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Ultrastructural changes of *Acanthamoeba* cyst of clinical isolates after treatment with minimal cysticidal concentration of polyhexamethylene biguanide

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Abstract: In order to understand the action mechanism of polyhexamethylene biguanide (PHMB) to the cyst of *Acanthamoeba* on the morphological basis, the cysts of four corneal isolates of *Acanthamoeba* were treated with minimal cysticidal concentration (MCC) of PHMB and their ultrastructural changes were examined by transmission electron microscopy. The most striking change of cysts treated with PHMB compared with normal cysts was the shrinkage of intracystic amoebae, which resulted in the separation of the plasma membrane of intracystic amoeba from endocystic wall. Subplasmalemmal lipid droplets became irregularly shaped. In severely damaged cysts, cytoplasm was aggregated and organelles were severely deformed. Cytoplasmic materials were leaked out through the damaged plasma membrane. Most cysts showed aggregation of nuclear chromatin material. Number of mitochondrial cristae was also reduced. Ecto- and endo-cystic walls were relatively well tolerated. Findings in the present study revealed that PHMB affected mainly on plasma membrane, but lesser on organellar membrane of intracystic amoeba. It seemed likely that PHMB might kill cystic forms of *Acanthamoeba* by similar mechanism in which this environmental biocide can damage the cell wall of *Escherichia* coli by binding with acidic phospholipids

Key words: *Acanthamoeba* cyst, PHMB, ultrastructural changes, MCC, plasma membrane

INTRODUCTION

Acanthamoeba spp. of which medical significance as human pathogen was recognized recently (Callicot, 1968; Jones *et al.*, 1975), have been isolated from various environmental sources (De Jonckheere, 1991). The first human acanthamoebic keratitis was reported in 1973 from a South Texas rancher who had an insidious, painful, and progressive keratitis (Jones *et al.* 1975). In the next decade, only ten cases had been reported sporadically and the amoebic keratitis was known to be associated with trauma and inoculation of contaminated material in the cornea (Larkin, 1991). However, there has been an exponential increase in incidence of the amoebic keratitis in recent years (Wilhelmus, 1991). The majority of the patients with acanthamoebic keratitis were contact lens wearers and the association between *Acanthamoeba* keratitis and contact lens wear is now firmly established (Moore *et al.*, 1985; CDC, 1986).

Acanthamoeba keratitis typically has a progressive chronic course, often with periods

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of remission (Theodore *et al.*, 1985). Medical cure is the goal in management of the amoebic keratitis, avoiding the requirement for corneal transplantation, at least in the setting of uncontrolled infection. Recently, successful medical treatments of *Acanthamoeba* keratitis with poyhexamethylene biguanide (PHMB) was reported intermittently (Larkin *et al.*, 1992; Mills *et al.*, 1993; Yee *et al.*, 1993; Gray *et al.*, 1994). *In vitro* cysticidal test confirmed the efficacy of this biocide on killing of resistant form of the amoeba (Mills *et al.*, 1993; Tirado-Angel *et al.*, 1995; Shin *et al.*, 1996). But the action mechanism of PHMB on pathogenic *Acanthamoeba* cyst is still unclear.

In the present study, we observed ultrastructural changes of *Acanthamoeba* cysts of clinical isolates after treatment with minimal cysticidal concentration (MCC) of PHMB to understand the action mechanism on the morphological basis.

MATERIALS AND METHODS

Clinical isolates of *Acanthamoeba*

Four isolates of *Acanthamoeba* from Korean amoebic keratitis patients diagnosed in 1996 were used *in vitro* cysticidal test and subsequent ultrastructural study.

Axenization and isolate typing of *Acanthamoeba*

Axenization of *Acanthamoeba* isolates were followed as previously described (Kong *et al.*, 1995). Mitochondrial DNA RFLP described by Yagita and Endo (1990) was applied to typing of the isolates.

Preparation of *Acanthamoeba* cyst

Acanthamoeba trophozoites were cultivated in PYG (proteose peptone, yeast extract and glucose) medium for more than three weeks at 25°C. The encystment was confirmed under an inverted microscope.

According to the method described by Shin *et al.* (1996), MCC of PHMB for 8 hr (MCC8) and 48 hr (MCC48) against individual isolates was determined by quadriplicated assay. Briefly, amoebic cysts (1×10^6/ml) of each isolate were treated with PHMB of serial dilution. After 8 or 48 hr, the cysts were washed with sterile phosphate buffered saline (PBS) and spread on an 1.5% agar plate covered with heat-inactivated *Escherichia coli*. The plate were examined under an inverted microscope every 4 hr. The MCC was the lowest concentration of diluted PHMB that resulted in neither excystment nor trophozoite replication on subsequent cultivation.

Observation of ultrastructural changes of *Acanthamoeba* cysts after treatment with MCC of PHMB

The amoebic cysts were treated with PHMB for 2, 4 and 8 hr at MCC8 and 48 hr at MCC48. The cyst suspension treated with PHMB was centrifuged for 10 min at 800 *g* and sediment was washed 3 times in PBS. The amoebic cysts were prefixed with 4% glutaraldehyde in 0.2 M cacodylate buffer, pH 7.2-7.4 for more than 2 hr. After rinse with 0.1 M cacodylate buffer, the sediment was postfixed with 1% osmium tetroxide for 3 hr. The cysts were rinsed twice with 0.1 M maleate buffer, pH 5.2 and dehydrated with 50%, 70%, 80%, 90% and 100% ethanol serially. After treatment with propylene oxide for 30 min, the pellet was immersed in propylene oxide-resin (1:1) mixture overnight with continuous shaking. The pellet was placed and embedded in capsule with fresh resin and incubated at 60°C for more than 48 hr. Ultrathin sections by an ultramicrotome (Reichert-Jung) were stained with uranyl acetate and lead citrate. The sections were observed under a transmission electron microscope (Hitachi H-7000).

RESULTS

Isolate typing

Four isolates from infected corneas used in this study showed different mitochondrial DNA RFLP by 2 kinds of restriction enzymes (Fig. 1) and they were identified as different types of *Acanthamoeba*.

MCC of PHMB

Table 1 shows the average MCC of PHMB after treatment for 8 hr (MCC8) and 48 hr (MCC48) against 4 isolates of *Acanthamoeba*. The isolates were similar in average MCC

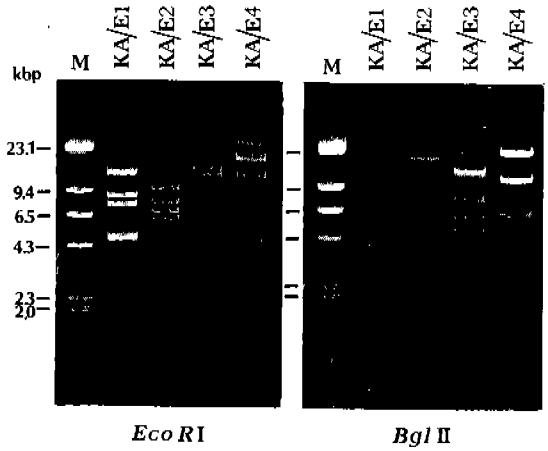


Fig. 1. Agarose gel electrophoretic restriction fragment patterns for mitochondria DNA of 4 Korean corneal isolates of *Acanthamoeba*. M: *Hind* III digested λ phage DNA as DNA molecular size standard.

Table 1. Average MCC (μg/ml) of PHMB against four *Acanthamoeba* isolates

strain	KA/E1	KA/E2	KA/E3	KA/E4
MCC8	14.32	22.45	21.54	23.59
MCC48	3.97	7.49	6.40	4.92

except for KA/E1 isolate with lower level of MCC than those of the others.

Transmission electron microscopic findings

The untreated normal cysts showed thin endocyst and somewhat thick ectocyst. The plasma membrane of intracystic amoeba lined interiorly along the endocystic wall (Figs. 2 & 3). The mitochondria had 4-10 cristae. Some mitochondriae came into contact with peroxisomes (Fig. 3). The lipid droplets were oval or round-shaped. The nucleus had a central karyosome without aggregation of chromatin material (Fig. 4).

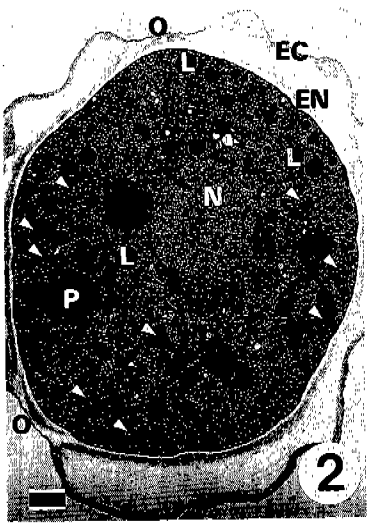
The electron microscopic findings were almost same among the cysts of four *Acanthamoeba* isolates treated with MCC8 or MCC48 of PHMB. Compared with the normal cysts, the most remarkable change on cysts treated with PHMB was shrinkage of intracystic amoebae. The shrinkage of the amoebae resulted in the separation of the plasma membrane of intracystic amoeba from endocystic wall (Figs. 5 & 6). The shape of subplasmalemmal lipid droplets became irregular (Fig. 7). The cytoplasm was aggregated and the organelles were deformed in severely damaged cysts (Fig. 8). Number of mitochondrial cristae was also decreased (Fig. 9). Lipid droplets and cytoplasmic materials were leaked through the damaged plasma membrane (Fig. 10). Most cysts showed aggregation of nuclear chromatin (Fig. 11).

In the case of the cysts treated with MCC8 of PHMB for 2 or 4 hr, invaginations of some parts of the plasma membrane were observed in some cysts (Fig. 12). In these cysts, intracellular organelles and nucleus were also relatively well preserved (Fig. 13). But most cysts showed severe shrinkage and cytoplasmic aggregations.

The shape of ecto- and endo-cystic walls were well preserved after treatment with MCC of PHMB.

DISCUSSION

Polyhexamethylene biguanide (PHMB), one of the environmental biocide, is a mixture of synthetic polymeric biguanides with an average polymer length (n) of 5. It has high antimicrobial activities against a wide range of microbes, such as bacteria, fungi and yeasts with low mammalian toxicity (Davies *et al.*, 1968; Davies and Field, 1969). The target site of PHMB is the cytoplasmic membrane of microbes, especially acidic phospholipids such as phosphatydyl-glycerol (Ikeda *et al.*, 1983 & 1984). Its adsorption to the membranes results in their disruption followed by rapid release of potassium and cytoplasmic constituents which leads to the death of microbes (Davies *et al.*, 1968; Davies and Field, 1969). The ultrastructures of untreated control cysts of *Acanthamoeba* coincided well with the description by Bowers and Korn (1969). The cysts treated with PHMB showed severe cellular shrinkage and separation of cytoplasmic membrane from endocystic wall with leakage of cytoplasmic components. These findings indicated that PHMB affected mainly on plasma membrane of intracystic amoeba, but lesser on organellar membrane.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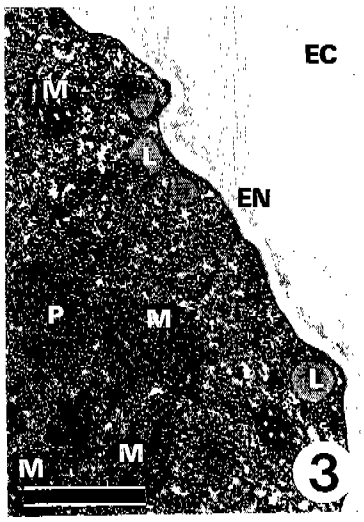
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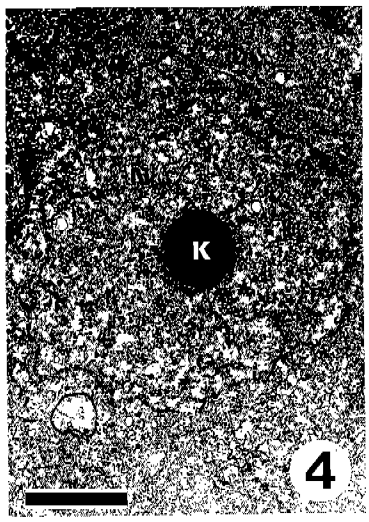
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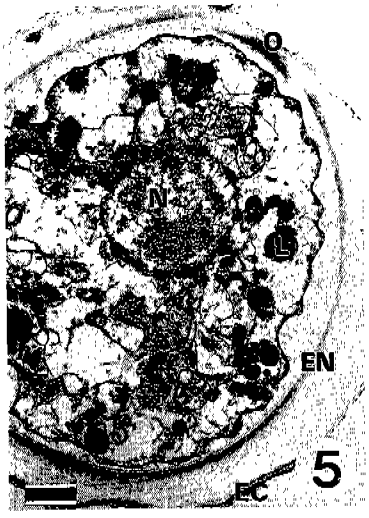
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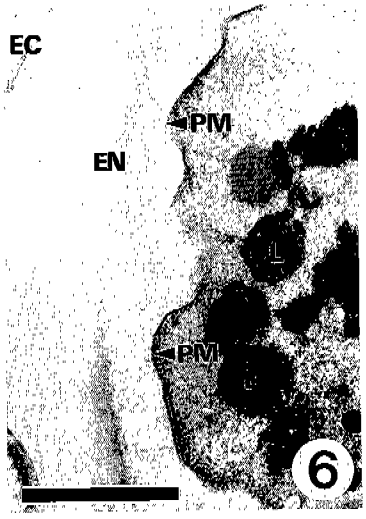
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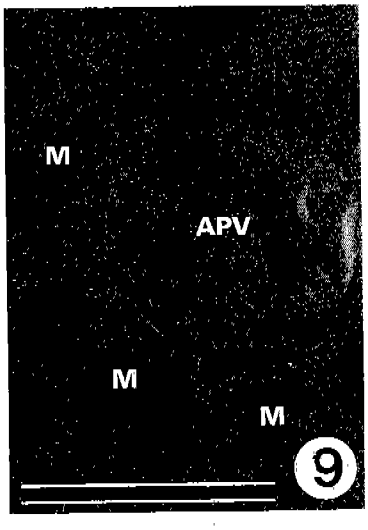
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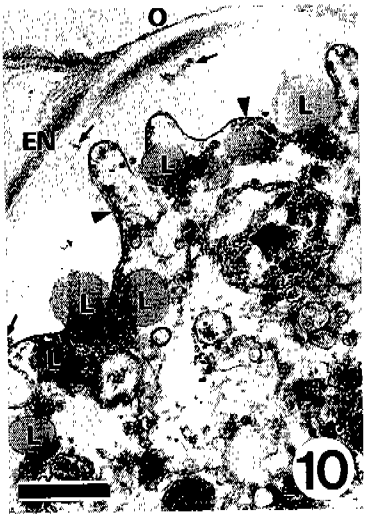
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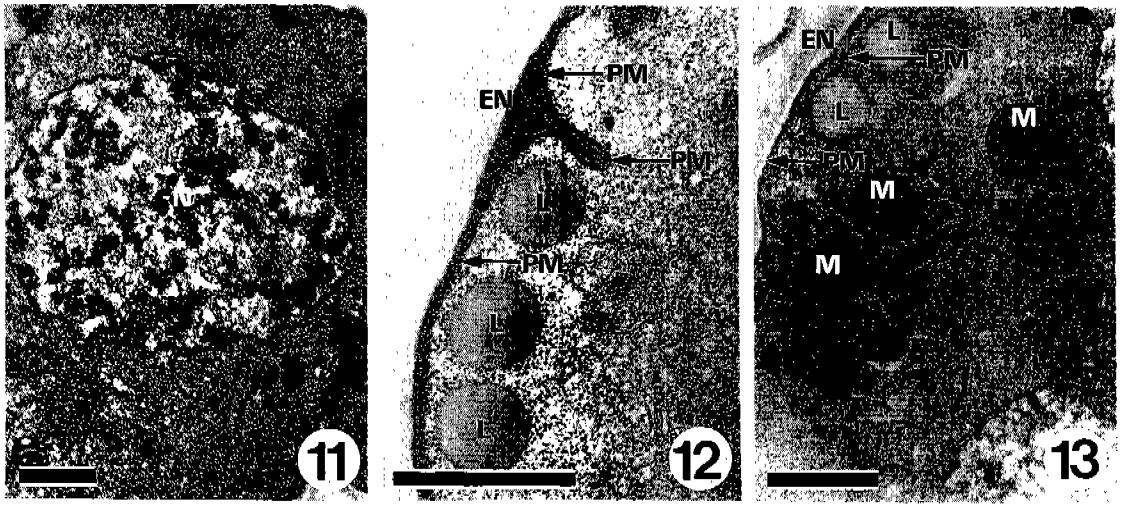

Figs. 2 & 3. Normal cyst of *Acanthamoeba* KA/E2 isolate. The plasma membrane (arrows) is lined along with endocystic wall. The morphology of nucleus, mitochondria (arrow heads) and other intracellular organelles are normal. The mitochondria had 4-10 cristae. Fig. 2, × 5,500; Fig. 3, × 18,500. Bars = 1 μm. **Fig. 4.** The nucleus with typical central karyosome of control cyst of KA/E3 isolate. × 16,500. Bar = 1 μm. **Fig. 5.** Severely damaged cyst of KA/E1 strain treated with MCC8. × 7,600. Bar = 1 μm. **Fig. 6.** Separation of plasma membrane from endocyst of KA/E2 cyst treated with MCC8. × 23,700. Bar = 1 μm. **Fig. 7.** Irregular shaped subplasmalemmal lipid droplet of KA/E4 cyst treated with MCC48. × 75,000. Bar = 0.1 μm. **Fig. 8.** Severely deformed intracellular organelles and aggregation of intracytoplasmic materials of KA/E3 cyst treated with MCC48. × 9,600. Bar = 1 μm. **Fig. 9.** The number of mitochondrial cristae was decreased, and autophagic vacuole was observed in the cyst of KA/E4 treated with MCC8. × 38,300. Bar = 1 μm. **Fig. 10.** Leakage of intracellular materials (arrows) and lipid droplets through plasma membrane (arrow heads) in KA/E3 cyst treated with MCC8. × 14,000. Bar = 1 μm. **Fig. 11.** Intranuclear aggregation of chromatin materials of KA/E1 cyst treated with MCC48. × 11,300. Bar = 1 μm. **Figs. 12 & 13.** In the cyst treated with MCC8 for 2 hr, invagination of plasma membrane was observed, and mitochondria and other intracellular organelles were not deformed. Fig. 12, × 27,300; Fig. 13, × 16,900. Bar = 1 μm. APV, autophagic vacuole; CW, cyst wall; EC, ectocyst; EN, endocyst; K, karyosome; L, lipid droplet; M, mitochondria; N, nucleus; O, ostyole; P, peroxisome; PM, plasma membrane.

Although lipid composition of *Acanthamoeba* plasma membrane is still unknown, the membrane might compose of acidic and neutral phospholipid as other eukaryotic cells. It was suggested that the cysticidal effects of PHMB resulted from the similar mechanism in which this environmental biocide can damage the cell wall of *E. coli* by binding with acidic phospholipids (Ikeda *et al.*, 1983 & 1984; Broxton *et al.*, 1984).

The absence of deformity in ecto- and endocystic walls indicated relative tolerance of these structures against PHMB treatment.

Although investigators usually determine MCC48 for chemicals, MCC8 has more importance in the use of PHMB as a component of contact lens disinfectant because most of contact lens wearers disinfect lens during sleeping time, approximately 8 hr.

According to Burgers *et al.* (1994), the killing effect of PHMB on *Acanthamoeba* cysts occured very rapidly and most cysts affected within 2 hr. In this study, not all but most cysts treated with MCC8 for 2 or 4 hr showed severe ultrastructural changes. Cysts with relatively preserved structures or with the initiation of the structural changes were rarely found. In order to know the serial changes of ultrastructure of cysts, the effect of PHMB treatment in less than 2 hr should be evaluated further.

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=초록=

최저살충농도의 PHMB로 처리한 각막염 유래 가시아메바 세포 미세구조 변화의 투과전자현미경적 관찰

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최근 가시아메바 각막염의 치료제로 새롭게 주목을 받고 있는 polyhexamethylene biguanide (PHMB)의 가시아메바에 대한 작용 기전을 알기 위해 각막염 유래 가시아메바 4 분리주에 대한 PHMB의 최저 살충 농도 (minimal cysticidal concentration; MCC)를 결정하고, 이 MCC의 PHMB로 처리한 포낭의 형태학적 변화를 투과전자현미경으로 관찰하였다. PHMB의 8시간과 48시간에 대한 MCC를 결정하였는데, 8시간 MCC는 KA/E1 14.32 μg/ml, KA/E2 22.45 μg/ml, KA/E3 21.54 μg/ml, 및 KA/E4 23.59 μg/ml였다. 48시간 MCC는 KA/E1 3.97 μg/ml, KA/E2 7.49 μg/ml, KA/E3 6.40 μg/ml 및 KA/E4 4.92 μg/ml로 나타났다. PHMB를 처리한 포낭에서는 포낭 내 amoeba가 심하게 수축되고 이로 인해 포낭 내벽과 분리되는 형태학적 변화가 관찰되었다. 세포막 아래의 subplasmalemmal lipid droplet의 모양이 불규칙해졌다. 심한 경우 세포질이 응집되고, 미토콘드리아의 cristae도 크게 감소하는 등 세포 내 소기관들도 모양이 심하게 변하였다. 포낭 내외벽은 그 형태가 비교적 잘 유지되었다. 이상의 변화들로 볼 때, PHMB는 세포막에 주로 작용하며, 세포 내 소기관들의 막에도 영향을 미치는 것을 알 수 있었다. 가시아메바의 세포막의 lipid composition은 아직 잘 알려져 있지 않지만, PHMB에 의한 가시아메바 포낭에 대한 살충 효과는 environmental biocide로서 *Escherichia coli*의 세포벽에 있는 acidic phospholipids에 작용하여 살균 효과를 나타내는 것과 유사한 기전으로 일어날 것으로 사료된다.

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