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Pathogenicity of Korean isolates of *Acanthamoeba* by observing the experimental infection and zymodemes of five isoenzymes

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Abstract: To determine the pathogenicity of *Acanthamoeba* spp. isolated in Korea and to develop a isoenzymatic maker, the mortality rate of infected mice, in vitro cytotoxicity against target cells and isoenzyme band patterns were observed. Five isolates of *Acanthamoeba* spp. (YM-2, YM-3, YM-4, YM-5, and YM-7) were used in this study as well as three reference *Acanthamoeba* spp. (*A. culbertsoni, A. hatchetti,* and *A. royreba*). According to the mortality rate of infected mice, Korean isolates could be categorized into three groups: high virulent (YM-4), low virulent (YM-2, YM-5, YM-7) and the nonpathogenic group (YM-3). In addition, the virulence of *Acanthamoeba* spp. was enhanced by brain passage in mice. In the cytotoxicity assay against chinese hamster ovary cells, especially, the cytotoxicity of brain-passaged amoebae was relatively higher than the long-term cultivated ones. The zymodeme patterns of glucose-6-phosphate dehydrogenase (G6PD), malate dehydrogenase (MDH), hexokinase (HK), glutamate oxaloacetate transaminase (GOT) and malic enzyme (ME) of *Acanthamoeba* spp. were different among each isolate, and also between long-term cultured amoebae and brain passaged ones. In spites of the polymorphic zymodemes, a slow band of G6PD and HK, and an intermediate band of MDH were only observed in pathogenic *Acanthamoeba* spp., which should be used as isoenzymatic makers.

Key words: *Acanthamoeba* sp., Korean isolate, pathogenicity, virulence, isoenzyme, cytotoxicity

INTRODUCTION

Acanthamoeba species that exist in atmosphere, fresh water, sewages, cooling towers, and swimming pools are known as the causative protozoa of the glanulomatous amoebic encephalitis (GAE) and keratitis (Fowler and Carter, 1965; Hwang et al., 1976; Stehr-Green et al., 1989; Srikanth and Berk, 1993; Vesaluoma et al., 1995; Mathers et al, 1996). Most of the strains isolated from the environment are not pathogenic to the experimental animals and to humans. However, recently, *Acanthamoeba* spp. were isolated from contact lens containers and a few amoebic keratitis cases were reported in

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Korea (Kim et al., 1995).

In order to differentiate the pathogenicity of *Acanthamoeba* spp., an experimental meningoencephalitis was demonstrated by intranasal or intracaecal inoculation into various animals (Culbertson et al., 1966). When the temperature tolerance of the free-living pathogenic amoebae was compared to that of the nonpathogenic amoebae, the pathogenic amoebae showed a higher temperature tolerance (Griffin, 1972). In addition, pathogenic *Acanthamoeba* spp. showed an induced agglutination in the presence of lectin (Kim et al., 1980). Cytotoxicity test using tissue cell lines, such as Vero cell, chinese hamster ovary (CHO) cell, rat neuroblastoma cell, and HeLa cell, was used for the pathogenicity determination of *Acanthamoeba* sp. (Marciano-Cabral et al., 1982; Lee et al., 1986). When using CHO cells as target cells, high virulent *Acanthamoeba* spp. showed a higher cytotoxicity than low virulent amoebae, which was revealed by the crystal violet staining method (Shin et al, 1992).

Sargeaunt and Williams (1979) verified isoenzyme (phosphoglucomutase, glucose phosphate isomerase, etc.) patterns that are important tools in differentiating the pathogenicity of *Entamoeba histolytica*. When cultured with *Escherichia coli* for a short period of time, *E. histolytica* showed an increasing virulence, but Mirelman et al. (1984) reported that the bacterial association did not cause any change in the electrophoretic isoenzyme patterns of *E. histolytica*.

In this study, in order to differentiate the pathogenic *Acanthamoeba* spp. from the nonpathogenic ones and to develop a isoenzymatic maker, the pathogenicity of *Acanthamoeba* spp. including the Korean isolates was demonstrated by observing the mortality rate of the infected mice, the degree of cytotoxicity levels against CHO cells and the comparison of band patterns of five isoenzymes. In addition, the virulence of brain-passaged amoebae was compared to that of amoebae cultured for a longer period of time by three above categories.

MATERIALS AND METHODS

Amoebae

The amoebae were axenically cultured and maintained at 37°C in CGV medium (Willaert, 1976) in plastic culture flasks. Five Korean isolates of *Acanthamoeba* spp. and three reference *Acanthamoeba* strains used in the present study are listed in Table 1.

Preparation of amoebae lysate

Live trophozoites were washed twice with 0.85% saline solution, centrifuged and suspended in the equal volume of enzyme stabilizer (1 mM dithiothreithol, 1 mM ε-aminocaproic acid, 1 mM ethylene diaminotetraacetic acid). Then, the amoebae were subjected to 3 to 4 cycles of freezing and thawing by the method of Moura et al. (1992). The lysate was prepared by centrifugation at 24,000 *g* at 4°C for 30 min, and stored at -70°C until use. The protein concentration of the lysate was determined by the method of Bradford (1976).

Table 1. *Acanthamoeba* spp. used in this study

Species	Strain	Isolated	
		from	in
Acanthamoeba sp.	YM-2	water puddle	1975
Acanthamoeba sp.	YM-3	reservoir	1975
Acanthamoeba sp.	YM-4	fish gill	1975
Acanthamoeba sp.	YM-5	sewage	1993
Acanthamoeba sp.	YM-7	corneal washing	1994
Acanthamoeba culbertsoni		human	1976
Acanthamoeba hatchetti		brackish water	1977
Acanthamoeba royreba		cell culture	1977

Observation of experimental GAE development

Mice used for the experimental infection were outbred ICR mice each weighing 15 to 18 g. Trophozoites of *Acanthamoeba* spp. were harvested, washed twice with saline and suspended in saline (1×10^5 trophozoites per 5 μl saline). Suspended trophozoites were inoculated intranasally into mice under anesthesia that was induced by intraperitoneal injection of secobarbital, 0.06 mg per gram of mouse body weight. The death of mice was observed after day 30 post-inoculation (PI). Autopsy was performed immediately after the death, and pieces of the forebrain tissue were placed into the culture medium for amoebae. In order to compare the virulence between long-term cultured (subcultured since the primary isolation) and brain-passaged amoebae (subcultured from the brain tissue of infected mice), the isolating amoebae were also cultured for subsequent infection.

In vitro cytotoxicity

The chinese hamster ovary (CHO) cells were used as target cells. The CHO cells were cultured and maintained in a Eagle's minimal essential medium (Gibco BRL, NY, USA) in a 5% CO_2 incubator at 37°C and harvested by treatment of trypsin-EDTA solution (Gibco BRL). After the cells were suspended in 0.4% trypan blue, the number of viable cell was counted using the hemocytometer.

The non-radioactive cytotoxicity assay (Promega, WI, USA) was used to determine the cytopathic effect of live trophozoites of *Acanthamoeba* and their lysates. The target cells (1×10^4) and various concentrations of lysates were added to each well of 96-well round-bottomed plates. The plates were spun at 250 *g* for 4 min and incubated at 37°C in a 5% CO_2 incubator for 4 hr. After centrifugation, 50 μl of supernatant fluid was harvested from each well, transferred to the fresh 96-well microtiter plates containing equal volume of substrate mixture and incubated at the room temperatute for 30 min. The absorbance of red colored formazan, which was developed by lactic dehydrogenase released from the dead target cells, was read at 490 nm by ELISA reader (Dynatech MR 5000, USA). The percent of cytotoxicity was calculated by using the following equation;

$$\text{cytotoxicity (\%)} = \frac{[(\text{exp} - \text{c5}) - (\text{c3} - \text{c5})] - (\text{c1} - \text{c5})}{(\text{c2} - \text{c4}) - (\text{c1} - \text{c5})} \times 100$$

exp: amoeba lysates + CHO cells in EMEM + lysis buffer
c1: CHO cells in EMEM
c2: CHO cells in EMEM + lysis buffer
c3: amoeba lysates
c4: EMEM + lysis buffer
c5: EMEM

Observation of isoenzyme band patterns

The lysates were subjected to thin-layer starch-gel electrophoresis as previously described by Soliman et al. (1982) with some modifications. A constant 75 mA of power was supplied to each gel for 4 hr. Chemicals used for the assay include glutamate oxaloacetate transaminase (GOT), malate dehydrogenase (MDH), malic enzyme (ME), glucose-6-phosphate dehydrogenase (G6PD), and hexokinase (HK). Preparation of buffers, coenzymes, substrates, and other staining procedures are listed in Table 2.

RESULTS

Pathogenicity in mice

Mice inoculated intranasally with amoeba began to die after day 4 PI, but no death was observed from mice inoculated with *Acanthamoeba* sp. YM-3. The mortality rate varied among different strains or isolates of *Acanthamoeba*, but the majority of mice died from day 6 to day 15 PI (Table 3). The mortality rate was the highest in mice infected with *Acanthamoeba* sp. YM-4 (Fig. 1). Although some of the infected mice survived, the amoebae were detected from the brains of mice after sacrifice. The virulence between the long-term cultured and second brain-passaged amoeba (especially *Acanthamoeba* sp. YM-4, YM-5, and *A. hatchetti*) revealed a significant difference in intensification. Mice inoculated with brain-passaged amoebae died earlier than mice with long-term cultured amoebae.

Table 2. Staining reagent buffers used for visualizing isoenzymes

Enzyme	Buffer	Coenzyme	Substrate	Linking enzyme	Dye	Other Reagent	Agar (2.0%)
GOT[a] (EC 2.1.1.1)[d]	0.2M Tris-HCl PH 8.0, 25 ml	P5P[b] 2.5 mg	α-Ketoglutarate 50 mg α-Aspartic acid 75 mg	—	FBB[c] 50 mg	—	—
MDH[e] (EC 1.1.1.37)	0.2M Tris-HCl PH 8.0, 8 ml	NAD[f] (1%) 1 ml	Sol. A[g], 5 ml	—	NBT[h] (1%)1 ml PMS[i] (1%)0.5 ml	—	5 ml
ME[j] (EC 1.1.1.40)	0.2M Tris-HCl PH 8.0, 8 ml	NADP[k] (1%) 1 ml	Sol. A, 5 ml	—	NBT (1%)1 ml PMS (1%)0.5 ml	$MgCl_2$ (1%) 1 ml	5 ml
G6PD[l] (EC 1.1.1.49)	0.2M Tris-HCl PH 8.0, 10 ml	NADP (1%) 1 ml	G6P[m] 20 mg	—	NBT (1%)1 ml PMS (1%)0.5 ml	EDTA 25 mg	5 ml
HK[n] (EC 2.7.1.1)	0.2M Tris-HCl PH 8.0, 10 ml	NADP (1%) 0.5 ml	Glucose 40 mg ATP 15 mg	G6PD 50IU, 20 μg	NBT (1%)1 ml PMS (1%)0.5 ml	$MgCl_2$ (1%) 1 ml	5 ml

[a]glutamate oxaloacetate transaminase; [b]pyridoxal-5-phosphate; [c]Fast Blue BB Salt; [d]enzyme commission number; [e]malate dehydrogenase; [f]diphosphopyridine nucleotide; [g]malic acid+Na_2CO_3; [h]nitro blue tetrazolium; [i]phenazine methosulfate; [j]malic enzyme; [k]triphosphopyridine nucleotide; [l]glucose-6-phosphate dehydrogenase; [m]glucose-6-phosphate; [n]hexokinase.

Table 3. Mortality of the mice infected intranasally with *Acanthamoeba* spp.

Amoeba Strains	Brain passage	No. of mice	Number of dead mice in each post-inoculation day																								Total	Mortality (%)
			4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30		
YM-2	N[a]	35																	1									1	2	5.7
	1st[b]	28				2	1			1		1	2	2		1	2			1									13	46.4
	2nd[c]	19				3	1	1					1			1			2										9	47.4
YM-3	N	35																												0	0
	1st	30												0																0	0
	2nd	20																												0	0
YM-4	N	35								1																				1	2.9
	1st	33									3			2	1					1										7	21.2
	2nd	29		3	5	4				2	2	2		1																19	65.5
YM-5	N	30																												0	0
	1st	25		1		4	1			1																				7	28.0
	2nd	32			1	3	4	2				1		1	1			1												14	43.8
YM-7	N	26						1	1									1												3	11.5
	1st	30					1				1				1				1		1					0		2		7	23.3
	2nd	18	1	1	1		2							1		1														7	38.9
A.	N	30							1																					1	3.3
culbertsoni	1st	29		1	1	1	1	1		1		1	1													0		1		9	31.0
	2nd	30		2	1	2	2	1		1	1		4	1	1											0		2		18	60.0
A.	N	20																	1											1	5.0
hatchetti	1st	20										1		1																2	10.0
	2nd	20			1	3	7		2		1																			15	75.0
A.	N	21				1								1																2	9.5
royreba	1st	18					1	1					1																	3	16.7

[a]long term cultivated amoebae in culture tube; [b]amoebae of first brain passage; [c]amoebae of second brain passage

Especially, *Acanthamoeba* sp. YM-4 produced a low mortality rate (2.8 %) in mice inoculated with the long-term cultured amoeba, but they induced a higher mortality rate (65.5 %) when they went through the brain passages twice. *Acanthamoeba culbertsoni*, *A. hatchetti*, *Acanthamoeba* sp. YM-2, and YM-5 also exhibited the enhanced virulence ($P<0.001$). According to the above results, the Korean isolates and reference *Acanthamoeba* spp. can be divided into three groups based on the mortality rate of infected mice: highly virulent (*Acanthamoeba* sp. YM-4, *A. culbertsoni* and *A. hatchetti*), low virulent (*Acanthamoeba* sp. YM-2, YM-5, YM-7 and *A. royreba*), and the non-pathogenic groups (*Acanthamoeba* sp. YM-3).

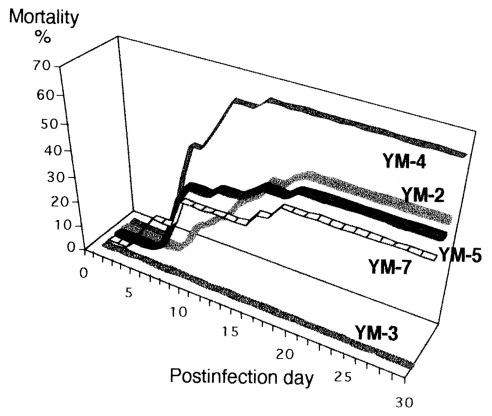

Fig. 1. The mortality of mice infected intranasally with Korean isolates of *Acanthamoeba* sp. YM-2, YM-3, YM-4, YM-5, and YM-7.

Cytotoxicity against CHO cells

Cytotoxicity of the Korean isolates of *Acanthamoeba* spp. did not agree with the level of pathogenicity demonstrated by the mortality rate of the infected mice (Table 4.). Nevertheless, the cytotoxicity of the nonpathogenic YM-3 was relatively low when compared to that of the pathogenic Korean isolates, *Acanthamoeba* sp. YM-4 or YM-7; in addition, the cytotoxicity of the brain-passaged amoeba was relatively higher than that of the long-term cultivated ones.

Isoenzyme patterns

Three migrating bands (slow, intermediate, and fast) of different electrophoretic mobility in each isoenzyme were observed (Table 5). All strains or isolates of *Acanthamoeba* spp. exhibited multiple forms of each isoenzyme patterns. But the isoenzyme band patterns were correlated with the pathogenicity or virulence, which was demonstrated by the mortality of the infected mice in this experiment. In case of G6PD, all pathogenic isolates or strains showed a slow band with the same mobility. And, all pathogenic or virulent isolates or strains revealed an intermediate band in migrating pattern of MDH, but did not show a slow band in HK. The isoenzyme band patterns of brain-passaged amoeba was distinctly different from those of long-term cultivated ones (Fig 2) On the other hand, the isoenzyme band patterns of brain-passaged *Acanthamoeba* spp. changed to those of long-term cultured ones several

Table 4. Cytotoxicity (%) of *Acanthamoeba* spp. against the chinese hamster ovary cells

Amoebae	Non-passage group				Brain-passage group			
	2.0[a)]	1.0	0.5	0.25	2.0	1.0	0.5	0.25
Acanthamoeba sp. YM-3	56.4	40.4	31.9	21.3	66.7	36.9	29.8	21.7
Acanthamoeba sp. YM-2	22.3	14.9	12.7	4.2	99.9	48.4	32.3	19.7
Acanthamoeba sp. YM-4	59.6	25.5	19.1	9.6	94.0	54.4	32.7	30.9
Acanthamoeba sp. YM-5	50.0	23.4	17.0	14.9	62.7	23.4	21.2	12.2
Acanthamoeba sp. YM-7	48.9	24.5	13.8	13.8	82.5	38.8	23.1	18.9
Acanthamoeba culbertsoni	31.9	12.7	14.9	13.8	44.3	21.3	19.9	26.4
Acanthamoeba hatchetti	35.1	15.9	11.7	11.7	93.4	47.4	36.8	26.4
Acanthamoeba royreba	47.9	35.1	31.9	37.2	72.4	41.5	36.7	27.3

[a)]protein concentration of lysate (mg/ml).

Table 5. Rate of flow values of fraction of *Acanthamoeba* spp. separated electrophoretically

Bands of fraction	Isoenzymes				
	G6PD[a]	MDH[b]	HK[c]	GOT[d]	ME[e]
Slow	0.14	0.10	0.34	–0.02	0.10
Intermediate	0.24	0.20	0.42	0.08	0.36
Fast		0.28		0.16	0.40

[a]glutamate oxaloacetate transaminase; [b]malate dehydrogenase; [c]hexokinase; [d]glucose-6-phosphate dehydrogenase; [e]malic enzyme.

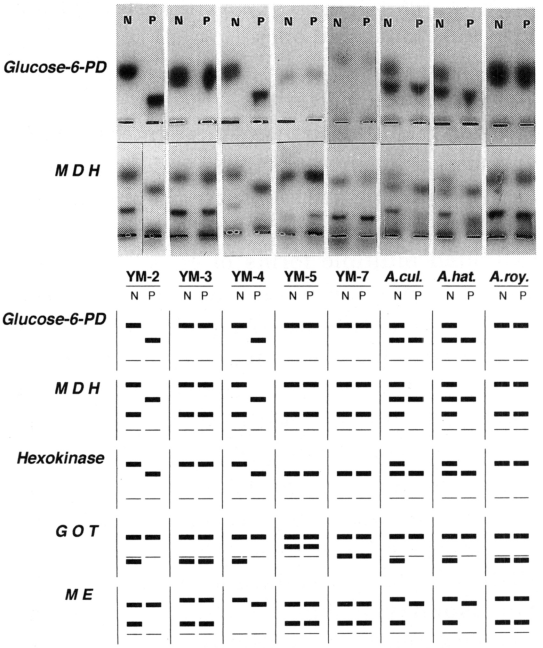

Fig. 2. Photographic and diagrammatic representation of isozyme zymodemes of Korean isolates and reference strains of *Acanthamoeba* spp. YM-2, YM-3, YM-4, YM-5, and YM-7 represent Korean isolates of *Acanthamaeba* spp. A. cul, *Acanthamoeba culbertsoni*; A. hat, *A. hatchetti*; A. roy, *A. royreba*; Glucose-6-PD, glucose-6-phosphate dehydrogenase; MDH, malate dehydrogenase; HK, Hexokinase; GOT, glutamate oxaloacetate transaminase; ME, malic enzyme; N, non-passaged amoeba; P, passaged amoeba.

months later; five and ten months later in case of *Acanthamoeba* sp. YM-2 and *A. culbertsoni*, respectively (Table 6). This period was not related to the virulence degree of amoeba.

DISCUSSION

For the determination of the pathogenicity, several studies have been conducted using the morphological, biochemical, immunological, and genetical tools (Kim et al, 1984). In this study, the Korean isolates and the reference strains of *Acanthamoeba* spp. were differentiated based on the mortality of the mice intranasally inoculated with amoebae. Also, the possible use of electrophoretic analysis for separating pathogenic strains was exploited in the present paper. Four out of five Korean isolates were proven to be virulent, and the remaining isolate, *Acanthamoeba* sp. YM-3, was nonpathogenic to mice.

The degree of amoeba virulence is not constant, but changes with respect to the duration of culture time which also depends on other factors. Lee et al. (1983) compared the difference of virulence between axenically maintained strains of *Naegleria fowleri* and those obtained by a serial brain passage in mice. They demonstrated that the virulence of amoeba was enhanced through the brain passage in mice. In this study, in order to elucidate the virulence of brain-passaged amoebae, the inoculation and isolation of amoebae into/from mice were examined monthly. The virulence of Korean isolates which showed the low mortality rate of mice could be enhanced by brain passage. These present results verify a general rule that the actual virulence of pathogenic strains of *Acanthamoeba* can be restored by the intranasal infection in mice, although this is not the case for the nonpathogenic strains.

To differentiate the pathogenic strains from the nonpathogenic *Acanthamoeba*, enzymatic

Table 6. Changes of isoenzyme band patterns of *Acanthamoeba* spp. subcultured several months after brain passage

Amoebae	Culture duration after brain passage (months)	Changes of band pattern
Acanthamoeba sp. YM-2	5	Yes
Acanthamoeba sp. YM-3	13	No
Acanthamoeba sp. YM-4	15	No
Acanthamoeba sp. YM-5	11	No
Acanthamoeba culbertsoni	10	Yes
Acanthamoeba hatchetti	15	No
Acanthamoeba royreba	27	No

experiments which include trypsin, esterase, phosphatase, phospholipase, peroxidase, and other hydrolase treatments have been proven to be important (Kim et al., 1988; Mazur and Hadas, 1994). Mazur and Hadas (1994) indicated that the increased levels of peroxidase and proteinase activities thoroughly correlated with the increasing virulence of *Acanthamoeba*. It would be of important value if the isoenzyme electrophoretic patterns could separate the pathogens from the nonpathogens. In the present study, three (G6PD, MDH and HK) out of the five systems were demonstrated to be useful for differentiation of the pathogenic strains.

It was very interesting that isoenzyme band patterns of brain-passaged amoebae were clearly different from those of the long-term cultivated amoebae. It was also verified that isoenzyme patterns of the brain passaged amoeba returned to those of long-term cultured ones several months later. The functional significance of this difference is not clear. The correlation between the pathogenicity of amoeba and its isoenzyme patterns is not elucidated at the present time.

On the other hand, in vitro cytotoxicity assay measuring LDH release from the target cells was used for the pathogenicity differentiation of Korean isolates of *Acanthamoeba* spp. in this experiment. The cytotoxic effect of Korean isolates was not related with the amoeba pathogenicity. But the degree of cytotoxicity of brain-passaged amoebae was relatively higher than that of long-term cultivated amoebae. By the ^{51}Cr release assay, Lee et al. (1986) observed the cytotoxicity differences between the pathogenic and nonpathogenic amoeba in culture systems using CHO cells as target cells. And it was reported that the lysate of *A. culbertsoni* was proven to be cytotoxic to CHO cells (Shin et al., 1993). Further work needs to be performed to set up the standard tool for the pathogenicity determination of *Acanthamoeba* spp.

Finally, the present study showed that the Korean isolates of *Acanthamoeba* spp. could be determined as virulent (YM-2. YM-4, YM-5 and YM-7) and nonpathogenic (YM-3) strains. The virulence of long-term cultured *Acanthamoeba* spp. including Korean isolates was enhanced by the mouse brain passage. And it was observed that isoenzyme band patterns of brain-passaged amoebae returned to those of long-term cultured ones several months later. In addition, the slow band of G6PD and HK, and the intermediate band of MDH could be very helpful markers to differentiate the pathogenicity of *Acanthamoeba* spp.

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