

***In vitro* assessment of cytokines interactions with *Balamuthia mandrillaris* using human brain microvascular endothelial cells**

Abdul Matin^{1,4*}, Suk-Yul Jung² and Salik Nawaz³

¹Department of Medical Lab Technology, University of Haripur, Hattar Road, Haripur, Khyber Pakhtunkhwa, Pakistan

²Department of Biomedical Laboratory Science, Molecular Diagnosis Research Institute, Namseoul University, Cheonan, Korea

³Department of Pharmaceutical Sciences, University of the Punjab, Lahore, Pakistan

⁴Institute of Biomedical & Genetic Engineering, PO Box: 2891, Sector G-9/1, Islamabad, Pakistan

Abstract: *Balamuthia* amoebic encephalitis (BAE) is a life threatening human disease which, always lead to death. Amoebae invasion of the bloodstream is considered an important step in BAE followed by their haematogenous spread. It is more likely that *Balamuthia mandrillaris* enters into the central nervous system through blood–brain barrier (BBB) sites. The objective of the present study was to determine the impact of cytokines on biological properties of *Balamuthia in vitro*. Human brain microvascular endothelial cells (HBMEC), which constitutes the BBB were used *in vitro* test model for the present investigation. It was observed that *Balamuthia* exhibited >90 % binding and >70% cytotoxicity to HBMEC. However, cytokines did not affect amoebic binding and cytotoxicity except lipopolysaccharide (LPS) which reduced *Balamuthia*-mediated HBMEC cytotoxicity. It is also important to note that amoebic numbers were reduced in the presence of LPS within 24 h. We have shown previously the bacterial uptake by *Balamuthia* is very limited which is further investigated in the presence of cytokines and observed a slight reduction of bacterial uptake during phagocytosis assay. Zymography assays revealed there is no effect of cytokines on proteolytic activity of *Balamuthia*. Overall we described for the first time that cytokines has no inhibitory effects on biological properties of *Balamuthia in vitro*.

Keywords: *Balamuthia mandrillaris*, cytokines, cytotoxicity, pathogenicity.

INTRODUCTION

Balamuthia mandrillaris is newly emerged protozoa and belongs to free-living amoebae family. It can cause serious and fatal human infections i.e. cutaneous and encephalitis, involving the skin and central nervous system (CNS) respectively (Anzil *et al.*, 1991; Visvesvara *et al.*, 1993). Single identified specie of *B. mandrillaris* exists in two stages life cycle i.e. cyst (dormant) and trophozoite (active/pathogenic) in the environment. The pathogenic potential of *Balamuthia* trophozoite relay on ability of the amoebae to bind to mucosal surfaces, migrate through tissue to tissue, and additionally release proteases which can destroy the host connective tissue (Matin *et al.*, 2006). Moreover, a galactose binding protein 100 kDa add the pathogenic potential of the parasite to target cells *in vitro* (Matin *et al.*, 2007).

There have been a lot of current developments in BAE diagnosis (Booton *et al.*, 2003; Tavares *et al.*, 2006), even then the pathogenesis and pathophysiology of this life threatening disease (BAE) remains unclear. During BAE infection process which involves *Balamuthia* invasion of intravascular space either through respiratory tract or skin lesions, followed by haematogenous spread. *Balamuthia* entry into the CNS most probably occurs through BBB (Schuster and Visvesvara, 2004a). Compared to the rest of the human body, the CNS is considered sterile; this environment is maintained by the BBB, which consists of

a continuous layer of vascular endothelial cells joined by tight junctions. This barrier is highly selective to all components of the blood and consequently, the brain is inaccessible to many dyes, drugs and microbes. In BAE, *Balamuthia* produce a disseminated blood-borne infection in immunocompromised as well as immunocompetent hosts. Since there are no lymphatic channels in the brain, amoebae most likely enter the CNS via the BBB. However, there is insufficient information available regarding *Balamuthia* interactions with HBMEC, which constitute the BBB. *Balamuthia* adhesion to the host cell is a key step in its pathogenesis, leading to invasion of deeper tissues. Given the complexity of pathogenesis, it would not be surprising if *Balamuthia* interaction with the host cell would result in stimulation of various signaling pathways, which eventually results into host cell damage (Matin *et al.*, 2006b; 2007; 2008) allowing amoebae translocation of the BBB.

Balamuthia granulomatous encephalitis (BGE) is distinguished by headache, fever, characteristic skin lesions, stiff neck, nausea, vomiting, acute confused state, with cerebral haemorrhagic necrotizing lesions detected by neuroimaging scans, cranial nerve palsies, seizures and finally death (Schuster and Visvesvara, 2004b). Presently there is no specific or even recommended treatment against BAE and hence the death rate is more than 98% (Healy, 2002; Deetz *et al.*, 2003; Jung *et al.*, 2004). There is a possibility of very limited rate of success which also totally depend on the early diagnosis followed by

*Corresponding author: e-mail: amawan@live.co.uk

aggressive treatment. Due to complexity and very little knowledge about BAE treatment, clinicians are forced to use a mixture of antimicrobials with a little hope for a successful outcome. However, such therapy methods may have adverse side effects on the patient (Schuster and Visvesvara, 2004).

Previously reports have already established the role of the somatic immune system in host defences against *Acanthamoeba* spp. Phagocytes, cytokines, antibodies and complement emerge to be the first-line of defense against *Acanthamoeba* infections in human (Ferrante, 1991; Toney and Marciano-Cabral, 1998). The cell types and mediators are involved in host defense mechanism within CNS, however, it have not been evidently clear. In contrast, it has been previously shown that murine microglia is suitable host cells for *T. gondii*. However, when primed by TNF- α and IFN- γ plus lipopolysaccharide are capable of reduction of parasitic increase (Chao *et al.*, 1993; Benedetto *et al.*, 2001). On the other hand, microglia derived from human fetuses which were activated by cytokines like TNF- α or IL-6, with or without IFN- γ emerge to diminish *T. gondii* growth (Chao *et al.*, 1994). Genus *Balamuthia* and *Acanthamoeba* belong to the same family, i.e., Acanthamoebidae. Being a member of free-living amoeba and a close relative of *Acanthamoeba* we can assume that *Balamuthia* may also exploit the similar strategies for infection as *Acanthamoeba* does and we might use the same strategy to control it. It is evident that proinflammatory cytokines could be involved in the immunopathogenesis of BAE, thus the aim of the present study was to investigate the impact of cytokines (TNF- α , TGF- β , IL-6 and INF- γ) and LPS on the biological properties of *Balamuthia mandrillaris* *in vitro*.

MATERIALS AND METHODS

All chemicals used in the present study were purchased from Sigma unless otherwise stated.

Human brain microvascular endothelial cell cultures

Primary human brain microvascular endothelial cells (HBMEC) were cultured as described previously (Stins *et al.*, 1997; Matin *et al.*, 2006a). Briefly, HBMEC were grown in 20% heat-inactivated fetal bovine serum, streptomycin (100 μ g/ml), 1 mM pyruvate, non-essential amino acids, 2 mM glutamine, penicillin (100 U/ml), vitamins and RPMI-1640 (Invitrogen, Paisley, UK). For experiments, HBMEC (5×10^5 cells/ml/well) were grown in 24-well plates and incubated at 37 °C in the presence of 5% CO₂. This cell density forms confluent monolayer within 24 h, which were used for experiments afterwards.

Cytokines

Recombinant human tumor necrosis factor- α (TNF- α), Interferon- γ (INF- γ), transforming growth factor- β 1

(TGF- β 1) and Interleukin-6 (IL-6) were purchased from Roche (Germany). Cytokines were used to determine whether exhibited cytolytic activity for *Balamuthia* (Fischer-Stenger *et al.*, 1990; Fischer-Stenger and Marciano-Cabral, 1992). In addition *E. coli* lipopolysaccharide (LPS) is also used as inflammatory stimuli on *B. mandrillaris*.

Cultures of Balamuthia mandrillaris

Balamuthia mandrillaris was isolated from the brain of a mandrill baboon (ATCC 50209). *Balamuthia* were cultured on HBMEC monolayer (which served as a food source for amoeba) as described previously (Matin *et al.*, 2006a). Briefly, *Balamuthia* (10^6 parasites in 10 ml of RPMI-1640) were inoculated with HBMEC in T-75 tissue culture flasks. *Balamuthia* consumed HBMEC within 48 h and approximately produced $5-8 \times 10^6$ parasites/10ml (approximately >99 % in trophozoite forms), which were used for experiments afterwards.

Cultures of bacteria

Bacterial isolate used in the present study was Gram-negative bacteria *Escherichia coli* K1. This clinical strain was isolate from a cerebrospinal fluid of a neonate with meningitis. Furthermore laboratory non-invasive *E. coli* K12 strain was also used in the present study. Bacteria were grown in Luria- Bertini (LB) medium for overnight containing 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 1% (w/v) NaCl.

Adhesion assays

To establish whether cytokines affects *Balamuthia* binding to HBMEC, adhesion assays were performed as described previously (Matin *et al.*, 2007a). Briefly HBMEC were grown until confluent in 24-well plates with or without various cytokines {INF- γ , TGF- β , TNF- α and IL-6 (1 and 10 ng/ml final concentration) and LPS (10 μ g/ml)}. *Balamuthia* (2×10^5 amoebae) were incubated with HBMEC in 24-wells plates for 60 min at 37°C in the presence of 5% CO₂. In some experiments *Balamuthia* was pre-incubated with various cytokines for 1 h followed by incubation with HBMEC. The unbound amoebae were counted by haemocytometer and the numbers of bound amoebae were calculated by the following formula:

$$\frac{\text{No. of unbound amoebae}}{\text{Total number of amoebae}} \times 100 = \% \text{ unbound amoebae}$$

While the numbers of bound amoebae were deduced as follows:

$$\% \text{ unbound amoebae} - 100 = \% \text{ bound amoebae}$$

Cytotoxicity assays

Cytotoxicity assays were performed as described previously (Matin *et al.*, 2007b). Briefly, *Balamuthia* were incubated with HBMEC in 24-well plates with or without various cytokines as described for adhesion assays. Plates were incubated for 24 h at 37°C in the

presence of 5% CO₂. After this incubation, cell free supernatants were collected and cytotoxicity was determined by measuring lactate dehydrogenase (LDH) release (cytotoxicity detection kit; Roche Applied Science, Lewes, East Sussex, UK). Briefly, conditioned media of co-cultures of amoebae and HBMEC were collected and percentage LDH was detected by the following formula:

$$\frac{\text{Sample value} - \text{control value}}{\text{Total LDH release} - \text{control value}} \times 100 = \% \text{ Cytotoxicity}$$

Control values were obtained from HBMEC incubated in RPMI alone. Total LDH release was determined from HBMEC treated with 1% Triton X-100 for 30 min at 37°C.

Growth assays

To determine whether *Balamuthia* increases in numbers on HBMEC monolayer in the presence of various cytokines growth assays were performed as described previously (Matin et al., 2006a). Briefly HBMEC were grown in 24-well plates with or without various cytokines. *Balamuthia* (2x10⁵ amoebae) were incubated with HBMEC in 24-wells plates as described in cytotoxicity assay. After 24 h incubation, the amoebae were counted using a haemocytometer and total numbers of amoebae were calculated by the following formula:

$$\frac{\text{No. of haemocytometer counted amoebae}}{\text{Total number of amoebae}} = \text{Amoebae growth or increased amoebae}$$

Phagocytosis assays

To determine whether cytokines influence the ability of *Balamuthia* to engulf bacteria, phagocytosis assays were performed as described previously (Alsam et al., 2005). Briefly, *Balamuthia* was pre-treated with various cytokines as described above. Furthermore pre-treated *Balamuthia* (5 x 10⁵/ 200 µl) were incubated with 5 x 10⁶ *E. coli* K1 and K12 at 37 °C for 1 h. After this, *Balamuthia* were centrifuged at 1,000 x g for 5 min. Pellets were resuspended with PBS. This process was repeated for 3 times (to drain out the outer surface bacteria), followed by the addition of gentamicin (100 µg/ml, final concentration for 45 min) to kill extracellular bacteria if any left. The samples were washed again as described above. Supernatants were removed and pellets were resuspended in 0.5% sodium dodecyl sulfate (SDS) in PBS to lyse *Balamuthia*. *E. coli* are insensitive to SDS at this concentration as described previously (Alsam et al., 2005). Finally, bacterial colonies were counted by plating on nutrient agar plates.

The percent bacterial invasion/uptake was calculated by the following formula.

$$\frac{\text{Recovered E. coli (cfu)}}{\text{Total E. coli (cfu)}} \times 100 = \% \text{ intracellular E. coli}$$

Zymography assays

To determine the impact of various cytokines on *Balamuthia* proteases, zymography assays were

performed as described previously (Matin et al., 2006b). Briefly, *Balamuthia* was pre-incubated with various cytokines for 1 h. Next amoebae were incubated with HBMEC for 24 h at 37°C in the presence of 5 % CO₂. Next day cultures were centrifuged at 13,000 rpm for 5 min. Supernatant was collected and used as a conditioned medium. *Balamuthia* pallet (5 x 10⁵ parasites, was lysed by freeze-thawed 3 times) and their conditioned media were mixed (1:1) with sample buffer (which contain 4 % SDS and electrophoresed on SDS-polyacrylamide gel electrophoresis (SDS-PAGE) containing gelatin (1 mg/ml) (Sigma Laboratories, Poole, Dorset, England). Electrophoresed gels were soaked in 2.5 % Triton X-100 (w/v) solution for 1h to remove SDS. Finally, the gels were incubated in a developing buffer (50 mM Tris-HCl, pH 7.5, containing 10 mM CaCl₂) for overnight at 37°C. Next day gels were rinsed and stained with Coomassie brilliant blue and areas of gelatin digestion were visualized as non-staining regions in the gel which represent the protease activity.

RESULTS

Cytokines exhibited insignificant effects on *Balamuthia* binding and cytotoxicity to HBMEC cells

It is well established notion that *Balamuthia* binds with HBMEC which is considered to be the primary step of its invasion to CNS. In this study, we investigated the impact of various cytokines in *Balamuthia* binding to HBMEC. Our results discovered that *Balamuthia* showed more than 90% binding to HBMEC. Furthermore to determine whether cytokines are involved in *Balamuthia* binding to HBMEC, adhesion assays were performed with or without various cytokines TNF-α, TGF-β, IL-6 and INF-γ (1, 10ng/ml final concentrations) and LPS (10µg/ml). We observed that cytokines exhibited slight increase in amoebae binding when incubated with HBMEC. *Balamuthia* alone with HBMEC (without cytokines) is considered as a control (table 1).

Next to determine the role of cytokines on *Balamuthia*-mediated HBMEC death, cytotoxicity assays were performed. Our results revealed that *Balamuthia* caused sever HBMEC cytotoxicity i.e. >70% within 24 h. Furthermore it has been observed that TNF-α, and IL-6 slightly reduced *Balamuthia*-mediated HBMEC cytotoxicity while LPS showed minimal as compare to the control (table 1). In contrast TGF-β and INF-γ did not show any increase or decrease in cytotoxicity when compared with control.

Cytokines exhibited slight increase in *Balamuthia* number in vitro

Moreover to determine the effects of cytokines on *Balamuthia* numbers, growth assays were performed. Our findings disclosed *Balamuthia* numbers were doubled within 24 h when incubated together with HBMEC.

However amoebae numbers reached to its maximum in the presence of cytokines TNF- α and TGF- β (fig. 1), in contrast interestingly LPS exhibited decrease in amoebic numbers as compared to control (amoeba alone).

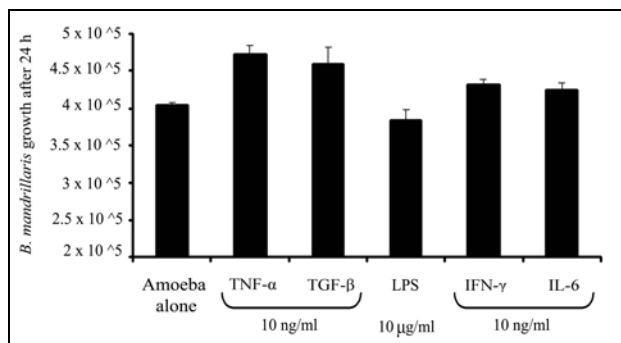


Fig. 1: Cytokines exhibited slight increase in *Balamuthia* number *in vitro*.

To determine the impact of cytokines on *Balamuthia*, assay was performed as described in “Materials and Methods”. Cytokines TNF- α and TGF- β (10 ng/ml) enhanced amoebic numbers in contrast LPS reduced the amoebae numbers within 24 h on HBMEC. *Balamuthia* with HBMEC is considered as a standard (A). Results are representative of three independent experiments performed in duplicate. Bars represent standard error.

Cytokines exhibited slight inhibition of bacterial ingestion by *Balamuthia*

We have shown previously in the plating assays (Matin *et al.*, 2006a) that *Balamuthia* displayed limited ability to uptake bacteria but remain in the trophozoite form for

longer period. Here to determine whether cytokines influence the amoebae ability of bacterial uptake, phagocytosis assays were performed using *E. coli* K1 and K12. We observed that all cytokines tested have demonstrated inhibitory effects on the ability of bacterial uptake by *Balamuthia* with maximum inhibition with LPS (figs. 2A and 2B).

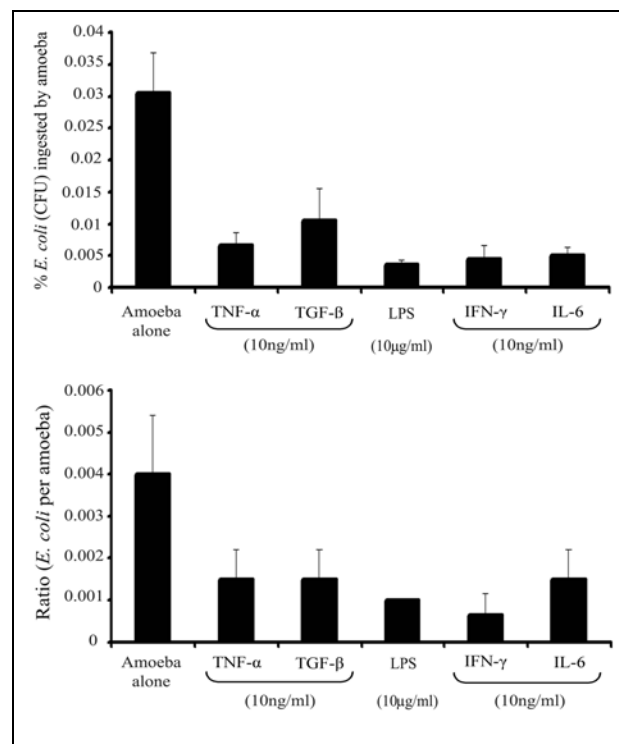


Table 1: Cytokines exhibited insignificant effects on *B. mandrillaris* binding and cytotoxicity to HBMEC cells

		<i>B. mandrillaris</i> (%) binding to HBMEC cells within 1 h incubation	<i>B. mandrillaris</i> -mediated (%) HBMEC cytotoxicity (cell death) after 24 h
<i>B. mandrillaris</i> incubated with HBMEC without cytokines (control)		88.5 $\pm$ 4.3	75.5 $\pm$ 5.3
<i>B. mandrillaris</i> incubated with HBMEC in the presence of different cytokines			
1.	Tumor necrosis factor- α (TNF- α)	<i>B. mandrillaris</i> + 1 ng/ml	90.15 $\pm$ 3.4
		<i>B. mandrillaris</i> + 10 ng/ml	92.3 $\pm$ 1.12
2.	Transforming growth factor- β 1 (TGF- β 1)	<i>B. mandrillaris</i> + 1 ng/ml	90.5 $\pm$ 5.8
		<i>B. mandrillaris</i> + 10 ng/ml	94.8 $\pm$ 2.6
3.	Interferon- γ (INF- γ)	<i>B. mandrillaris</i> + 1 ng/ml	89.2 $\pm$ 2.16
		<i>B. mandrillaris</i> + 10 ng/ml	93.85 $\pm$ 0.5
4.	Interleukin-6 (IL-6)	<i>B. mandrillaris</i> + 1 ng/ml	91.19 $\pm$ 6.3
		<i>B. mandrillaris</i> + 10 ng/ml	89.4 $\pm$ 6.7
5.	Lipopolysaccharide (LPS)	<i>B. mandrillaris</i> + 10 μ g/ml	92.68 $\pm$ 6.5

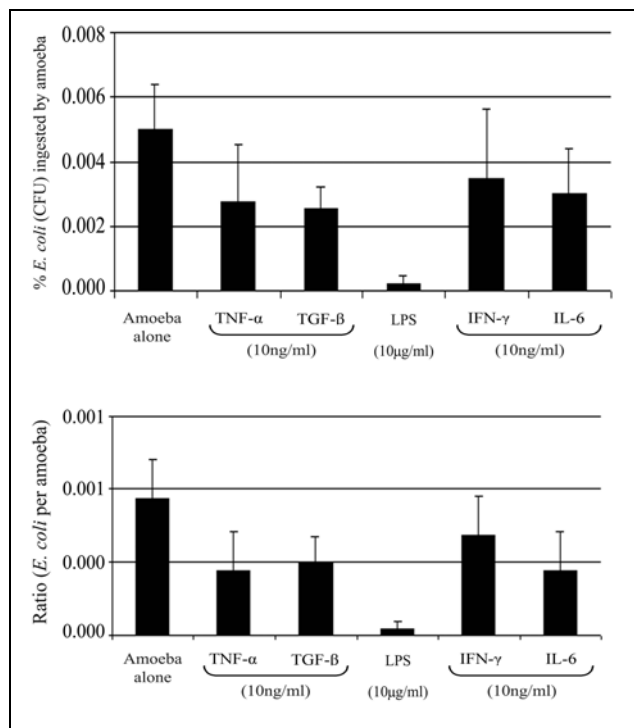


Fig. 2: Cytokines exhibited slight inhibition of bacterial ingestion by *Balamuthia*.

To investigate the ability of bacterial uptake by *Balamuthia* in the presence of various cytokines including INF- γ , TGF- β 1, TNF- α and IL-6 and LPS, phagocytosis assays were performed as described in “Materials and Methods”. Note that *Balamuthia* exhibited significant reduction in bacterial uptake in the presence of all cytokines tested and LPS. (A) represents the percentage of bacterial uptake by *Balamuthia*, while (B) represents the ratio of bacteria per amoeba. Results are representative of three independent experiments.

Cytokines exhibited no effects on proteolytic ability of *Balamuthia*

To determine whether cytokines influence the proteolytic ability of *B. mandrillaris* or their conditioned media, zymography assays were performed. Our results revealed; the lysates of *B. mandrillaris* exhibited several gelatin degradation activities (protease bands) below 48 kDa (i.e. ~47, 42 and 40 kDa) which is not affected by cytokines (fig. 3A) when compared with control (*B. mandrillaris* alone), whereas the conditioned media (*B. mandrillaris* plus HBMEC) showed proteolytic activity below 82 kDa (i.e. ~76 kDa) (fig. 3B) which is quite similar to our previous findings (Matin et al., 2006). Another protease band is also observed at ~96 kDa which appears almost in all CM suggesting *B. mandrillaris* lysates do express protease activity (fig. 3A) while CM don't (fig. 3B). Overall, these results suggest that cytokines have no major affect on proteolytic ability of *B. mandrillaris* in vitro.

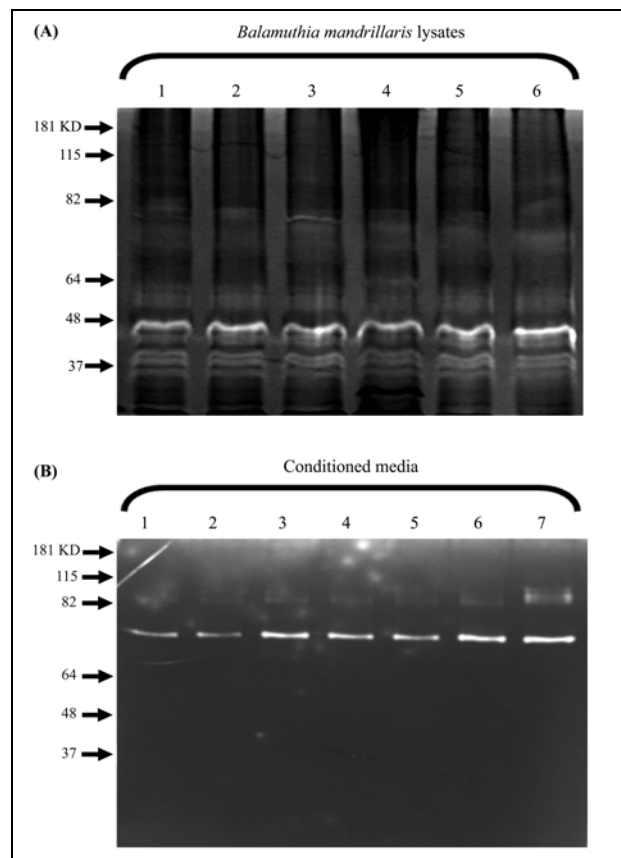


Fig. 3: Cytokines exhibited no effects on proteolytic ability of *Balamuthia*.

To determine the proteolytic ability of *B. mandrillaris* lysates or their conditioned media, zymography assays were performed as described in “Materials and Methods”. (A) Lane 1: *B. mandrillaris* without cytokines (control); Lane 2: *B. mandrillaris* + TNF- α ; Lane 3: *B. mandrillaris* + TGF- β ; Lane 4: *B. mandrillaris* + LPS; Lane 5: *B. mandrillaris* + IFN- γ ; and Lane 6: *B. mandrillaris* + IL-6. (B) Lane 1: *B. mandrillaris* without cytokines (control); Lane 2: *B. mandrillaris* + TNF- α ; Lane 3: *B. mandrillaris* + TGF- β ; Lane 4: *B. mandrillaris* + LPS; Lane 5: *B. mandrillaris* + IFN- γ ; and Lane 6: *B. mandrillaris* + IL-6 and Lane 7: HBMEC without *B. mandrillaris* and cytokines (control). We observed cytokines don't have any effect on either proteolytic activity of *B. mandrillaris* lysates (A) or the conditioned media (*B. mandrillaris* plus HBMEC) (B). Results are representative of three independent experiments.

DISSUSSION

Balamuthia mandrillaris is a newly recognized opportunistic protozoan pathogen that may cause serious human infections. Cutaneous infections can last for months but encephalitis (BAE) which involves CNS always ends up the patient's death within few days. This may be due to the failure of the host immune system to

control infections in the CNS sterile environment. The pathophysiological complications which mostly involve CNS perhaps include invasion of the amoebae to the host connective tissues, stimulation of broad proinflammatory responses and neuronal damage which ultimately leads to brain dysfunction. Furthermore cytokines such as INF- γ , TNF- α , IL-1 and IL-6, as well as LPS are considered to be the important mediators of cellular resistance against parasites. The importance of the above mentioned factors (cytokines and microglia) in protective immunity against parasitic brain infections is well documented (Chao *et al.*, 1994; Benedetto *et al.*, 2001), but their precise role in cerebral protozoal infection is unclear yet. Recently, it has been reported that rat microglia exhibited the ability to ingest *Acanthamoeba castellanii* and elicit IL- α , IL- β and TNF- α (Marciano-Cabral *et al.*, 2000). It has also been previous studies that *T. gondii* replicate in murine microglia (Benedetto *et al.*, 2001); it is assumed that glial cells and proinflammatory cytokines perhaps involved in the cerebral anti-amebic activity.

With the mounting deadly diseases like HIV, it is reasonable to speculate the increasing numbers of opportunistic infections like BAE. This is specifically more worrying for the developing countries like Pakistan where HIV patients have no or very inadequate approach to novel-antiretroviral therapies. Therefore there is an urgent need for sustained efforts to i) increase public awareness, ii) develop rapid diagnostic methods and iii) understand basic molecular mechanisms of host-parasite interactions, which may help us to design preventative and therapeutic strategies. During the last two decades, among the opportunistic protozoan pathogens, *Balamuthia* has gained particular attention in recent years and recognized as human pathogens causing serious as well as life threatening infections. This may be due to its ability to produce BAE infections in both immunocompromised as well as immunocompetent individuals (reviewed by Matin *et al.*, 2008).

Although BAE has been reported in immunocompetent populations (individuals having no previous history of syphilis, fungal, malignancies, diabetes mellitus, HIV-1, HIV-2 and mycobacterial infections as well as enclosed normal CD4- and CD8-positive T-lymphocytes counts and B-lymphocytes), however BAE is a rare disease and it is hard to believe that there are no predisposing factors in contracting BAE. It will be crucial to illustrate whether the predisposing factors might be the other primary infections, underlying genetic factors, exposure to the *Balamuthia* distributed environment or a combination of the above is still need to be evaluated. For instance, most of BAE cases have been reported from America (>90% cases), with majority of them are from the warmer regions (Schuster and Visvesvara, 2004). Among them, a large number of BAE cases were reported among the Hispanic origin (Schuster *et al.*, 2004).

CONCLUSIONS

In the present study cytokines were used to investigate their impact on biological properties of *Balamuthia* using HBMEC *in vitro*. Overall, we demonstrated for the first time that cytokines have no significant effect on *Balamuthia* binding and cytotoxicity to HBMEC and amoebic proteolytic activity. In contrast cytokines exhibited limited inhibition of amoebic growth and phagocytosis, which suggests the virulence potential of the amoebae. That might be one of the reasons *Balamuthia* can cause infections in both immunocompetent and immunocompromised individuals. Future studies will focus on the role of macrophages primed with cytokines like INF- γ or immune serum *Balamuthia* biological properties with special interest in pathogenesis.

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