	++
	H-1		-	+	+	++	++	++	++	++
	W4		-	-	-	-	-	-	-	-
10^4	IB-1-7		-	-	-	-	+	+	+	++
	H-1		-	-	-	-	+	+	+	++
	W4		-	-	-	-	-	-	-	-
10^3	IB-1-7		-	-	-	-	-	-	-	+
	H-1		-	-	-	-	-	-	-	+

(-) No effect
(+) Partially detached or disintegrated
(++) Completely detached or disintegrated

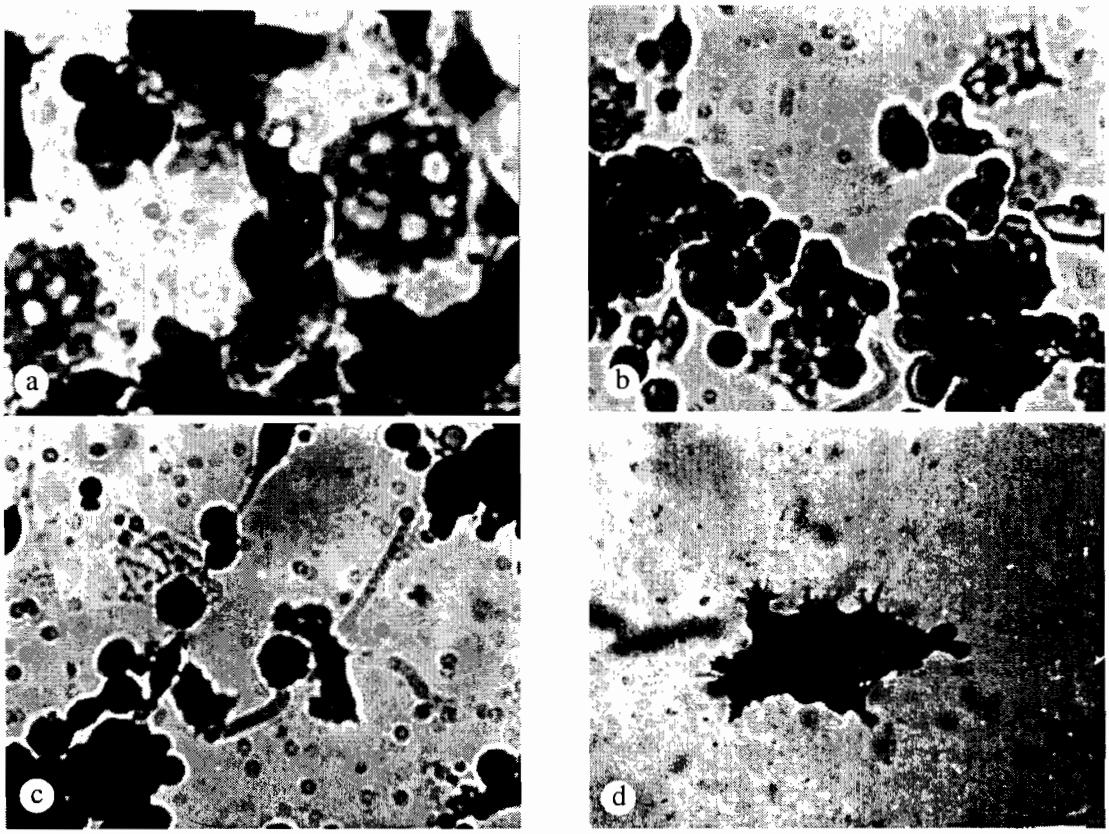

Fig 2–Light micrographs showing the sequence of events of CPE produced by trophozoites of the *Acanthamoeba* clones on rat glial C6 cell monolayers after co-cultivation for a) 24 hours (contraction of cell processes and formation of gaps in the monolayer), b) 36 hours (loss of contact of cells with their neighbors), c) 36 hours (extension of pseudopodia by the ameba on the glial cell fragments), d) 48 hours (phagocytocis). Bar = 5 μm.

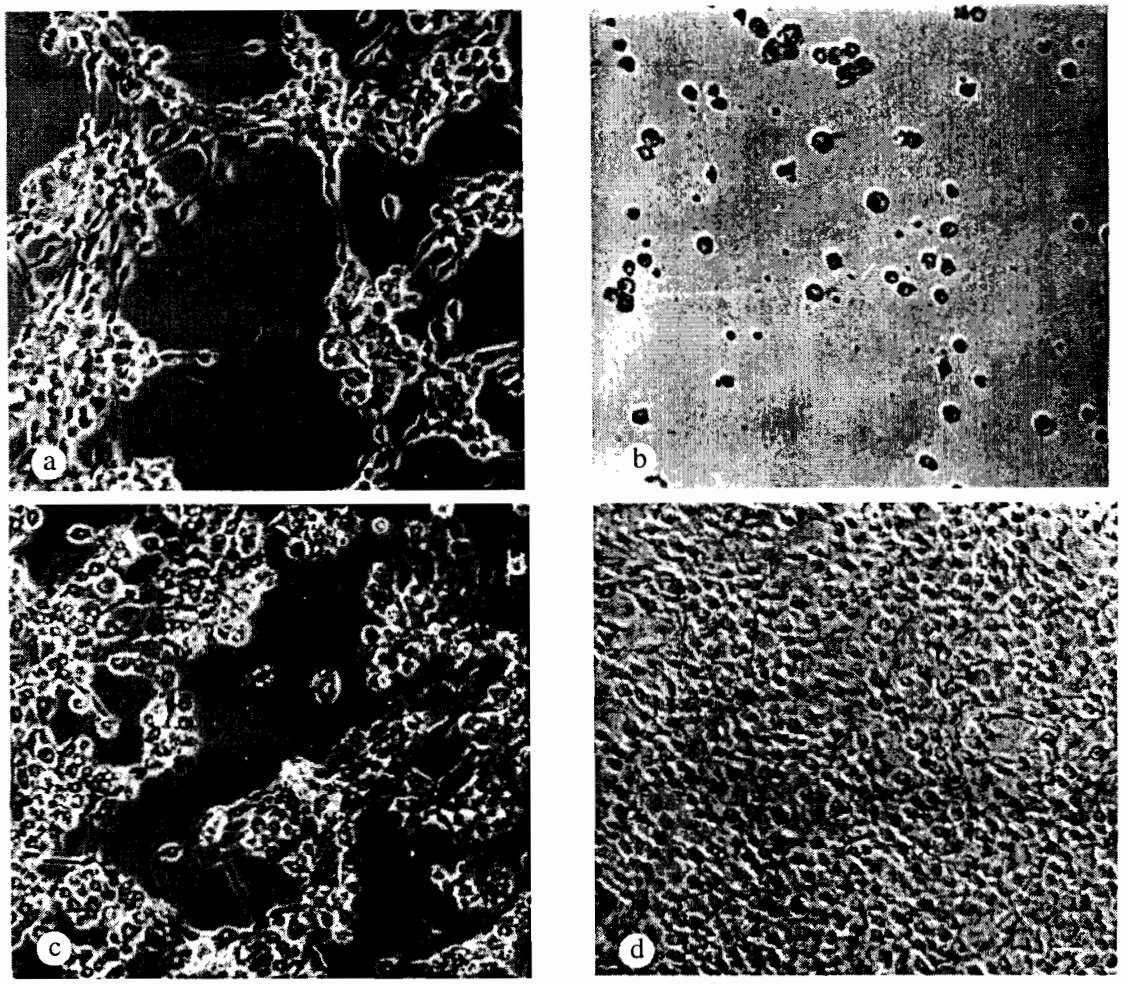

Fig 3–Light micrographs showing the cytolytic effects of cell-free supernatants from *Acanthamoeba* clones on rat glial C6 cell monolayers after coincubation for a) 1 hour, and b) 3 hours with IB-1-7 & H-1, c) 3 hours with W4, d) control. Bar = 2 μm.

opment of CPE as detected by light microscopy started at 24 hours with the contraction of cell processes and formation of gaps in the monolayer, loss of contact of some cells with their neighbors at 36 hours, extension of pseudopodia by the ameba on the glial cell fragments, subsequent ingestion of glial cell fragments by the trophozoites at 48 hours, and replacement of the cell monolayers by the trophozoites (Fig 2). Amebae at first surround target C6 cells, which then become rounded and are finally destroyed.

Cytotoxic effects

The responses elicited by the target C6 cells to amebic supernatants were concentration-and time-dependent. A concentration of 0.30 μg protein /μl of both H-1 and IB-1-7 clones produced partial and

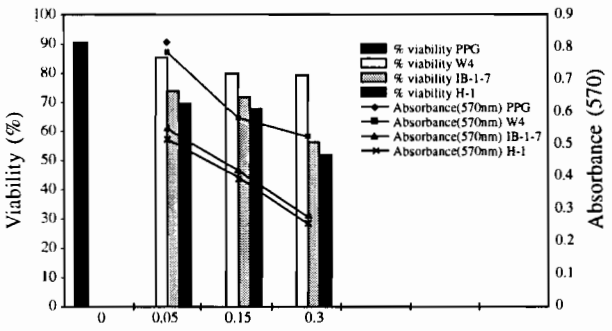

Fig 4–Cytotoxic and cytolytic effects of cell-free supernatants from *Acanthamoeba* clones on rat glial C6 cells as measured by TBE and MTT assays. Histogram and line graph represent values obtained from TBE and MTT assays, respectively.

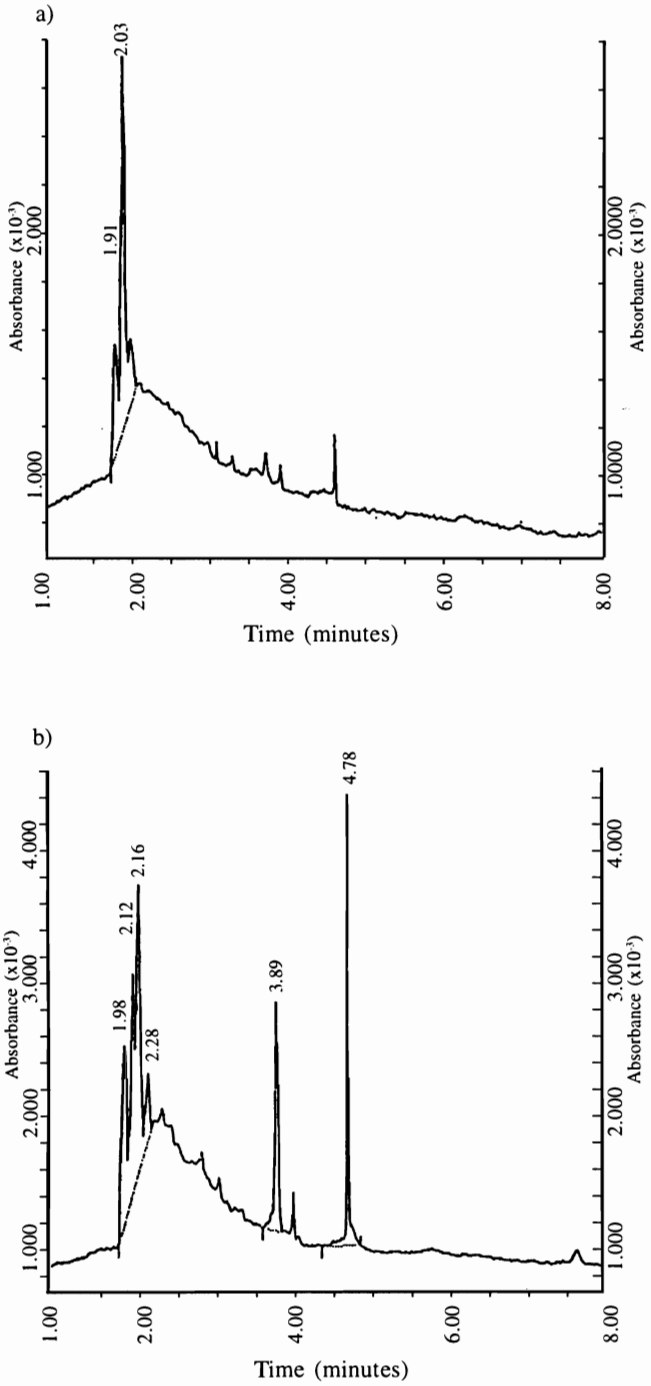

Fig 5–Free zone capillary electrophoresis profiles of a) Fresh PPG medium, and b) cell-free supernatant from H-1 clone. Running conditions: 25 KV in 1 M borate buffer, pH 8.35, 57 cm capillary tube, 214 µm.

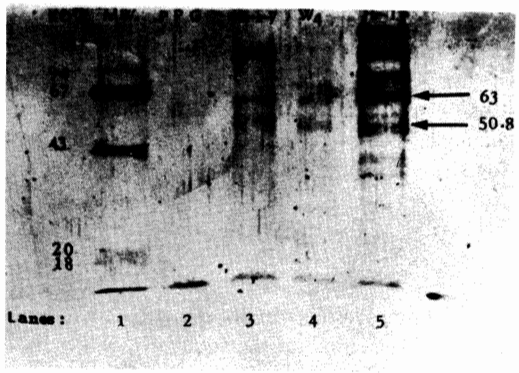

Fig 6–SDS-PAGE protein profile of cell-free supernatants from 96 hours *Acanthamoeba* cultures stained with silver. Protein band at 63 kDa region is common to all three isolates, while the band at 50.8 kDa is found in both H-1 and W4 clones. H-1 and IB-1-7 have bands not seen in the W4 isolate. 1) Molecular weight marker, 2) Fresh PPG medium, 3) IB-1-7, 4) W4, 5) H-1.

maximum destruction of the target cell monolayers after 1 hour and 2 hours of incubation, respectively. The same concentration of conditioned culture medium from W4 clone resulted in partial detachment of the cell monolayers from 2 hours to 3 hours. Such effect was also observed at lower concentrations (0.05 and 0.15 µg protein/µl) of cell-free supernatants from both H-1 and IB-1-7 clones. Control cells maintained their structural integrity up to 96 hours (Fig 3).

Results of the trypan blue exclusion assay correlate well with MTT assay (Fig 4). Highest % of cell death occurred in C6 cells incubated with 0.30 µg protein/µl of conditioned culture media from both H-1 and IB-1-7 clones. Conversely, incubation with a similar concentration of W4 supernatant did not result in significant cell death with 80% viable cells. Moreover, untreated cells showed 91% viability.

Free zone capillary electrophoresis

Figs 5 a and b show profiles of the fresh PPG medium and amebae-conditioned culture medium, respectively. Two prominent peaks (at 3.89 and 4.78 minutes) were detected in the culture supernatant from H-1 clone but these were not found in the fresh PPG medium.

SDS-PAGE analysis

Silver staining after SDS-PAGE (Fig 6) shows two bands in IB-1-7 clone, 3 in W4 clone, and 10

in H-1 clone. The molecular weights of these bands range from 36 kDa to 75 kDa. A common band appears in all three *Acanthamoeba* culture supernatants at 63 kDa. W4 and H-1 supernatants have a common band at 50.8 kDa region. No bands were found in the control.

DISCUSSION

The present data indicate that both live amoebae and amebic culture supernatants produced CPE on rat glial C6 cells in a dose-and-time-dependent process. A dose of 10^5 cells/ml of H-1 and IB-1-7 destroyed the target cell monolayers after 48 hours of incubation, whereas 10^4 cells/ml caused similar effects only after 96 hours. However, amebic trophozoites at 10^3 dose level did not only fail to induce CPE on the cell monolayers but also failed to grow after 96 hours coincubation. These document previous findings that successful infection *in vivo* is a consequence of high dose (Ferrante, 1991; Lagmay, 1995).

The pattern of virulence seen with both human isolates on rat glial C6 cells was in general agreement with the CPE on human keratocytes (Badenoch *et al*, 1990).

The observed recognition and adherence of amebic trophozoites to target C6 cells that culminated in phagocytocis conform with past studies. Morton *et al* (1991) cited that the recognition of host cell glycolipids and/or glycoproteins by a variety of microbial pathogens appears to be significant in adherence of microorganisms to their target tissues. *Acanthamoeba* adhesion to the cornea is a prerequisite for colonization and invasion of the corneal epithelium.

Light microscopic studies of the interaction between both axenically grown amebae and conditioned culture media with rat glial C6 cells infer the release of amebic factors, which were observed to coincide with the log-phase of growth of the amebae as one of the major mechanisms for the cytopathic effects leading to target cell death. This supposition has been shown in several studies (Fulford *et al*, 1985; Pellegrin *et al*, 1991; Pidherney *et al*, 1993; Alizadeh *et al*, 1994). Such findings are in agreement with the data obtained from both capillary electrophoresis and SDS-PAGE. These show two major peaks in the ameba-conditioned culture medium and the presence of protein bands in all three *Acanthamoeba* clones that are not found in the fresh PPG medium.

The appearance of a common protein band with an approximate molecular weight of 63 kDa in the three *Acanthamoeba* isolates is of particular interest since the protein product may play a role in the disruption as well as death of C6 cells. Similar results were obtained by Mitro *et al* (1994) from the excretory and secretory products of *A. polyphaga* trophozoites. The additional protein bands that were not observed from the environmental isolate could probably contribute to the more marked CPE produced by the human isolates.

While the present investigation strongly suggests a role for *Acanthamoeba*-released soluble factors as one of the major mechanisms for the CPE on the target cells, the possibility of additive effects of contact-dependent lysis by amebic trophozoites cannot be excluded. Trogocytocis of Vero cells by means of sucker-like structures termed amebostomes was observed with two strains of *Acanthamoeba* (Diaz *et al,* 1991). This mechanism has also been elucidated in 4 species of *Acanthamoeba* that destroyed rat B103 neuroblastoma cells *in vitro* (Dove Pettit *et al,* 1996).

Moreover, Alizadeh *et al* (1994) reported that *Acanthamoeba castellanii* lysed tumor cells by a process involving apoptosis or programed cell death. Dove Pettit *et al* (1996) documented such findings when they observed *Acanthamoeba* trophozoites in contact with the target cell to have played a critical role in eliciting target cell death. The effector cell-target cell contact was associated with margination of chromatin and membrane blebbing, morphological changes characteristic of apoptosis.

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REFERENCES

Alizadeh H, Pidherney MS, McCulley JP, Neiderkorn JY. Apoptosis as a mechanism of cytolysis of tumor cells by a pathogenic free-living amoeba. *Infect Immun* 1994; 62: 1298-303.

Badenoch PR, Johnson AM, Christy PE, Coster DJ. Pathogenicity of *Acanthamoeba* and a *Corynebacterium*

in the rat cornea. *Arch Ophthalmol* 1990; 108: 107-12.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye-binding. *Anal Biochem* 1976; 72: 248-54.

Diaz J, Osuna A, Rosales MJ, Cifuentes J, Mascaro C. Sucker-like structures in two strains of *Acanthamoeba*: Scanning electron microscopy study. *Int J Parasitol* 1991; 21: 365-7.

Dove Pettit DA, Williamson J, Cabral GA, Marciano-Cabral F. *In vitro* destruction of nerve cell cultures by *Acanthamoeba* spp: A transmission and scanning electron microscopy study. *J Parasitol* 1996; 82: 769-77.

Ferrante A, Bates EJ. Elastase in the pathogenic free-living amoebae *Naegleria* and *Acanthamoeba* spp. *Infect Immun* 1988; 56: 3320-1.

Ferrante A. Free-living amoebae: pathogenicity and immunity. *Parasite Immunol* 1991; 13: 31-47.

Fulford DE, Bradley SG, Marciano-Cabral F. Cytopathogenicity of *Naegleria fowleri* for cultured rat neuroblastoma cells. *J Protozool* 1985; 32: 176-180.

Hadas E, Mazur T. Proteolytic enzymes of pathogenic and nonpathogenic strains of *Acanthamoeba* spp. *Trop Med Parasitol* 1993; 44: 197-200.

He YG, Neiderkorn JY, McCulley JP, *et al. In vivo* and *in vitro* collagenolytic activity of *Acanthamoeba castellanii. Invest Ophthalmol Vis Sci* 1990; 31: 2235-40.

Lagmay JP. Pathogenicity of two human isolates of *Acanthamoeba* spp in mice. Institute of Biology, College of Science, UP Diliman, Quezon City, Philippines. 1995, PhD Dissertation.

Matias RR, Schottelius J, Raddatz Ch F, Michel R. Species identification and characterization of an *Acanthamoeba* strain from human cornea. *Parasitol Res* 1991; 77: 469-74.

Mitro K, Bhagavathiammai A, Zhou O, *et al.* Partial characterization of the proteolytic secretions of *Acanthamoeba* polyphaga. *Exp Parasitol* 1994; 78: 377-85.

Morton LD, McLaughlin GL, Whitely HE. Effects of temperature, amebic strain, and carbohydrates on *Acanthamoeba* adherence to corneal epithelium *in vitro*. *Infect Immun* 1991; 59: 3819-22.

Pellegrin JJ, Ortega-Barria E, Barza M, Baum J, Perevia MEA. Neuraminidase activity in *Acanthamoeba* species trophozoites and cysts. *Invest Ophthalmol Vis Sci* 1991; 32: 3061-6.

Pidherney MS, Alizadeh H, Stewart GL, McCulley JP, Neiderkorn JY. *In vitro* and *in vivo* tumoricidal properties of a pathogenic/free-living amoeba. *Cancer Lett* 1993; 72: 91-8.