EUROPEAN ACADEMIC RESEARCH
Vol. X, Issue 7/ October 2022

Impact Factor: 3.4546 (UIF)
DRJI Value: 5.9 (B+)

ISSN 2286-4822
www.euacademic.org

Pseudomonas aeruginosa induces programmed cell death in *Acanthamoeba castellanii* and human U-937 cells

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Abstract

Pseudomonas aeruginosa is a gram-negative and aerobic free-living bacterium that is found in many habitats. This bacterium produces several extracellular enzymes and toxins causing serious nosocomial infections to become a human opportunistic pathogen. It possesses type III secretion system (T3SS) that allow bacteria to inject the effector proteins directly into the cytoplasm of the host cell inducing both apoptosis and necrosis in human as well as environmental macrophages. Therefore, aims of this article were 1) to study interaction between A. castellanii and P. aeruginosa 01 at 30℃ to highlight rule of KS095 wildtype strain producing T3SS effector proteins as well as elastase, staphylolytic enzyme and exotoxin A, compared to the extracellular protein defective (xcp) strain KS911; 2) to get an insight into the cytotoxicity of P. aeruginosa 103 before and after incubation with the A. castellanii on U-937 cells, using viable cell count, Annexin V propidium iodide staining and caspase-3 activity assay. In the result, survival time of A. castellanii co-cultivated with either the wildtype PA01 strain KS905 producing elastase, staphylolytic enzyme and exotoxin A, or with its extracellular protein defective (xcp) strains KS911was compared to alone A. castellanii at 30 °C uncovered that co-cultivated A. castellanii with either wildtype PA01 or the extracellular proteins defective strain survived 4 days compared to alone cultivated A. castellanii that survived more than 14 days, respectively. Viability of U-937 decreased in co-cultivation with P. aeruginosa alone and that viability decrease was significantly higher in co-cultivation with P. aeruginosa pre-incubated with A. castellanii. The FACS found percentages of

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necrotic/late apoptotic U-937 cells that co-cultivated with P. aeruginosa pre-incubated with A. castellanii compared to the U-937 cells cultivated with P. aeruginosa 103 alone but the difference was not significant. The caspase activity assay of U-937 cells showed a high significant activity when these cells cultivated with P. aeruginosa pre-incubated with A. castellanii compared to the cultivation with P. aeruginosa 103 alone after 24 hours. We can conclude that interaction of P. aeruginosa 01strains KS905 and KS911 with A. castellanii was lethal to the amoeba population and might lead to increased cytotoxic properties of this bacterium due to T3SS effectors proteins. Furthermore, the caspase-3 activity induced by the bacteria at 24 hours was with a higher rate, leading the U-937 cells to undergo apoptosis, but the diversity of P. aeruginosa virulence factors seems to simultaneously activate a necrotic pathway also.

Keywords: *Pseudomonas aeruginosa*, *Acanthamoeba castellanii*, U-937 cells, Apoptosis, Necrosis, Caspase-3 activity

INTRODUCTION

Pseudomonas aeruginosa is an aerobic, Gram-negative, and free-living bacterium that is found in many habitats as an opportunistic pathogen for both human and plants (1). *P. aeruginosa* has become a human pathogen causing nosocomial infection such as pneumonia, urinary tract infections, surgical wounds, and sepsis in immune suppressed individuals with cystic fibrosis (CF), cancer, burn wounds and bone marrow transplantation or individual with acquired immune-deficiency syndrome (AIDS) (2, 3). *P. aeruginosa* produces several extracellular enzymes and toxins (1), such as exotoxin A (4), elastase, and alkaline protease (5), which can cause extensive tissue damage (6). However, it was shown that rhamnolipids, phenazines, pyocyanin, or elastase secreted by *P. aeruginosa* were not involved in killing the amoeba *Dictyostelium discoideum* (7). To facilitate colonization in host cells, *P. aeruginosa* can utilize some substantial virulence factors including both cell-associated factors such as single polar flagellum, pilus, non-pilus adhesions, alginate/biofilm, lipopolysaccharide (LPS) and secreted (extracellular) virulence factors like hemolysin, lipases and proteases, exotoxin A that make *P. aeruginosa* a complex pathogen able to defend itself against eukaryotic cells. Furthermore ADP-ribosyl transferase as well as type III secretion system (T3SS) that allow bacteria to inject the effector proteins (exoenzymes) described ExoS. ExoT, ExoU and ExoY (8, 9, 10) directly into the cytoplasm of the host cell (11). *P. aeruginosa* can survive in nearly any environment and can be found in most aquatic and terrestrial environment (3) sharing this environment with other microorganisms such as diversity of protozoa. *Acanthamoeba castellanii* is an environmental eukaryote found worldwide in soil and fresh or salt water. Its life cycle has a reproductive trophozoite and a dormant cyst. *A. castellanii* is an amphizoic protozoon that live as a free-living amoeba (FLA) in environment or as parasite in humans and animals. It is an opportunistic pathogen having an increased role as human pathogen causing encephalitis in the nervous system (CNS) or inflammation in eye's cornea called keratitis. *A. castellanii* interacts commonly with bacteria as a host to *Vibrio cholerae* either predator to *Escherichia coli* or a victim to *P. aeruginosa* that induced T3SS related apoptosis and necrosis to kill *A. castellanii* (12, 13, 14). Research on cell death in U-937 cells that derived from a human monocyte like cell line, has shown apoptosis in these cells when the cells were treated with several bacterial

toxins such as diphtheria toxin (DT) and *P. aeruginosa* exotoxin A (15). The exotoxin A is also shown to be able to induce activation of caspase 8 and 3 and finally apoptosis (16). Chai et al, 2008 showed that the rate of apoptosis on U-937 cell after exposing to *P. aeruginosa*, were increased with increasing of bacterial infection time. They have also showed that apoptosis in U-937 cells may be related with the regulated expression of Bax and Bcl-2, mediating factors of mitochondrial pathway in apoptosis and furthermore that caspases 3 and 9 are activated in *P. aeruginosa*- induced U-937 cell death, leading to apoptosis (17).

It is unclear whether the interaction of *P. aeruginosa* with *A. castellanii* or with U-937 cells can increase the virulence of this bacterium, therefore, aims of this article were 1) to study interaction between *A. castellanii* and *P. aeruginosa* 01 at 30℃ to highlight rule of KS095 wildtype strain producing elastase, staphylolytic enzyme and exotoxin A, compared to the extracellular protein defective (xcp) strain KS911; 2) to get an insight into the cytotoxicity of *P. aeruginosa* 103 before and after incubation with the amoeba on U-937 cells.

MATERIALS AND METHODS

Microorganisms

Acanthamoeba castellanii (ATCC 30234) was obtained from the American Type Culture Collection (ATCC, Manassas, VA), USA, and the human histiocytic U-937 (ATCC- CRL- 1593.2) cells were purchased from the American Type Culture Collection (ATCC).

Pseudomonas aeruginosa strains 01, 103 and their mutant strains (Table 1) were kindly supplied by Professor Bengt Wretlind, Department of Laboratory Medicine, Division of Clinical Microbiology, Karolinska Institute and Karolinska University Hospital, Stockholm, Sweden, and Dr. Dara Frank, Medical College of Wisconsin, USA.

Table 1: Strains of *Pseudomonas aeuroginosa* strains used in this study

Strain	Description	Reference
PA01, KS095	Wildtype strain producing Exo S, Exo T, and ExoY in addition to extracellular factors; elastase, staphylolytic enzyme and exotoxin A	18
PA01, KS911	An extracellular protein defective (xcp) strain KS911	
PA 103	Wildtype produces significant amounts of ExoT, ExoU and exotoxin A, but fails to express ExoY and does not possess exoS	19

Culture media and growth conditions.

A. castellanii was grown at 30 ºC to a final concentration of 10^6 cell/ml in ATCC medium no. 712 (ATCC, Manassas, VA) and U-937 cells were cultured in RPMI medium no. 1640 with 10 % bovine serum and 2 mmol/L L-glutamine at 37 ºC, 5 % CO2. To infect *A. castellanii*, *P. aeruginosa* was grown on blood agar plates at 37 ºC, thereafter, bacteria were grown in Luria–Bertani broth (Merck) to an absorbance of 0.6 at 600 nm. Co-cultures of each bacterial strain and *A. castellanii* were incubated in 75 cm^2 cell culture flasks (Corning Incorporated Costar, NY) filled with 50-ml ATCC medium 712 containing an initial concentration of 10^5 cell/ml of *A. castellanii* and 10^6 cell/ml of each *P. aeruginosa* isolate.

Control flask for *A. castellanii* was cultivated separately and prepared in the same way and with the same initial concentration of *A. castellanii*. All the flasks were

incubated without shaking at 30 ºC or 37℃ and samples were withdrawn for daily analysis until death of all *A. castellanii* cells in each co-cultivation flask. The dead *A. castellanii* cells were determined qualitatively by erythrosine stain from three different samples from each flask by a light microscope (Carl Zeiss, Stockholm, Sweden) within 15 min of sample removal.

METHODS

1. Assessment of U-937 cell viability using Erythrosine B staining

To examine if there is any difference in cytotoxic effect of wildtype *P. aeruginosa* before and after co-cultivation with amoeba on the U-937 cells, the cell viability assay was performed as following.

Cultivation of only the wildtype of *P. aeruginosa*103 and co-cultivation with *A. castellanii* were performed in ATCC medium no. 712 for 24 h at 30 ºC at an initial concentration of 10^6 cell/ml of *A. castellanii* as described in the method section. Then the co-cultured bacteria were separated from *A. castellanii* by centrifugation for 10 min at 500 g.

The U-937 cells were cultured in RPMI medium no. 1640 with 10 % bovine serum and 2 mmol/L L-glutamine at 37 ºC, 5 % CO2 and then were co-cultured with either wildtype *P. aeruginosa*103 or with *P. aeruginosa* 103 that pre-incubated with *A. castellanii*.

The co-cultivation occurred at an absorbance of approximately 0.6 optical density unit at 600 nm for bacteria and an initial concentration of 10^6 cell/ml of the U-937 cells. Co-culturing was performed in RPMI medium in each well of 24-well tissue culture plate. The plate was then incubated at 37 ºC, 5 % CO2. U-937 cells in RPMI medium was used as a control. U-937 cells were then collected and stained with Erythrosine B after 0, 2, 4, 6 and 24 hours. Viable and dead cells were counted at mentioned times in 3 A-squares of a Bürker counting chamber.

2. FACS analysis of Propidium iodide- and Annexin V staining

To quantify necrosis and apoptosis in U-937 cells after co-culturing with bacteria Annexin V/Propidium iodide staining (PI) was performed. Culturing of bacteria with amoeba and culturing of only bacteria was performed as described in the cell viability assay.

Either wildtype *P. aeruginosa* 103 or *P. aeruginosa* 103 that pre-incubated with *A. castellanii* were co-cultivated with U-937 cells that were performed in 5 wells of a 24-well tissue culture plate. U-937 cells in RPMI medium was used as a control and two wells containing U-937 cells and PA103 were used as controls for Annexin-V-Fluos labeling reagent and propidium iodide solution respectively. The plate was then incubated at 37 ºC, 5 % CO_2 at an initial concentration of 10^6cell/ml of the U-937 cells. Preparation of the Annexin-V-FLOUS labeling solution was done by predilution of Annexin-V-Fluos labeling reagent and PI solution in incubation buffer. The U-937 cells were then collected after 4 as well as 24 hours of incubation and were centrifuged for 10 minutes at 500×g. Pellet from the following samples were resuspend in the Annexin-V-FLOUS/PI labeling solution: U-937 cells (control), U-937 cells co-cultivated with wildtype *P. aeruginosa* 103 or with *P. aeruginosa* 103 that pre-incubated with *A. castellanii*.

Annexin-V-Fluos reagent was added only to the pellet of fourth sample containing the U-937 cells and PA103 as well as Propidium iodide solution, only to the fifth sample containing U-937 cells and PA103 (controls). The samples were incubated at 15 min in the dark at room temperature. The samples were then diluted in incubation buffer and analyzed by Fluorescence Activated Cell Sorting (FACS) using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA).

3. Caspase-3 activity assay

For analysis of caspase-3 activity, the CaspASE colorimetric Assay System (Promega Biotech AB, Nacka Sweden) was used. The following samples were prepared: U-937 cells in RPMI medium was used as a control. U-937 cells and carbonyl cyanide was used as a positive control. Co-cultivation of U-937 cells with *P. aeruginosa* 103 as well as U-937 cells with *P. aeruginosa* 103 that pre-incubated with *A. castellanii* were also prepared as described in the cell viability and FACS assay. The U-937 cells were then collected after 4 as well as 24 hours incubation and were washed once in PBS. The lysis buffer was then added to the cell extracts. The cells were then frozen at -20 °C. The following solution was prepared, at next day to each well of a 96-well microtiter plate: Assay buffer, dimethyl sulfoxide (DMSO), Dithiothreitol (DTT), distilled H2O. The cell extracts were then thawed and centrifuged for 10 min at 3000 g. The supernatant was then added to the plate. Lysis buffer was used as blank. The Ac-DEVD-AMC (substrate solution) was then added to each well. The plate was incubated at 37 ºC, 5 % CO_2 for 4 hours. The absorbance was measured at 405 nm with a Thermo Lab Systems Multiskan EX. Protein concentration in each sample was measured using Pierce Microplate BCA protein assay kit (Fisher Scientific, Götenberg) according to the below: Protein standards A, B, C, D, E, F and G were prepared by diluting the contents of Albumin Standard (BSA) with PBS using different concentrations. PBS was used as blank. Working reagent was also prepared by mixing 50 parts BSA Reagent A with 1 part of BSA Reagent B (50:1, Reagent A: B). The working solution was then added to each well of a 96-well microtiter plate. The protein standard samples (blank, A, B, C, D, E, F and G) and the samples from caspase activity assay with unknown protein concentration (control, positive control, *P. aeruginosa* 103+U937 cells as well as *P. aeruginosa* 103 that pre-incubated with *A. castellanii* +U937 cells) were added to the wells containing working solution. The plate was then incubated at 37 ºC, in 30 min and the absorbance was measured at 595 nm.

RSULT AND DISCUSSION

1. Survival time of both alone ***A. castellanii*** **and co-cultivated with wildtype** ***P. aeuroginosa*** **O1 strain KS905 or its extracellular protein defective (xcp) strain KS911**

Survival time of *A. castellanii* co-cultivated with either the wildtype PA01 strain KS905 producing ExoS, ExoT and ExoY together with elastase, staphylolytic enzyme and exotoxin A, or with its extracellular protein defective (xcp) strains KS911was compared to alone *A. castellanii* at 30°C uncovered that co-cultivated *A. castellanii* with either wildtype PA01 or the extracellular proteins defective strain survived 4 days compared to alone cultivated *A. castellanii* that survived more than 14 days, respectively (Fig. 1).

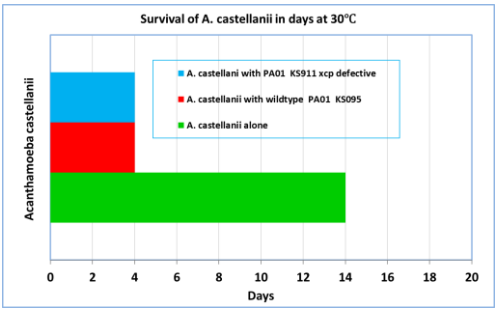

Fig.1: Survival time of A. castellanii alone (green colour), A. castellanii co-cultivated with PA01, KS095 wildtype strain (red colour) producing elastase, staphylolytic enzyme and exotoxin A and A. castellanii co-cultivated with the extracellular protein defective (xcp) strain PA01, KS911(blue colour) at 30℃.

Discussion of *P. aeruginosa* 01 interaction with *A. castellanii* in this partial result has pointed out clearly the important role of T3SS effector proteins of *P. aeuroginosa* 01 wildtype strain and the extracellular protein defective (xcp) strain in killing of *A. castellanii* in co-cultivation at 30℃. It is well known that both strains of this bacterium produce ExoS, ExoT and ExoY effectors proteins causing rounding of lung epithelial like cell line and lysis of macrophage cell line (20) in accordance with the previously published research article about interaction between *P. aeruginosa* 103 and *A. castellanii* at 30℃ (21). Abd et al., 2008 found that survival time of alone *A. castellanii*, in the presence of wildtype *P. aeruginosa* 103 producing ExoT and ExoU in the presence of the defective bacterium in production of ExoT and ExoU *P. aeruginosa* 103 was >12 days, 4 days and >12 days, respectively (21). Furthermore, apoptosis and cytotoxic vacuolation in *A. castellanii* were induced by *P. aeruginosa* 103 producing ExoS after one day of co-cultivation compared to *P. aeruginosa* 103 possessing ExoY that induced cytotoxic vacuolation at day 3 of co-cultivation (21) as in figure 2 below.

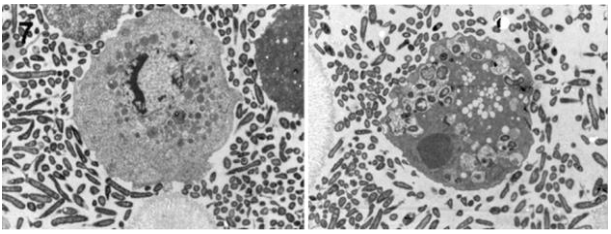

Fig. 2. Transmission electron microscope analysis, magnification 3700x.
A. castellanii **showing apoptotic nuclear fragmentation (left panel) and cytotoxic vacuolation (right panel), the micrographs were from Abd et al., 2008 (21).**

However, co-cultivated amoebae with wildtype or with the extracellular protein defective strains of *P. aeruginosa* 01producing ExoS, ExoT and ExoY effectors proteins, survived only 4 days at 30℃ uncovering that extracellular virulence factors might not affect the viability of the amoebae since these factors would be activated at 37℃, thus, *P. aeruginosa* becomes more invasively virulent against different eukaryotes at 37℃.

2. ***P. aeruginosa*** **103 decreases viability of U-937 cells**

To analyze differences in the cytotoxic effects on U-937 cell co-cultivated with alone *P. aeuroginosa* 103 or with pre-incubated *P. aeuroginosa* 103 with *A. castellani*, U-937

cells viability assay was carried out as described in methods section. The result found that the U-937 cells viability was decreased after co-cultivation with either alone *P. aeuroginosa*103 or that pre-incubated with *A. castellanii* compared to U-937 cells control that cultivated alone (Fig. 3). Compared to the control cells, the viability of U-937 cells co-cultivated *P. aeuroginosa*103 decreased from 90% to 40% and with the U-973 cells cultivated with pre-incubated with *A. castellanii* decreased from 90% to 25% (Fig. 3). The viability of the U-937 cells cultivated alone, co-cultivated with *P. aeruginosa*103, and co-cultivated with pre-incubated with *A. castellanii,* was reduced significantly since *P* value of $\chi 2$ was 0.000001.

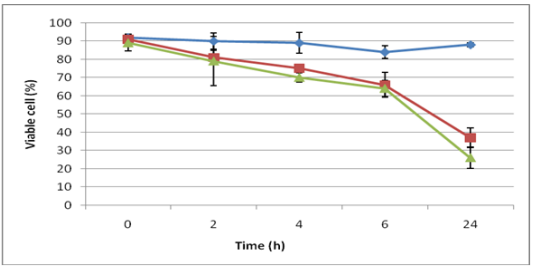

Fig. 3. Viability of U-937 cells. U-937 cells cultivated alone as control (♦); U-937 cells co-cultivated with PA103 (■); U-937 cells pre-cultivated *A. castellanii* PA103 (▲).

Surprisingly, this larger decrease in viability of U-937 cells, caused by *P. aeruginosa* after pre-incubation with *A. castellanii* compared to that decrease caused by co-cultivation with *P. aeruginosa* alone might be due to a synergically virulence of *P. aeruginosa* interacted previously with *A. castellanii*. Moreover, this decrease in viability would agree with Chai et al, 2008 who previously showed a time dependent increase of apoptosis in U-937 cell after exposure to P. aeruginosa (17).

3. *P. aeruginosa*103 induces necrotic/late apoptotic cell death in U-937 cell

FACS analysis of Annexin V/ Propidium iodide-stained U-937 cells incubated with *P. aeruginosa* or with *P. aeruginosa* pre-incubated with *A. castellanii* for 4 or 24 hours uncovered presence of a high number double stained necrotic/late apoptotic cells that increased with time and mostly in the U-937 cells that co-cultivated with *P. aeruginosa* pre-cultivated with *A. castellanii*. The percentages of necrotic/late apoptotic U-937 cells were 18, 44 after 4h and 24h, compared to 24 and 52, respectively. Difference between the percentages was not significant according to $\chi 2$ statistical analysis method since *P* was 0.98, as shown in table 2.

Table 2: Percentage of U-937 cells after incubation with the wildtype PA103 or the pre-incubated *A. castellanii* PA103

Morphology of U-937 cells					
U-973 cells morphology	**Incubation types of U-937 cells**				
	Alone	with PA103		with amoebae pre-incubated PA103	
		4 h	24 h	4h	24 h
Viable cells	83.5 ± 5.4	73.6 ± 14.7	41.0 ±17.5	66.8 ±11.4	31.1±11.3
Necrotic cells	0.5 ± 0.4	1.4 ± 0.4	1.5 ± 0.0	1.8 ± 1.1	2.1 ± 1.6
Necrotic or late apoptotic	7.32 ± 3.5	18.1 ± 7.7	44.3 ± 2.8	23.7 ± 5.2	52.3 ± 1.5
Apoptotic cells	5.7 ± 5.1	3.7 ± 2.6	8.0 ± 7.3	4.1 ± 2.2	8.0 ± 5.1

Interestingly, fluorescence activated cell sorting (FACS) analysis utilized in the current article emphases killing of U-973 and gave more details than viability and defined killing type of the killing as necrotic/late apoptotic cell death. Surprisingly, it was shown that *P. aeruginosa* 103 induced either cytotoxic vacuolation together with apoptosis or injured cell membrane and karyolysis as characters for necrosis in *A. castellanii* (21).

4. Caspase-3 activation in U-937 cells after co-cultivation with *P. aeruginosa* 103

Caspase activity assay was performed to investigate activation of caspase-3 in U-937 cells upon co-cultivation with *P. aeruginosa* 103 or with *P. aeruginosa* 103 that pre-incubated with *A. castellanii* for 4 or 24 hours, compared to the control U-937 cells without bacteria.

The result showed that caspase-3 activity of U-937 cells incubated with *P. aeruginosa* 103 as well as with the *P. aeruginosa* 103 that pre-incubated with *A. castellanii* increased after 24 hours of incubation but not after 4 h as shown in (Fig. 4) and the increase was significant according to statistical analysis since p of $\chi 2$ was < 0.000001.

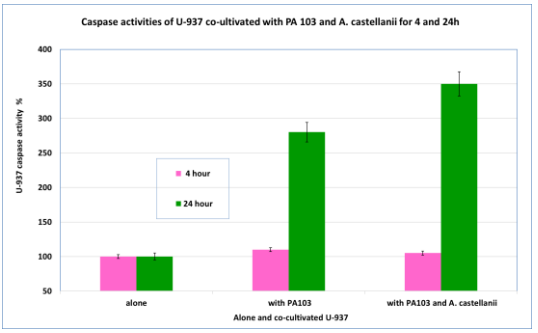

Fig. 4. Caspase-3 activity of U-937 cells co-cultivated with *P. aeruginosa*103 and the *P. aeruginosa* 103 that pre-incubated with *A. castellanii* compared U-937 cells without bacteria that were used as control. The assay was carried out after both 4 hours (pink colour) and 24 hours (green colour).

In discussion, the result indicates that *P. aeruginosa* 103 increases its cytotoxicity after exposing to the *A. castellanii*, inducing caspase-3 activity with a higher rate after 24 hours. But since the FACS assay showed no difference in apoptotic cells and only a limited increase of necrotic/late apoptotic cells after infection with *P. aeruginosa* 103 that pre-incubated with *A. castellanii* compared to alone *P. aeruginosa* 103 (at both 4 and 24 hours) this pathway might not seem be crucial for *P. aeruginosa* mediated cell death in U-937 cells.

From result of FACS assay and caspase-3 activity assay, we can conclude that the caspase-3 activity is induced by the bacteria at 24 hours with a higher rate, leading the cells to undergo apoptosis, but the diversity of *P. aeruginosa* virulence factors seems too simultaneously activate a necrotic pathway.

In summary, our result found that *P. aeruginosa* interaction with *A. castellanii* might be lethal to the amoeba population and might lead to increased cytotoxic properties of this bacterium. Viability of U-937 decreased in co-cultivation

with *P. aeruginosa* alone and that viability decrease was statistically higher in co-cultivation with *P. aeruginosa* pre-incubated with *A. castellanii*.

The FACS found notable percentages of necrotic/late apoptotic U-973 cells that co-cultivated with *P. aeruginosa* pre-cultivated with *A. castellanii* compared to the U-973 cells cultivated with *P. aeruginosa* 103 alone but the difference was not statistically by $\chi 2$ test.

Caspase activity assay of U-937 cells showed a high significant activity when pre-incubated with *A. castellanii* compared to the incubation with *P. aeruginosa* 103 alone after 24 hours.

CONCLUSIONS

Bacteria-amoebae interaction in this study has pointed out clearly, the important rule of T3SS effector proteins of *P. aeuroginosa* 01 wildtype strain and the extracellular protein defective (xcp) strain, in killing of *A. castellanii* in co-cultivation at 30 ℃ since this bacterium produces ExoS, ExoT and ExoY effectors proteins causing apoptosis and necrosis to the amoebae.

The result of FACS assay and caspase-3 activity assay uncovered that a higher rate of the caspase-3 activity induced by the *P. aeruginosa* 103 at 24 hours, led the U-937 cells to undergo apoptosis, but the diversity of *P. aeruginosa* virulence factors seemed to simultaneously activate a necrotic pathway also.

ACKNOLEDGEMENT

The authors acknowledge Professor Bengt Wretlind, Karolinska Institute, Laboratory Medicine Department, Division of Clinical Microbiology and Karolinska University Hospital Huddinge, Sweden and Dr. Dara Frank, Medical College of Wisconsin, USA, for *Pseudomonas aeruginosa* isolates early providing.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest.

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