Phospholipase Activities in Clinical and Environmental Isolates of Acanthamoeba

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Abstract: The pathogenesis and pathophysiology of Acanthamoeba infections remain incompletely understood. Phospholipases are known to cleave phospholipids, suggesting their possible involvement in the host cell plasma membrane disruption leading to host cell penetration and lysis. The aims of the present study were to determine phospholipase activities in Acanthamoeba and to determine their roles in the pathogenesis of Acanthamoeba. Using an encephalitis isolate (T1 genotype), a keratitis isolate (T4 genotype), and an environmental isolate (T7 genotype), we demonstrated that Acanthamoeba exhibited phospholipase A₁ (PLA₁) and phospholipase D (PLD) activities in a spectrophotometry-based assay. Interestingly, the encephalitis isolates of Acanthamoeba exhibited higher phospholipase activities as compared with the keratitis isolates, but the environmental isolates exhibited the highest phospholipase activities. Moreover, Acanthamoeba isolates exhibited higher PLD activities compared with the PLA₁. Acanthamoeba exhibited optimal phospholipase activities at 37°C and at neutral pH indicating their physiological relevance. The functional role of phospholipases was determined by in vitro assays using human brain microvascular endothelial cells (HBMEC), which constitute the blood-brain barrier. We observed that a PLD-specific inhibitor, i.e., compound 48/80, partially inhibited Acanthamoeba encephalitis isolate cytotoxicity of the host cells, while PLA₁-specific inhibitor, i.e., cytidine 5’diphosphocholine, had no effect on parasite-mediated HBMEC cytotoxicity. Overall, the T7 exhibited higher phospholipase activities as compared to the T4. In contrast, the T7 exhibited minimal binding to or cytotoxicity of, HBMEC.

Key words: Acanthamoeba, human brain microvascular endothelial cell, phospholipase A2, phospholipase D, adhesion, cytotoxicity

INTRODUCTION

Acanthamoeba is a free-living protozoan and has been isolated from diverse environments, including air, soil, tap water, or swimming pools [1]. It is recognized as one of the most ubiquitous protozoans [1]. Acanthamoeba is an opportunistic human pathogen and can cause a rare form of granulomatous encephalitis which almost always results in death or a common keratitis with consequences of blindness [2-4]. However, the pathogenesis and pathophysiology associated with Acanthamoeba infections remain incompletely understood. Acanthamoeba has been shown to demonstrate several extracellular toxins, particularly extracellular proteases which might contribute towards its pathogenicity. Previous studies have shown the presence of phospholipases in Acanthamoeba; however, our knowledge of the role of phospholipases in the virulence of Acanthamoeba is unclear [5-7]. Besides their role in remodelling of the plasma membrane of the parasite itself, based on the fact that phospholipases are known to cleave phospholipids, they may involve in the host cell plasma membrane disruptions leading to penetration of the host cells, and cell lysis [5-7]. This suggests that an understanding of the phospholipases in the biology and pathogenesis of Acanthamoeba may identify targets for therapeutic interventions.

Phospholipases are a diverse group of enzymes that hydrolyze ester linkage in glycerophospholipids and are involved in the biosynthesis and degradation of membrane lipids. Thus, the activities of phospholipases can result in membrane dysfunction. The 5 major known phospholipases are A₁, A₂, B, C, and D, each having the ability to cleave a specific ester bond in the phospholipid substrate of the target membrane [8]. For example, PLA₁ hydrolyses the fatty acyl ester bond at the sn-1 position of the glycerol moiety, while PLA₂ removes the fatty acid at the sn-2 position of glycerol [8]. The results of both the PLA₁ and PLA₂ action are free fatty acids or acyl lysophospholipid. The latter are further cleaved by the action of lysophospholi-
pases. Phospholipase B has both hydrolase activities, such as cleaving fatty acids from phospholipids and lysophospholipids as well as acyltransferase activity, for instance, the production of phospholipids by transferring a free fatty acid to a lysophospholipid [8]. Phospholipase C hydrolyses phosphodiester bond to yield 1,2-diacylglycerol depending on the phospholipid class involved, while PLD hydrolises the phosphodiester bond between the phosphate and the polar head group, producing phosphatidic acid and choline [8-10]. All phospholipases are present in multiple forms. For example, approximately 20 different PLA₂ enzymes have been identified in mammalian tissues and have been broadly classified into 3 families based on their calcium requirement for catalytic activity [8,11,12]. Different parts of the breakages by PLA₂ and PLD are focused on this study. Victoria and Korn [6] observed that PLA of Acanthamoeba castellanii was located in the plasma membrane [6]. It may be affected on binding of Acanthamoeba to target cells. The aims of the present study were to determine PLA₂ and PLD activities in Acanthamoeba and to determine their role in Acanthamoeba binding to, and cytotoxicity of, human brain microvascular endothelial cells (HBMEC).

**MATERIALS AND METHODS**

**Culture of Acanthamoeba**

All chemicals were purchased from Sigma Laboratories (Poole, Dorset, UK), unless otherwise stated. The following Acanthamoeba isolates of different pathogenicity were used; (1) a clinical isolate of A. castellanii belonging to T4 genotype, isolated from a keratitis patient (ATCC 50492) (American Type Culture Collection, Manassas, USA), (2) a clinical isolate of A. castellanii belonging to T1 genotype, isolated from an encephalitis patient (ATCC 50494), and (3) an environmental isolate of Acanthamoeba astronyxis belonging to T7 genotype, isolated from soil (ATCC 30137). All amoebae were grown in 15 ml of PYG medium (proteose peptone 0.75% w/v, yeast extract 0.75% w/v, and glucose 1.5% w/v) at 30°C and the media was refreshed 17-20 hr prior to experiments as previously described [13]. This resulted in more than 99% amoebae in trophozoite forms, which were used for subsequent experiments.

**Human brain microvascular endothelial cells (HBMEC)**

The primary HBMEC were isolated from the human brain tissue and cultured as previously described [14,15]. In brief, HBMEC were purified by fluorescent activated cell sorting (FA-
Sussex, UK). In brief, the supernatants of co-cultures of Acanthamoeba and the host cells were assessed for the presence of LDH, the release of which is considered as an estimate of cell death. The percentage LDH release was calculated as follows: (LDH activity in experimental sample as measured by optical density at 492 nm–LDH activity in control samples)/(total LDH activity release–LDH activity in control samples) × 100 = % cytotoxicity. Control samples were obtained from host cells or Acanthamoeba incubated alone. Total LDH activity release was determined by total host cell lysis with 1% Triton X-100 for 30 min at 37°C.

Phospholipase A2 assay
To determine the activity of phospholipases, the characteristics of L-α-glycerophosphate oxidase for PLA2 and choline oxidase for PLD were used. Acanthamoeba PLA2 activities were determined using parasite lysates and their conditioned medium in a spectrophotometric-based assay. To prepare lysates, amoebae were lysed using freeze-thaw method as previously described [16]. In brief, various numbers of amoebae were resuspended in 100 µl of PBS, followed by freeze-thaw for 3 times. To prepare the conditioned medium, Acanthamoeba were incubated in RPMI-1640 for 24 hr. Next day, the cell-free conditioned medium was collected and used for PLA2 assays. A 100 µl of amoebae lysate or conditioned medium was incubated with 900 µl of PLA2 substrate solution (0.2 M D, L-α-glycerophosphate, 0.1% 4-aminoantipyrine, 0.1% phenol, and 0.025% peroxidase dissolved in 0.1 M Tris-HCl buffer, pH 8.0). The reaction mixtures were incubated for 2 hr. After this incubation, the mixtures were centrifuged at 900 g for 5 min, and supernatants were collected and their optical density determined at 500 nm. The PLD activity was calculated as follows: (OD test–OD blank)/min × Vt × df/12.0 × 0.5 × 1.0 × Vs = enzymatic activity (units per ml). Here, Vt is total volume; Vs is sample volume; 12.0 is the millimolar extinction coefficient of quinoneimine dye under the assay condition; 0.5 is the factor based on the fact that 1 mole of H2O2 produces half a mole of quinoneimine dye; t is reaction time; 1.0 is light path (cm); and df is the dilution factor.

RESULTS
Acanthamoeba exhibit PLA2 activities
Both clinical and environmental isolates of Acanthamoeba exhibited PLA2 activities (Fig. 1). As expected, optimal PLA2 activities were observed with increasing numbers of Acanthamoeba. Interestingly, the environmental isolate belonging to T7 genotype exhibited the maximum PLA2 activities, followed by the encephalitis isolate belonging to the T1 genotype, while the keratitis isolate belonging to the T4 genotype exhibited the least PLA2 activities (Fig. 1). Of interest, Acanthamoeba condi-
tioned medium did not exhibit PLA<sub>2</sub> activities in the assay conditions tested in the present study, suggesting that PLA<sub>2</sub> are cell-associated and/or not released (data not shown).

**PLD activities**

The results revealed that both clinical and environmental isolates of *Acanthamoeba* exhibit PLD activities (Fig. 2). Again, the encephalitis isolate (T1) and environmental isolate (T7) exhibited the highest levels of PLD activities compared with the keratitis isolate (T4) (Fig. 2). It was interesting to note that

![Fig. 2. Clinical and environmental Acanthamoeba isolates exhibiting PLD activities.](image)

**Optimal phospholipase activities at 37˚C**

To determine the optimum phospholipase activities, assays were performed at various temperatures from 4˚C to 65˚C. The optimal phospholipase activities were observed at 37˚C suggesting their physiological relevance (Fig. 3). Similar findings were observed both with PLA<sub>2</sub> and PLD activities. Activities were also observed at 65˚C, suggesting that *Acanthamoeba* PLA<sub>2</sub> and PLD activities are stable at higher temperatures (Fig. 3).

**Optimal phospholipase activities at neutral pH**

Next to determine the pH for optimal phospholipase activities, assays were performed at various pH. Our findings revealed that *Acanthamoeba* exhibit optimal PLA<sub>2</sub> and PLD activities at neutral pH indicating their physiological relevance (Fig. 4). Both clinical and environmental isolates exhibited optimal phospholipase activities at pH 7.5.

**Phospholipase inhibitors blocked the environmental isolate binding to HBMEC, but not clinical isolates**

To determine whether phospholipases play a role in *Acan-
Phospholipase activities in Acanthamoeba isolates

Matin and Jung: Phospholipase activities in Acanthamoeba isolates

Adhesion assays were performed using the PLA$_2$ inhibitor, i.e., cytidine 5'-diphosphocholine, and the PLC/PLD inhibitor, i.e., compound 48/80. Our findings revealed that neither phospholipase inhibitor had any significant effects on the adhesion of clinical isolates of Acanthamoeba to HBMEC (Fig. 5). In contrast, adhesion of the environmental isolate of Acanthamoeba was completely abolished in the presence of phospholipase inhibitors (Fig. 5).

Phospholipase A$_2$ inhibitor had no effect on Acanthamoeba-mediated HBMEC cytotoxicity

To determine the role of PLA$_2$ in Acanthamoeba-mediated HBMEC cytotoxicity, adhesion assays were performed using the PLA$_2$ inhibitor, i.e., cytidine 5'-diphosphocholine, and the PLC/PLD inhibitor, i.e., compound 48/80. Our findings revealed that neither phospholipase inhibitor had any significant effects on the adhesion of clinical isolates of Acanthamoeba to HBMEC (Fig. 5). In contrast, adhesion of the environmental isolate of Acanthamoeba was completely abolished in the presence of phospholipase inhibitors (Fig. 5).

Phospholipase A$_2$ inhibitor had no effect on Acanthamoeba-mediated HBMEC cytotoxicity

To determine the role of PLA$_2$ in Acanthamoeba-mediated HBMEC cytotoxicity.
HBMEC death, cytotoxicity assays were performed in the presence of cytidine 5'-diphosphocholine, a PLA2-specific inhibitor. The results revealed that cytidine 5'-diphosphocholine had no significant effect on parasite-mediated HBMEC cytotoxicity in all Acanthamoeba isolates tested (Fig. 6).

Phospholipase C/D inhibitor inhibited HBMEC cytotoxicities mediated by the encephalitis isolate but not by the keratitis isolate

To determine whether PLC/PLD plays a role in Acanthamoeba-mediated HBMEC death, cytotoxicity assays were performed in the presence of compound 48/80. Interestingly, compound 48/80 partially blocked the encephalitis isolate-mediated HBMEC cytotoxicity, i.e., 49% cell death in the presence of the inhibitor compared with 73% in the absence of the inhibitor (Fig. 6). However, it had no effect on the keratitis isolate-mediated HBMEC cytotoxicity (Fig. 6). In contrast, the effects of phospholipase inhibitors on the environmental isolate-mediated HBMEC cytotoxicity remained unclear. This was due to the fact that the environmental isolate exhibited minimal HBMEC cytotoxicity even in the absence of inhibitors.

**DISCUSSION**

Infections due to Acanthamoeba have increased over the years. This is most likely due to the increasing numbers of contact lens wearers, increasing populations of immunocompromised patients, and global warming. This has led to a remarkable interest in the field of Acanthamoeba pathogenesis and to design strategies to control and prevent Acanthamoeba infections. In particular, over the last 2 decades, research in Acanthamoeba virulence factors has gained significant attention to identify novel approaches for therapeutic interventions. Acanthamoeba pathogenesis is attributed to their ability to bind to the host cells, induce host cell apoptosis, secrete proteases, exhibit ecto-ATPase activities, and switch phenotypes. The present study was undertaken to determine the roles of phospholipases (i.e., PLA2 and PLD) in Acanthamoeba and Acanthamoeba-mediated binding to, and cytotoxicity of HBMEC in vitro. Our findings revealed that Acanthamoeba show PLA2 and PLD activities. This is consistent with previous studies that showed the presence of phospholipase activities in Acanthamoeba [5-7].

It was interesting to note that the environmental isolate (belonging to the T7 genotype) exhibited higher phospholipase
activities as compared to the keratitis isolate (belonging to the T4 genotype). In contrast, the environmental isolate exhibited minimal binding to or cytotoxicity of HBMEC. This is not a surprising finding. Being a free-living amoeba, the primary functions of phospholipases in Acanthamoeba may be predominantly in the phospholipids turnover for membrane maintenance and remodelling. The fact that environmental isolate is significantly larger in size compared to the keratitis isolate, thus larger plasma membranes, may explain its optimal PLA2 and PLD activities, and thus rationalize these findings. In support, the size of keratitis isolate tested here is approximately 7 µm. The size of the encephalitis isolate is approximately 10 µm and the environmental isolate being the largest, approximately 17 µm. Another explanation may be that the environmental isolate exhibited distinct types or isoforms of phospholipases compared with the clinical isolates. For example, Sissons et al. [17] have shown that both clinical and environmental isolates of Acanthamoeba exhibit ecto-ATPase activities, however, clinical isolates exhibit distinct ecto-ATPases. This may be the case for phospholipases, which are normally required for routine cellular functions but pathogenic isolates may possess distinct phospholipases. The fact that compound 48/80 had no effect on keratitis isolate-mediated host cell death but partially inhibited encephalitis isolate-mediated HBMEC cytotoxicity further suggests possible differences in phospholipases in Acanthamoeba belonging to different genotypes. Future studies are in progress to determine the molecular basis of these differences.

Of interest was the observation that compound 48/80, only partially inhibited encephalitis isolate-mediated HBMEC cytotoxicity. It is noteworthy that Acanthamoeba pathogenesis is a multifactorial process involving adherence, phagocytosis, apoptosis, proteases, ecto-ATPases, and the inhibition of a single putative factor may not be sufficient to block their ability to produce host cell cytotoxicity. In support, previous studies have shown that inhibition of Acanthamoeba binding to host cells is not adequate to block host cell cytotoxicity [18]. At present, the inability of compound 48/80 to block keratitis isolate-mediated host cell death is unclear. To this end, the source of amoeba isolates, i.e., keratitis and encephalitis patients may be indicative of these differences. This possibly means that phospholipases from the encephalitis isolate may play a role in the pathogenesis of AGE. However, it is a subject which needs additional research and study.

The finding that phospholipase inhibitors did not clearly block Acanthamoeba-mediated HBMEC cytotoxicity does not rule out their involvement in Acanthamoeba pathogenesis. It is possible that cytotoxicity is a delayed event and that phospholipases are involved in the early events. Recent studies have shown that phospholipases may be involved in interference with the intracellular signalling pathways. For example, phospholipases generate lipids and lipid-derived products that act as mediators and second messengers, which may act as the modulators of signal transduction pathway [19,20]. Oishi et al. [21] showed that lysophospholipids, by-end products of PLA2 and PLB, induce activation of protein kinase C, which has diverse functions in host cell signalling pathways [21]. Phospholipase C of Clostridium perfringens induces expression of IL-8 synthesis in endothelial cells [22,23]. Phospholipase A facilitates host cell penetration of Toxoplasma gondii [24] and Entamoeba histolytica [25], while PLD has been shown to be an important virulence determinant of Corynebacterium pseudotuberculosis in the persistence and spread of bacteria within the host [26].

Overall, these studies suggest that Acanthamoeba phospholipases may play a role in causing host cell damage or affect other cellular functions, such as inducing inflammatory responses and thus facilitating the virulence of Acanthamoeba. The fact that phospholipases cleave phospholipids suggests their possible role in membrane disruptions and penetration into host cells. Future studies are needed to identify and characterize Acanthamoeba phospholipases, which should help determine their potential role for therapeutic interventions and in differentiation of Acanthamoeba isolates belonging to different genotypes. Some previous studies have shown that phospholipase C from C. perfringens induced protection against C. perfringens-mediated gas gangrene [27]. In addition, targeting phospholipases using synthetic compounds are shown to have the potential to prevent Candida infections [28].

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REFERENCES

1. Khan NA. Acanthamoeba: biology and increasing importance in human health. FEMS Microbiol Rev 2006; 30: 564-595.
2. Marciano-Cabral F, Cabral G. Acanthamoeba spp. as agents of disease in humans. Clin Microbiol Rev 2003; 16: 273-307.
3. Schuster FL, Visvesvara GS. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int J
4. Jung SY, Alsam S, Kim KS, Khan NA. Pathogen–pathogen interactions: a comparative study of *Escherichia coli* interactions with the clinical and environmental isolates of *Acanthamoeba*. World J Microbiol Biotechnol 2008; 24: 2339-2348.

5. Victoria EJ, Korn ED. Plasma membrane and soluble lysophospholipases of *Acanthamoeba castellanii*. Arch Biochem Biophys 1975; 171: 255-258.

6. Victoria EJ, Korn ED. Enzymes of phospholipid metabolism in the plasma membrane of *Acanthamoeba castellanii*. J Lipid Res 1975; 16: 54-60.

7. Cursons RTM, Brown TJ, Keys EA. Virulence of pathogenic free-living amoebae. J Parasitol 1978; 64: 744-745.

8. Ghannoum MA. Potential role of phospholipases in virulence and fungal pathogenesis. Clin Microbiol Rev 2000; 13: 122-143.

9. Exton JH. Phospholipase D–structure, regulation and function. Rev Physiol Biochem Pharmacol 2002; 144: 1-94.

10. McDermott M, Wakelam MJO, Morris AJ. Phospholipase D. Biochem Cell Biol 2004; 82: 225-253.

11. Akiba S, Sato T. Cellular function of calcium-independent phospholipase A2. Biol Pharm Bull 2004; 27: 1174-1178.

12. Kudo I. Diversity of phospholipase A2 enzymes. Foreword. Biol Pharm Bull 2004; 27: 1157.

13. Sissons J, Kim KS, Stins M, Jayasekera S, Alsam S, Khan NA. *Acanthamoeba castellanii* induces host cell death via a phosphatidylinositol 3-kinase-dependent mechanism. Infect Immun 2005; 73: 2704-2708.

14. Stins MF, Gilles J, Kim KS. Selective expression of adhesion molecules on human brain microvascular endothelial cells. J Neuroimmunol 1997; 76: 81-90.

15. Alsam S, Kim KS, Stins M, Rivas AO, Sissons J, Khan NA. *Acanthamoeba* interactions with human brain microvascular endothelial cells. Microb Pathog 2003; 35: 235-241.

16. Matin A, Stins M, Kim KS, Khan NA. *Balanamuthia mandrillaris* exhibits metalloprotease activities. FEMS Immunol Med Microbiol 2006; 47: 83-91.

17. Sissons J, Alsam S, Jayasekera S, Khan NA. Ecto-ATPases of clinical and non-clinical isolates of *Acanthamoeba*. Microb Pathog 2004; 37: 231-239.

18. Leher H, Silvany R, Alizadeh H, Huang J, Niederkorn JY. Mannose induces the release of cytopathic factors from *Acanthamoeba castellanii*. Infect Immun 1998; 66: 5-10.

19. Dennis EA, Rhee SG, Billah MM, Hannun YA. Role of phospholipase in generating lipid second messengers in signal transduction. FASEB J 1991; 5: 2068-2077.

20. Serhan CN, Håggström JZ, Leslie CC. Lipid mediator networks in cell signaling: update and impact of cytokines. FASEB J 1996; 10: 1147-1158.

21. Oishi K, Raynor RL, Chapp PA, Kuo JE. Regulation of protein kinase C by lipophospholipids. Potential role in signal transduction. J Biol Chem 1988; 263: 6865-6871.

22. Bryant AE, Stevens DL. Phospholipase C and perfringolysin O from *Clostridium perfringens* upregulate endothelial cell-leukocyte adherence molecule 1 and intercellular leukocyte adherence molecule 1 expression and induce interleukin-8 synthesis in cultured human umbilical vein endothelial cells. Infect Immun 1996; 64: 358-362.

23. Bunting M, Lorant DE, Bryant AE, Zimmerman GA, McIntyre TM, Stevens DL, Prescott SM. Alpha toxin from *Clostridium perfringens* induces proinflammatory changes in endothelial cells. J Clin Invest 1997; 100: 565-574.

24. Saffer LD, Long Krug SA, Schwartzman JD. The role of phospholipase in host cell penetration by *Toxoplasma gondii*. Am J Trop Med Hyg 1989; 40: 145-149.

25. Long-Krug SA, Fischer KJ, Hysmith RM, Ravdin JJ. Phospholipase A enzymes of *Entamoeba histolytica*: description and subcellular localization. J Infect Dis 1985; 152: 536-541.

26. McNamara PJ, Bradley GA, Songer JG. Targeted mutagenesis of the phospholipase D gene results in decreased virulence of *Corynebacterium pseudotuberculosis*. Mol Microbiol 1994; 12: 921-930.

27. Kameyama S, Sato H, Murata R. The role of alpha-toxin of *Clostridium perfringens* in experimental gas gangrene in guinea pigs. Jpn J Med Sci Biol 1972; 25: 200.

28. Hänel H, Kirsch R, Schmidts HL, Kottmann H. New systematically active antimycotics from the beta-blocker category. Mycoses 1995; 38: 251-264.