INTERACTION BETWEEN MONOCYTE AND PORPHYROMONAS GINGIVALIS SURVIVAL AND CELL RESPONSES

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A THESIS SUBMITTED IN PARTIAL FULLFILLMENT
OF THE REQUIREMENTS FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY
(ORAL BIOLOGY)
FACULTY OF GRADUATE STUDIES
MAHIDOL UNVERSITY
2007

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Thesis

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INTERACTION BETWEEN MONOCYTE AND PORPHYROMONAS GINGIVALIS SURVIVAL AND CELL RESPONSES

was submitted to the Faculty of Graduate Studies, Mahidol University
For the degree of Doctor of Philosophy (Oral Biology)
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ACKNOWLEDGEMENT

First of all, I would like to be grateful for the principal person of these studies, Assoc. Prof. Rudee Sirarit, who is my major advisor and also main director of these studies. In case of performing study without her support, guidance, and problem solving throughout working period, these studies could not reach this achievement. I would like to thank Prof. Steven Offenbacher and Asso. Prof. Ratchapin Srisatjalik, my co-advisor, for their valuable advice, support and guidance in these studies.

I would like to thank Assist. Prof. Panjit Chunhabundit and Assoc. Prof. Woranut Weerapradist, for their kindness in suggestions and support these studies.

I would like to thank Asso. Prof. Kleophanth Thakerngpol, personal of Department of Pathology and Department of Pathology, Siriraj Hospital, Mahidol University for their facility in Transmission electron microscope for these studies.

I would like to thank Mrs. Suwanna Korsuwannawong for her advice providing in data analysis.

I would like to thank Ms.Taniya Muadcheingka and the personal of Department of Microbiology, Faculty of dentistry, Mahidol University for their cheerfulness and kind support.

I would like to thank Royal Golden Jubilee Grant from Thailand Research Fund for fully support this studies.

Finally, I am grateful to my mother and family for all kind support, assistance, encouragement, entirely care and love.

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INTERACTION BETWEEN MONOCYTE AND PORPHYROMONAS GINGIVALIS SURVIVAL AND CELL RESPONSES

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ABSTRACT

P. gingivalis is considered to be an important periodontal pathogen in adult chronic periodontitis. In addition, these bacteria may be positively related to atherosclerotic lesion since DNA of *P. gingivalis* is present in atherosclerotic lesions. The aims of this study were to examine; 1) the localization and survival efficiency of *P. gingivalis* in human monocyte, 2) the cytokines production and 3) the infected human monocyte migration. Human monocyte cells were cocultured with *P. gingivalis*, after which the external bacteria were killed with the combination of metronidazole and gentamycin. After antibiotics exposure, the monocytes were resuspended in RPMI1640 and further incubated at various times. The infected monocytes were harvested and examined. A transmission electron microscope (TEM) was used to evaluate the localization of *P. gingivalis* in cells. The survival efficiency of *P. gingivalis* was determined by plating cells on blood agar and culturing under anaerobic conditions. Enzyme-linked immunosorbent assay (ELISA) and reverse transcriptase polymerase chain reaction (RT-PCR) using specific antibodies and primers for IL-1β and TNF-α were performed. The effect of *P. gingivalis* on migration of infected monocyte cells was studied by using transwell. TEM study showed that *P. gingivalis* were located within the autophagosome- like structure after coculturing of monocyte cells with *P. gingivalis* and treatment with a combination of antibiotics. No significant difference was found in the survival efficiency at 0, 4 and 8 h after infection. Using ELISA and RT-PCR techniques, it was found that IL-1β and TNF-α were present in the media after stimulation with *P. gingivalis.* The combination of antibiotics did not affect the IL-1β and TNF-α expression. Infected monocyte cells treated with monocyte chemotactic protein-1 (MCP-1) were able to migrate through the membrane. The migrated monocyte cells through membrane in the presence and absence of *P. gingivalis* were $18.64 \pm 2.33 \times 10^4$ cells and $19.11 \pm 2.33 \times 10^4$cells, respectively. No significant difference was found between these two groups. It may be concluded that; 1) *P. gingivalis* can survive in monocyte cells within the autophagosome, 2) *P. gingivalis* can stimulate IL-1β and TNF-α production from monocytes and 3) Internal *P. gingivalis* of infected monocytes did not affect monocyte migration.

KEY WORDS: MONOCYTE/ PORPHYROMONAS GINGIVALIS
TEM/ SURVIVAL EFFICIENCY/ IL-1β/ TNF-α/ ELISA/ RT-PCR
MONOCYTE MIGRATION/ MCP-1

85 P.

ปฏิกิริยาระหว่างเซลเม็ดเลือดขาวชนิดโมโนไซต์และแบคทีเรียชนิดพอร์ไฟโรโมแนสจินจิวาลิสใน
เรื่องการมีชีวิตอยู่ภายในเซลของแบคทีเรียและการตอบสนองของเซลโมโนไซต์
(INTERACTION BETWEEN MONOCYTE AND PORPHYROMONAS GINGIVALIS SURVIVAL AND CELL RESPONSES)

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บทคัดย่อ

เชื้อพอร์ไฟโรโมแนสจินจิวาลีสเป็นแบคทีเรียที่เชื่อว่าเป็นสาเหตุหลักของการเกิดโรคปริทันต์อักเสบในผู้ใหญ่ และเชื้อดังกล่าวยังอาจมีส่วนเกี่ยวข้องกับการเกิดโรคการหนาตัวของผนังหลอดเลือด วัตถุประสงค์ของการศึกษานี้คือ ศึกษาตำแหน่งและอัตราในการรอดชีวิตของเชื้อชนิดนี้ในเซลโมโนไซด์ การสร้างสารไซโตไคน์และการเคลื่อนที่ของเซลโมโนไซต์หลังการติดเชื้อ **วัสดุและวิธีการ** นำเซลโมโนไซต์มาเพาะเลี้ยงกับเชื้อข้างต้น แล้วกำจัดเชื้อที่อยู่นอกเซลด้วยยาปฏิชีวนะ ต่อมานำเซลโมโนไซต์ไปเพาะเลี้ยงต่อตามระยะเวลาที่กำหนด แล้วศึกษาด้วยกล้องจุลทรรศน์ชนิดส่องทะลุเพื่อศึกษาตำแหน่งของเชื้อ ศึกษาอัตราการรอดชีวิตของเชื้อโดยทำให้เซลโมโนไซต์แตกตามด้วยการเพาะเลี้ยงในสภาวะที่ไร้ออกซิเจน ศึกษาการสร้างสารไซโตไคน์ด้วยวิธีอิไลซาและอาร์ทีพีซีอาร์ และศึกษาการเคลื่อนที่ของเซลโมโนไซต์หลังการติดเชื้อ **ผลการศึกษา** จากการศึกษาด้วยกล้องจุลทรรศน์ชนิดส่องทะลุพบว่าเชื้อดังกล่าวอาศัยอยู่ในบริเวณที่คล้ายออโต้ฟาโกโซมของเซลโมโนไซต์และพบว่าอัตราการรอดชีวิตของเชื้อหลังนำเซลโมโนไซต์ไปเพาะเลี้ยงต่อเป็นเวลา 0, 4 และ 8 ชั่วโมงไม่มีความแตกต่างกัน จากการศึกษาโดยวิธีอิไลซาและอาร์ทีพีซีอาร์พบว่าเซลโมโนไซต์มีการสร้างสารอินเตอร์ลูคิน-1เบต้าและทีเอ็นเอฟ-อัลฟ่าภายหลังจากการกระตุ้นด้วยเชื้อแบคทีเรียโดยยาปฏิชีวนะที่ใช้ไม่มีผลต่อการสร้างไซโตไคน์ทั้งสองชนิดนี้ และพบว่าไม่มีความแตกต่างกันของการเคลื่อนที่ของเซลที่มีการติดเชื้อและไม่มีการติดเชื้อ **สรุป** แบคทีเรียชนิดพอร์ไฟโรโมแนสจินจิวาลีสสามารถมีชีวิตภายในเซลโมโนไซต์โดยอาศัยอยู่ในบริเวณที่คล้ายออโต้ฟาโกโซม เชื้อแบคทีเรียชนิดนี้สามารถกระตุ้นเซลโมโนไซต์ให้มีการสร้างสารอินเตอร์ลูคิน-1เบต้าและทีเอ็นเอฟ-อัลฟ่า และการติดเชื้อชนิดนี้ของเซลโมโนไซต์ไม่มีผลกระทบต่อการเคลื่อนที่ของเซลเม็ดเลือดขาวชนิดนี้

85 หน้า.

CONTENTS

	Page
ACKNOWLEDGEMENTS	iii
ABSTRACT	iv
LISTS OF TABLE	viii
LISTS OF FIGURE	ix
LISTS OF ABBREVIATIONS	xi
CHAPTER	
I INTRODUCTION	1
II OBJECTIVES	5
III LITERATURE REVIEW	6
Porphyrononas gingivalis	6
Porphyrononas gingivalis and periodontal disease	8
Cytokines	19
Periodontal pathogens and atherosclerosis	21
IV MATERIALS AND METHODS	23
Cell culture	23
Bacterial culture	23
Effect of gentamycin and metronidazole on viability of THP-1	24
Effect of gentamycin and metronidazole on viability of *P. gingivalis*	24
Effect of ddH_2O on viability of *P. gingivalis*	24
Effect of ddH_2O on THP-1 cells lysis	25
Multiplicity of infection (MOI) of *P. gingivalis* and incubation time	25
Bacterial localization in THP-1 cell	25
Survival *of P. gingivalis* in THP-1	26
IL-1β and TNF-α production	27
Monocytes migration	29

CONTENTS (Continue)

	Page
V RESULTS	32
Effect of gentamycin and metronidazole on viability of THP-1	32
Effect of gentamycin and metronidazole on viability of *P. gingivalis*	34
Effect of ddH_2O on viability of *P. gingivalis*	35
Effect of ddH_2O on THP-1 cells lysis	36
Multiplicity of infection (MOI) of *P. gingivalis* and incubation time	37
Bacterial localization in THP-1 cell	38
Survival *of P. gingivalis* in THP-1	48
IL-1β and TNF-α production after invasion THP-1 cells by *P. gingivalis*.	51
Monocytes migration	58
VI DISCUSSION	61
VII CONCLUSIONS	69
REFERENCES	70
APPENDIX	79
BIOGRAPHY	85

LIST OF TABLES

Table **Page**

3.1 Evidence supporting a role for *P. gingivalis* as pathogen in periodontal disease: Socransky's criteria. 9

3.2 Mediators of inflammatory and immune response from mononuclear phagocytic cell and their functions 18

3.3 Main biologic activities of IL-1 20

5.1 Effects of gentamycin and metronidazole on viability of THP-1. 32

5.2 Effect of gentamycin and metronidazole on viability of *P. gingivalis*. 34

5.3 Effect of ddH_2O on viablility of *P. gingivalis*. 35

5.4 The number of THP-1 cells after incubation in RPMI 1640 and ddH_2O. 36

5.5 The effect of MOI and incubation time on bacterial survival efficiency of *P. gingivalis* in THP-1 cells. 37

5.6 TNF-α and IL-1β production by THP-1 cells after treatment with *P. gingivalis* infection. 55

5.7 The number of THP-1 cells in lower compartment, when stimulated by different concentration of MCP-1. 58

5.8 Number of THP-1 in lower compartment and CFU of *P. gingivalis* per THP-1 cells following lyzed the THP-1 cells. 60

LIST OF FIGURES

Figure **Page**

3.1 EM picture of *Porphyromonas gingivalis* 7

3.2 Outer membrane of *Porphyromonas gingivalis* 10

3.3 Lipopolysaccharide of gram-negative bacteria 11

3.4 Fimbriae of *P. gingivalis* 12

5.1 The morphology of THP-1 cells under the phase contrast microscope. 33

5.2 Transmission electron microscopy (TEM) of infected THP-1 cells. 39

5.3 The cell surface of THP-1 presented with the electron dense (ED) and pseudopodia (P). 40

5.4 This TEM picture presented *P. gingivalis (Pg*) aggregated and contacted at certain area of THP-1 membrane. 41

5.5 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics. 42

5.6 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics. 43

5.7 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics in RPMI 1640 for 4 h. 44

5.8 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics and incubation in RPMI 1640 for 4 h. 45

5.9 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics and incubation in RPMI 1640 for 4 h. 46

5.10 Transmission electron microscopy (TEM) of infected THP-1 cells after incubation in RPMI 1640 for 8 h. 47

5.11 The survival efficiency was calculated from CFU of *P. gingivalis* recovered intracellular THP-1 cells as a percentage of total bacteria inoculated and incubated with THP-1 cells for 0, 4, 8 h. 49

5.12 Percent survival efficiency of *E. coli* in THP-1 cell following antibiotic treatment and varied in incubation time. 50

LIST OF FIGURES (Continue)

Figure **Page**

5.13 The expression of house keeping gene 18s rRNA and the IL-1β expression in THP-1 cells after *P. gingivalis* infection. 53

5.14 The expression of house keeping gene 18s rRNA and the TNF-α expression in THP-1 cells after *P. gingivalis* infection. 54

5.15 IL-1β production by THP-1 cells after *P. gingivalis* infection. 56

5.16 TNF-α production by THP-1 cells after *P. gingivalis* infection. 57

LIST OF ABBREVIATIONS

Abbreviations or symbols	Terms
ATCC	American Type Culture Collection
CFU	colony forming unit
ddH_2O	double distilled water
P.gingivalis	*Porphyromonas gingivalis*
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
dNTP	deoxynucleotide triphosphate
RT	reverse transcriptase
PCR	polymerase chain reaction
EDTA	ethylene diamine tetraacetic acid
MCP-1	monocyte chemotactic protein-1
et.al	Et alli (Latin), and other
e.g.,	example gratia (Latin) for example
g	gram
mg	milligram (10^{-3}g)
μg	microgram (10^{-6}g)
pg	picrogram (10^{-9}g)
ng	nanogram (10^{-12}g)
fg	femtogram (10^{-15}g)
l	litter
ml	milliter (10^{-3}l)
μl	microliter (10^{-6}l)
m	meter
nm	nanometer (10^{-9}m)
min	minute
h	hour

LIST OF ABBREVIATIONS (Continue)

Abbreviations or symbols	Terms
OD	Optical density
RT	room temperature
°C	degree celcius
CO_2	carbon dioxide
H_2	hydrogen
N_2	nitrogen
ELISA	enzyme-linked immunosorbent assay
%	percentage
α	alpha
β	beta
×g	centrifugal force

CHAPTER I
INTRODUCTION

Periodontal disease is a chronic inflammatory disease of the tooth supporting tissues[1]. The result of this disease leads to teeth supporting tissue destruction and finally teeth loss in the severe cases. Periodontal disease distributed widely in adult population. Previous reports presented that 35% of U.S. adult population were suffered from this disease[2] and periodontal pockets were seen in 37.3% of the 35-44 years old adult in Thailand[3](the 5th oral health survey of Thailand). This disease has also been reported to positive relate to other disease such as coronary heart disease, stroke, diabetes mellitus and low-birth-weight[4,5,6].

Periodontal disease has been classified according to the clinical signs and symptoms into two major groups, gingivitis and periodontitis. Gingivitis is defined as the inflammation of gingival margin due to the accumulation of dental plaque. It is characterized clinically by redness, swelling and bleeding gingiva. The underlying periodontal ligament and alveolar bone are not involved in this inflammation. The epithelium attachment does not extend apically beyond its normal position at the cemento-enamel junction.

Periodontitis is characterized by the destruction of periodontal ligament, apical migration of the epithelial attachment and loss of crestal alveolar bone. The loss of periodontal attachment is associated with the presence of an inflammation at the gingiva margin. Other commonly identified symptoms include periodontal pocket formation, recession of gingival margin and eventually radiographic alveolar bone loss.

Gingivitis has been accepted that it is the result of the accumulation of dental plaque in poor oral hygiene patients. It is reversible. Mechanical removal of dental plaque will resolve this gingival inflammation condition. On the other hand, periodontitis is associated with oral hygiene and it becomes irreversible once bone loss occurs. Periodontal disease has been accepted that it results from the interaction

between specific periodontal pathogens and their products and host response mechanism. Interaction of periodontal pathogens and host responses are believed to be the cause of the destruction of tooth supporting tissues[7].

Porphyromonas gingivalis, a gram negative anaerobic bacteria, is considered to be an important periodontal pathogen in adult chronic periodontitis[8,9]. This bacteria is classified as a member of black pigmented bacteroides group because this bacteria form convex, smooth glossy brownish-black colonies on the blood agar plate. *P. gingivalis* is a non-motile, bacteria with cocco-bacilli shape[9]. They were found to be increased in number at disease site[10], but lower or undetectable in healthy gingiva sites[11]. The elimination of *P. gingivalis* is one method for periodontal disease therapy[12]. Many virulence factors produce by *P. gingivalis* such as lipopolysaccharides[13] and proteases[14]. The bacterial proteases can degrade antibodies, complement components and collagen[14,15,16]. Besides proteases, *P. gingivalis* also produces 24 KDa and 75 KDa proteins that are able to induce bone resorption[17,18]. The bacteria has been shown to induce proinflammatory cytokine production such as IL-1β and TNF-α from host cells[19,20], which are important in periodontal tissue destruction process. In addition, there is a number of evidence indicating that *P. gingivalis* can invade and survive in some non-phagocytic cells, such as buccal epithelium cells[21], oral epithelium cells[22] and endothelial cells[23] *in vitro*. This is considered to be an important mechanism, which protects them from host immune system. Once within the cells, *P. gingivalis* has developed various mechanisms for survival. In those cells, *P. gingivalis* accesses and survives in the cell through autophagosomes[22,23]. Bacterial trafficking to the autophagosome has been proposed to be a mechanism to increase the concentration of free amino acids to be utilized by the bacteria for their biochemical pathways as well as reduced the cellular response to the pathogen[24]. However, no previous studies demonstrated the invasion and intracellular survival of phagocytic cells by *P. gingivalis* like the other intracellular bacteria, *i.e.*, *Mycobacterium tuberculosis* and *Legionella pneumophila*[25]. These bacteria were found to be able to reside in monocytes for and evaded from host response.

Monocyte is a phagocytic cell that performs many important functions in host response against bacterial infection. This cell is 10-15 μm in diameter, contains bean-

shaped nucleus and the fine granule cytoplasm contains lysosomes, phagocytic vacuoles and cytoskeleton filaments. The functions of monocytes include phagocytosis, secretion of biologically active molecules and surface antigen presentation. Monocytes release many factors including proinflammatory cytokines such as IL-1 β and TNF-α, which are soluble proteins produced by cells in response to bacterial infection. These cytokines can influence other cells activities such as activation of bone resorption, inhibition of bone formation, stimulation of neutrophil and stimulation of B-cell and T cell proliferation. Their biological activities associate with inflammatory process and immune response. The secreted cytokines have biological activity that associated with the pathological mechanism in periodontal disease. Among proinflammatory cytokines, IL-1β and TNF-α are considered to be important in periodontal disease. They are multifunctional cytokines that appear to be the main regulator of the inflammatory response. The biological activities of these cytokines include induction of prostaglandin E_2 secretion, stimulation of neutrophils, inhibition of bone formation and activation of bone resorption process[26]. The subcutaneous administration of TNF-α accelerated the progression of periodontitis in rats[27]. Early studies presented that the level of IL-1β in gingival crevicular fluid has significant correlation with clinical attachment loss[28] and the elevation level of IL-1β has been demonstrated in tissue and gingival crevicular fluid from periodontitis area[29]. Many cells types are capable of synthesizing IL-1β including mononuclear phagocytic cell[30]. Previous report showed that *P. gingivalis* can activate monocytes to release both IL-1β and TNF-α[31], but no previous studies reported the response of the mononuclear phagocytic cell to *P. gingivalis* infection.

Monocyte chemotactive protein 1 (MCP-1) is an inflammatory chemotactic cytokines that targets monocyte[32]. MCP-1 has been related as a key player in the recruitment of monocyte from the blood into the atherosclerotic lesion[33]. These circulating blood monocytes are precursors of foam cells at the atherosclerotic lesion[33]. Several studies demonstrated that DNA of *P. gingivalis* were present in atherosclerotic lesion[34,35,36] and the bacteria were able to promote atherosclerosis progression[37]. The migration of infected monocytes to the disease lesion may play an important role in this process. It has been shown that the extracellular bacteria are

able to stimulate the migration of monocytes possibly through bacterial products, which act as potent chemoattractants for human monocytes[38]. Therefore, it may be possible that the presence of intracellular *P. gingivalis* could promote migration of infected monocytes.

As mention above, periodontal disease is likely to be related to the cardiovascular disease and infant low birth weight. There was a study reported that DNA of *P. gingivalis* was found at the atherosclerosis plaque. Thus the understanding of their mechanism of invasion and survival of this intracellular bacteria in phagocytic cells may help to understand the relationship between periodontal disease and cardiovascular diseases. The monocytic cell may be a good candidate for such mechanism since it functions in host response mechanism and can transport freely in blood circulation.

Therefore, the aims of this study were to demonstrate the survival ability of *P. gingivalis* in the phagocytic cells and to show the response of these cells to this bacterial infection in terms of cytokines production and cell migration. Understanding such processes may help us to understand the relationship between periodontal disease and cardiovascular disease or the other systemic diseases, which may lead to preventive measure in the further.

CHAPTER II
OBJECTIVES

1. To study the survival ability of *P. gingivalis* in the monocytic cell line (THP-1).
2. To study the trafficking of *P. gingivalis* in the monocytic cell line (THP-1).
3. To determine the cytokines production of the monocytic cell line (THP-1) after *P. gingivalis* invasion.
4. To study the effect of *P. gingivalis* infection of the monocytic cell line on cell migration.

CHAPTER III
LITERATURE REVIEW

Periodontal disease is an infections disease that commonly found in humans. This inflammatory disease varies in severity from mild and reversible inflammation of gingiva (gingivitis) to chronic destruction of tooth supporting tissue (periodontitis). Nowadays, it is widely accepted that the formation of this disease is the result from interactions between periodontal pathogens and host responses. If this interaction shifts to hyper-inflammatory response, then there will be destruction of tooth supporting tissues. On the other hand, if this interaction shifts to non-inflammatory response, then mild progression of destruction will occur or even stop. The interaction between periodontal pathogens and host response is a complex process that involved many factors such as virulence factors of pathogenic bacteria, functions of leukocytes and cytokine secretion. This review will focus on *P. gingivalis*, monocytes and proinflammatory cytokine productions.

Porphyromonas gingivalis

Porphyromonas gingivalis is a gram-negative, non-motile, non-spore forming, anaerobic, and short rod bacteria (Fig 3.1). This bacteria was classified as a member of black-pigmented *Bacteroides* group because it forms brownish-black colonies on blood agar plates. Bacteria in the *Bacteroides* group were grouped by Oliver and Wherry in 1921. In the late 1970s, it was recognized that the black-pigment *Bacteoides* contain three subgroups namely: asaccharolytic group, intermediate level of carbohydrate fermentation group and high saccharolytic fermentation group. Recently, black-pigmented *Bacteroides* was reclassified base on 16s rRNA and subdivided into *Porphyromonas*, *Bacteroides* and *Prevotella* genus.

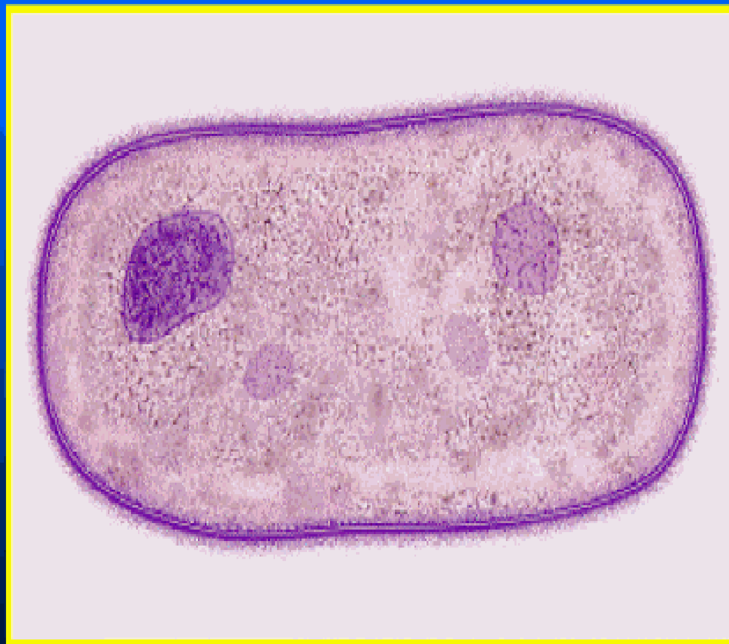

Figure 3.1 EM picture of *Porphyromonas gingivalis* (picture from www.altcorp.com)

Porphyromonas gingivalis and periodontal disease

Periodontal disease comprises a group of inflammatory conditions of tooth supporting tissues that are caused by periodontal pathogenic bacteria. *P. gingivalis* is considered one of bacterial species that is well accepted as periodontal pathogen in chronic periodontal disease in human. This hypothesis is supported by:

1) *P. gingivalis* increased in periodontitis sites[10], whereas, it was detected in lower number or undetectable from healthy gingiva sites[11].

2) The elimination of *P. gingivalis* has been associated with successful therapy, whereas persistence of the microorganism has been associated with disease recurrent[12].

3) *P. gingivalis* has been shown having virulence factors, such as collagenase[39], bone resorption-induction factor[40], proteases[41] and proinflammatory cytokine induction factor [19,20].

4) The systemic and local immune response to *P. gingivalis* in adult periodontitis patients are elevated [42].

5) *P. gingivalis* may also enter the junctional epithelium and multiply in that location [43].

6) In animal models, the initiation of progressive bone loss and clinical sign of periodontitis have been reported in a complex ecosystem, which consists of *P. gingivalis* [44].

These evidences were summarized and suggested as Socransky's criteria as shown in table 3.1.

Many products from *P. gingivalis* can function as virulent factors in inflammatory process and bone destruction factors, which cause periodontal disease. These include capsule, proteins of outer membrane, lipopolysaccharide, fimbriae and proteinases.

Table 3.1 Evidence supporting a role for *P. gingivalis* as pathogen in periodontal disease: Socransky's criteria.

Criterion	**P. gingivalis**
Association	Increased in periodontitis lesions Found associated with gingival sulcus epithelium
Elimination	Suppressed or eliminated in successful therapy Found in recurrent lesions
Host response	Increased systemic and local antibody levels in periodontitis
Animal studies	Found to be important in experimental mixed infections and periodontitis in animal model
Virulent factors	Products from *P. gingivalis* can function as virulent factors

Adapted from Socransky SS, Haffajee AD: The bacterial etiology of destructive periodontal disease: Current concepts .J Periodontol 1992;63: 322-331

1. Capsule

Capsule of *P. gingivalis* is composed of mucopolysaccharide, which surround the outer membrane of bacteria. The presence of capsule in *P. gingivalis* has been considered as an important anti-phagocytic factor and this may be part of the *P. gingivalis* , which contains ability to be considered as a periodontal pathogen. It was found that the increased thickness of *P. gingivalis* capsule is correlated with the increased in resistance to phagocytosis[45]. This effect may be the thick capsule functions as physiologically mask surrounding the outer membrane of bacteria preventing activation of the complement pathway. In summary, capsule enables bacteria cells to escape from complement activation and protects *P. gingivalis* from phagocytosis.

2. proteins of outer membrane

The cell wall of *P. gingivalis* is a complex multilayer structure (Figure 3.2). The cell envelope consists of the inner cytoplasmic membrane, a thin peptidoglycan, which attached to outer membrane by the lipoprotein. The outer membrane of *P. gingivalis* contains complex lipopolysaccharides, lipoproteins, peripheral and transport proteins. Porin protein in outer membrane functions as transport mechanism for movement of selective proteins in and out the cells. The virulence factor of *P. gingivalis* outer membrane has been reported[17,18] e.g. the purification of a 24-KDa protein from outer membrane of *P gingivalis* was found to stimulates bone resorption *in vitro*[17]. Watanabe *et al.*[18] found that a 75-KDa protein from outer membrane of *P. gingivalis* could also activate B-cell and induce IL-1 production or expression in mouse macrophage.

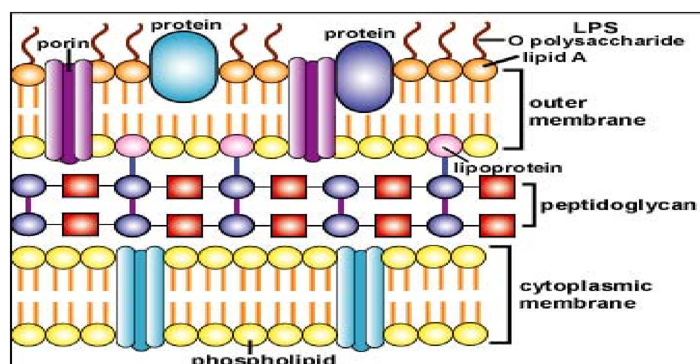

Figure 3.2 Outer membrane of *Porphyromonas gingivalis* (picture from www. cat. cc. md. us).

3. Lipopolysaccharide

The outer membrane of *P. gingivalis* contains the lipopolysaccharide (Figure 3.3) which is a 10-KDa molecule. Lipopolysaccharide is an amphiphatic molecule. It consists of polysaccharide, which projects toward to the outer environment on the membrane surface of bacteria. The hydrophobic lipid end is embedded within bacterial outer membrane. Several studies on *P. gingivalis* lipopolysaccharide showed that lipopolysaccharide or endotoxin, could act as an activator of proinflammatory cytokines, such as interleukin-1 (IL-1), tumor necrosis factor (TNF). These cytokines were significantly less toxic and produced at lower level of when compared to those activated by lipopolysaccharide of *Samonella typhimurium* and *E. coli* [13, 46,47,48].

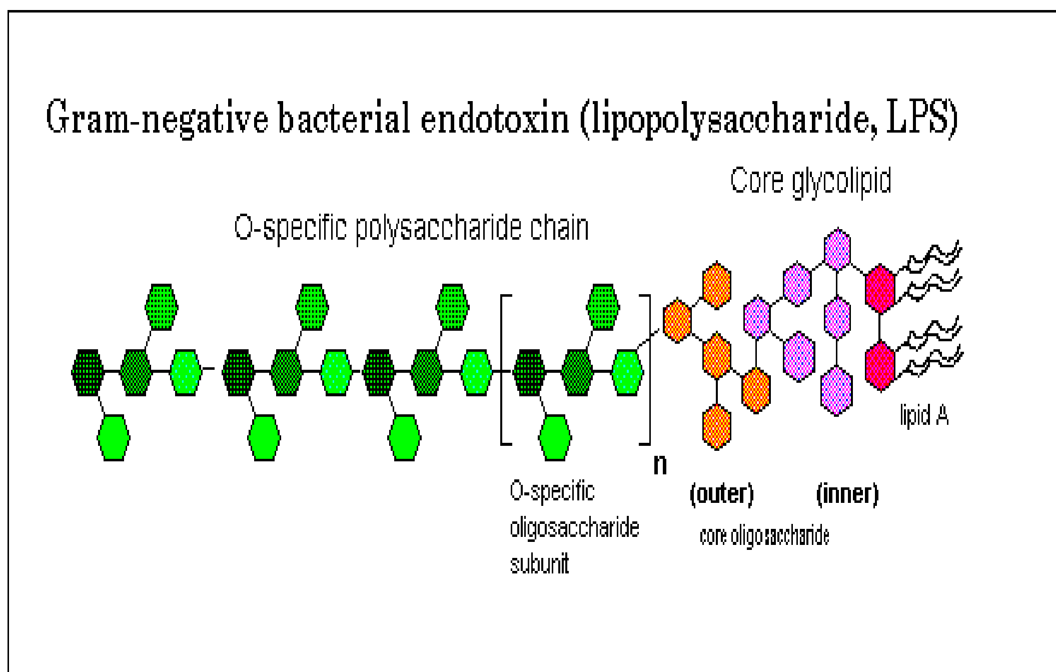

Figure 3.3 Lipopolysaccharide of gram-negative bacteria (picture from www.freespace.virgin.net)

4. Fimbriae

Fimbriae of *P. gingivalis* is approximately 0.5-1.6 μm in length and 5 nm in width. It is composed of fimbrillin monomers which are assembled into 9 units per turn of mature hair-like helix structure[49]. Fimbriae is arranged over the outer membrane surface of the cell. The previous evidence of biological properties of *P. gingivalis* fimbriae was demonstrated that it was the major factor for binding the bacteria to the host cells[50]. The antibody to *P. gingivalis* fimbriae was found to be able to inhibit adhesion of the bacteria to host cells[51]. In the *fim A* knockout mutant of *P. gingivalis* strain 381, the bacteria host cells only one half of the normal wild type strain and exhibited eight times lower in its ability to invade the host cells than the wild type strain[52]. Analysis of human serum from both healthy subjects and periodontal patients found that sera from periodontal patients had the higher antibody levels to *P. gingivalis* fimbriae than that from healthy subjects[53]. *P. gingivalis* fimbriae can stimulate monocytes to produce proinflammatory cytokines such as TNF-α and IL-6[54,55]. Therefore, it can be concluded that fimbriae is an important virulence factor of *P. gingivalis.*

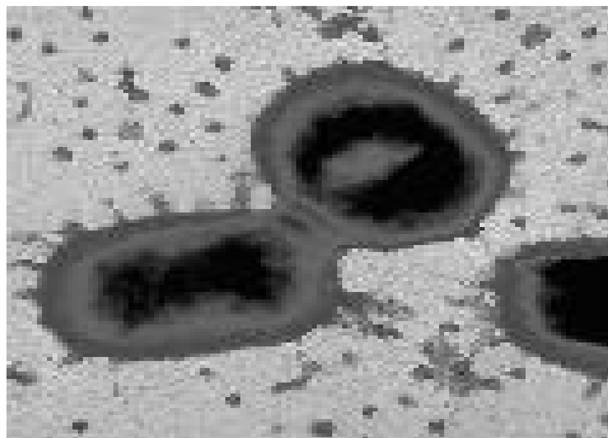

Figure 3.4 Fimbriae of *P. gingivalis* (picture from www.P. gingivalis.org)

5. Proteinases

Proteinases of *P. gingivalis* are considered to be one of the virulence factors. They are concentrated at the cell membrane of bacteria (outer surface of outer membrane, periplasmic space and outer membrane vesicles). The observations about the function of *P. gingivalis* proteinases have been reported. They are able to induce vascular permeability [56,57]. They are also capable of protecting *P. gingivalis* from host defense mechanism by degrading human IgA and IgG into smaller fragments [15,16]. Metabolically, the ability of *P. gingivalis* to produce a variety of proteinases is essential for its survival in host. Since they are asaccharolytic bacteria that cannot metabolize carbohydrates, they must use proteinases to obtain its carbon and energy sources. Thus, the ability of the trypsin-like proteinases to degrade large proteins into small peptides provides a potentially important mechanism for the growth and multiplication of bacteria in host[14,58].

Invasion and survival of *Porphyromonas gingivalis* in host cells

Porphyromonas gingivalis is considered to be a major pathogen of adult periodontitis. Interactions of *P gingivalis* with host cells are believed to be the basic for the destructive inflammatory response, which is the characteristic of periodontal disease. One of the possible pathogenesis of periodontal disease is bacterial invasion and survival in host cells. *P gingivalis* may use this mechanism to pass through epithelial barrier and to protect them from host immune response. Several studies have demonstrated the invasion ability of *P gingivalis in vitro*, such as invasion to human gingival epithelium cells[22], buccal oral epithelium cells[21] and coronary artery cells[23]. *P gingivalis* also has the capacity to pass through the epithelial barrier[45] and replicate within gingival epithelium cells[59] and oral epithelium cells[60].

Invasion of host cells is a common strategy of evading the immune system for *P. gingivalis*. *P. gingivalis* can invade into a variety of human cells in vitro. The intracellular location of *P. gingivalis* has not been singularly defined within the variety of host cells. Dorn et al have demonstrated that *P. gingivalis* resides within a vacuole that resemble autophagosome in human coronary artery cells[23]. *P. gingivalis* was reported to be free in cytoplasm in gingival epithelial cells[59]. The other studies also found evidence of *P. gingivalis* was free in cytoplasm and within endosome in

oral epithelial cells[22] and human junctional epithelium cells[43]. Although many studies demonstrated that *P. gingivalis* could invade and survive in variety of human nonphagocytic cells but the ability of *P. gingivalis* in invasion and survival in phagocytic cells has not yet been studied

Host response in periodontal inflammation

Many host cells are involved in host response mechanism including leukocytes, neutrophils, lymphocytes, T-cells, B-cells, NK cells and mononuclear phagocytic cells.

1. Leukocytes and host response in periodontal inflammation

Gingivitis and periodontitis are caused by microorganisms in dental plaque activating host inflammatory response, which lead to periodontal tissue destruction. The periodontal inflammatory response is a series of vascular changes and infiltration of leukocytes into gingival tissue. These changes result in the erythema, edema, heat, pain and dysfunction of gingiva.

The periodontal inflammatory response is controlled by leukocytes and may be classified into two stages, acute and chronic inflammation. Acute periodontal inflammation is characterized by migration of neutrophils to the inflammatory area after they exist the vascular system. If the problem is not solved, acute inflammation will progress to chronic inflammation. Chronic inflammation is dominated by the migration of lymphocytes and mononuclear phagocytic cells to the inflammatory area. These leukocytes in inflammation tissue are called inflammatory leukocytes.

2. Neutrophils (Polymorphonuclear Leukocytes or PMNs)

Neutrophils are the predominant phagocytic leukocytes in vascular system. Their numbers are about two thirds of all leukocytes in blood (4000-8000 cell/mm^3). They possess many lysosomes, within their cytoplasm. Their differentiation is completed in the bone marrow. This is because neutrophils play role in rapid responses in acute inflammation. Neutrophils possess receptor for complement components and antibodies. These receptors enable neutrophils to participate in

inflammatory response by ingesting foreign molecules into cells in the process of phagocytosis.

3. Lymphocytes

The three main types of lymphocytes in inflammatory response are T-lymphocytes, B-lymphocytes and Natural killer cells (NK cells). In blood, B-cells and T-cells are small and inactive (diameter of 8 to 10μm). NK cells are differentiated completely in bone marrow and appear in blood as large granular lymphocytes with a diameter of 15μm or grater. These cells are larger than any other leukocytes in the blood.\

4. T-cells

T-cells recognize antigens upon binding to T-cell antigen receptor (TAR). Antigens are recognized by T-cells in association with either major histocompatibility class I and class II (MHC class I and MHC class II) molecules on the surface of antigen presenting cells. T-cells are subdivided base on their receptors, into CD4+ T-cells and CD8+ T-cells. Via the CD4 receptor, CD4+ T-cells or T4 helper cells recognized antigen presented by MHC class II molecules that are found on dendritic cells, monocytes, macrophages and B-cells. The T4 helper cells produce lymphokines, such as IL-2 IL-4, IL-6 and IL-10, which play important roles in host responses. The CD8 receptor binds for MHC class I molecules. The CD8+ T cells or T8 killer cells are cytotoxic T-cells involved in controlling intracellular antigens (e.g. certain bacteria, fungi or viruses) by killing the infected cells or limit viral multiplication inside the infected cells.

5. B-cells

B-cells help controlling extracellular antigens, such as bacteria, fungi, yeasts and viruses. B-cells recognize antigens by using B-cell antigen receptors (BAR), which is a high-affinity antigen receptor. The high-affinity interaction between BAR and antigens enable the B-cells to bind and ingest antigens without antigens presenting cells. After exposure to antigens, some B-cells differentiate into plasma cells, which produce and secrete antibodies of the IgM against those antigens. Others

B-cells may differentiate and form memory B-cells. Memory B-cells are a B cell sub-type that is formed following primary infection. When a B cell is activated by recognizing a specific antigen, it proliferates to form antibody producing plasma cells and long-lived memory cells. The memory B cells are specific for the antigen that first stimulated their production. If this antigen is encountered again, memory B cells can recognize it and quickly proliferate. This forms a new generation of antibody-producing plasma cells. This means that the antibody response is much more rapid in subsequent infections than in primary infection.

6. Natural killer cells (NK cells)

NK-cells recognize and kill certain tumor and infected cells. The NK-cells possess several classes of antigen receptors including killer inhibitory receptor (KIR) and killer activating receptor (KAR). These receptors will recognize antigen associated with MHC class I molecules. Normally, cells possess MHC class I molecules and present antigens recognized as “self”. These self-antigens interact with KIR and protect the cells from NK-cells killing. But in tumor or viral infected cells, these cells do not present self-antigen. Then NK-cells will be activated and will kill tumor and infected cells.

7. Mononuclear phagocytic cells

Mononuclear phagocytic cells are originated from the multipotential stem cells in the bone marrow. These progenitor cells can differentiate into either the granulocytes or mononuclear phagocytic cells. Their differentiation depend on the microenvironment and host regulation. The mononuclear phagocytic cells then further differentiate to monocyte. Monocytes are 10-15 μm in diameter. They have bean shaped nuclei and fine granule cytoplasm containing lysosomes, phagocytic vacuoles and cytoskeleton filaments. Once they settle in tissues, these cells become macrophages. Macrophages are found in all connective tissues. They have been given special names to designate specific locations. In central nervous system, they are called the microgial cells. They are called Kupffer cells in liver and alveolar macrophages in pulmonary airway. Some mononuclear phagocytic cells may also

differentiated into other cell types such as osteoclasts. Mononuclear phagocytic cells have many functions in host inflammatory response as follows.

1). Mononuclear phagocytic cell releases many factors associated with host defense mechanism. These factors include IL-1, IL-6, IL-8, TNF-α, TGF-β, matrix metalloproteinases and prostaglandin E_2. Some of these factors were formed to play important roles in periodontal disease. (table 3.2)

2). After mononuclear phagocytic cells phagocytose bacteria, they can play role as antigen presenting cells. They present the antigen on their cell surface. The antigen then can be recognized by antigen-specific T lymphocyte. This process activates lymphocyte and made them more effective to response to antigens.

3). Mononuclear phagocytotic cells phagocytose foreign particles such as macromolecules and microorganisms. This process has shown to be enhanced by the presence of receptors on the surface of mononuclear phagocytic cell. The ability to internalize soluble substances supports the increased microbicidal. However, some bacteria such as *Mycobacterium tuberculosis, Legionella pneumophila* can invade and survive in mononuclear phagocytic cells[61,62]. It has been shown that the survival mechanism of *Mycobacterium tuberculosis* is the blockade of fusion of phagosomes and lysosome vacuoles[61] whereas of *Legionella pneumophila* is escaped to autophogosome pathway[62].

Human monocytic cell line (THP-1) has been developed and use in research. THP-1 monocytes were purchased from ATCC. THP-1 cells were isolated from the blood of a boy with acute monocytic leukemia. This cell line had Fc, complement and antibody receptors on their surfaces[63]. This cell line has the ability to produce lyzozymes, secrete cytokines, and phagocytose. These abilities play a major role in inflammation[63].

Table 3.2 Mediators of inflammatory and immune response from mononuclear phagocytic cell and their functions

Inflammatory/immune mediator	Functions
IL-1α, IL-1β	Co-stimulation of antigen presenting cells and T-cells; induction of acute phase protein synthesis
IL-6	Growth and differentiation of B and T cells; induction of acute phase protein synthesis
IL-8	Neutrophil chemotaxis
TNF-α	Pro-inflammatory
TGF-β	Anti-inflammatory
Prostaglandin E_2	Increased vascular permeability; vascular dilation, neutrophil chemotaxis; stimulation of bone resorption
Matrix metalloproteinase	Degradation of connective tissue

Cytokines

Cytokines are soluble proteins produced by specific cell types in responses to infections. They are cell products that influence activities of the other cells. They can act as cellular mediators of the inflammatory and immune responses. Cytokines also can activate T lymphocytes, B lymphocytes and mononuclear phagocytic cells for cell-mediated cytotoxicity reactions and increase antibody synthesis. Cytokines have biological activity that associated with the pathological mechanism in periodontal disease. The cytokines in diseased tissue also are correlated quantitatively with clinical parameters of periodontal disease. Past studies strongly suggest that IL-1 plays a role in periodontal tissue destruction[64, 65, 29].

IL-1

IL-1 is a potent multifunctional cytokine that appears to be the main regulation of inflammatory responses. IL-1 retains its biologic activity at picomolar (10^{-12} M) concentration. Almost every type of cells is able to produce and response to IL-1. These cells include mononuclear phagocytic cells, keratinocytes, epithelium cells endothelium cells, fibroblasts, T-lymphocytes, etc. Monocytes are considered as one of the important IL-1 producer cells in chronic inflammation. The production of IL-1 is a response of cells to stimulation by bacteria or injury. Bacterial lipopolysaccharide is a potent inducer of IL-1 production by mononuclear phagocytic cells.

There are two forms of this cytokine, IL-1α and IL-1β. Both forms are produced as 31 KDa precursor, which is cleaved by plasmin or elastase to the mature 17 KDa. IL-1α and IL-1β share only about one third of their amino-acid sequence, but their biological activities are very similar. The biological activities of this cytokine include induction of fever, stimulation of neutrophils, inhibition of bone formation, and activation of bone resorption process (table 3.3). The activation of bone resorption processed by IL-1β is very important for bone loss in periodontal disease. It is found that IL-1β is 15 folds more potent than IL-1α in stimulation of human bone resorption[65]. In addition, the elevation level of IL-1β has been demonstrated in tissue and gingival crevicular fluid from periodontitis area[29,66].

Table 3.3 Main biologic activities of IL-1

Biologic Activity
Activation of bone resorption process
Inhibition of bone formation
Stimulation of proteases and PGE_2
Stimulation of neutrophils
T-cells and B-cells proliferation
NK cells proliferation
Augmentation of IL-1 production
Augmentation of IL-2 production
Augmentation of TNF-α production

This table was modified from. Marginal periodontis and cytokines: A review of the literature. J Periodontol 1993:64:1013-1022

TNF-α

TNF is a soluble protein released from cells involveing in immunoinflammatory process, and modulates the activity of cells. TNF is secreted by numerous types of cells and acts on many cells types. TNF exists in two forms TNF-α and TNF-β, which are structurally related with 25% homology[67] but TNF-α is more related with periodontal disease than TNF-β. TNF-α is one of the important proinflammatory cytokines in periodontal tissue destruction. TNF-α is multifunctional cytokines that appears to be the main regulator of the inflammatory response. The biologic activities of this cytokine include induction of prostaglandin E_2 secretion, stimulation of neutrophils, inhibition of bone formation and activation of bone resorption process[26]. Early studies have presented that the level of TNF-α in gingival crevicular fluid has significant correlation with probing depth, gingival index and clinical attachment level[68]. In addition, the elevation level of TNF-α has been demonstrated in tissue and gingival crevicular fluid from periodontal disease patients[68]. The subcutaneous administration of TNF-α has been shown to accelerate the progression of periodontitis in rats[27]. Many cells types are capable of synthesizing TNF-α including mononuclear phagocytic cells[69]. The previous data have shown that *P. gingivalis* can activate monocytes and the result is TNF-α production[31]. However, no previous study reported the monocytes response after *P. gingivalis* survival within the monocyte cells.

Periodontal pathogens and atherosclerosis

Atherosclerosis is a focal thickening of the blood vessel. Early in the formation of atherosclerosis, monocytes in vascular system adhere to the vascular endothelium. This adherence is mediated by several adhesion molecules including intercellular adhesion molecule-1 (ICAM-1), endothelial leukocyte adhesion molecule-1 (ELAM-1) and vascular cell adhesion molecule-1 (VCAM-1)[70]. These adhesion molecules are up-regulated by pro-inflammatory cytokines, prostaglandin and bacterial lipopolysaccharide. After binding to the endothelial cells, monocytes penetrate the endothelium cell lining and migrate to arterial media. The migration process is mediated by monocyte chemotactic protein-1. In the arterial media,

monocytes may produce pro-inflammatory cytokines, *i.e.*, IL-1, TNF-α, PGE_2 and growth factor, *i.e.*, fibroblast growth factor (FGF) and platelet-derived growth factor (PDG). These pro-inflammatory cytokines and growth factors promote the atherosclerosis formation. This mechanism results in the thickening of the blood vessel wall[71]. At the same time, monocytes in the blood vessel ingest circulating low-density lipoprotein (LDL) and oxidize LDL. After this mechanism, monocytes change into foam cells, which are the characteristic of atheromatous plaque. Atheromatous plaque formation and the thickening of the blood vessel wall narrow the arterial lumen and decrease the blood flow through the vessel. Arterial thrombosis often occurs after atheromatous plaque ruptures. Plaque ruptures and tissue factors from monocytes can activate platelets and coagulation pathway. Platelet and fibrin accumulation forms thrombus. This thrombus may occlude the vessel and results in ischemic events such as angina or myocardial infraction.

Many past studies demonstrated the presence of *P. gingivalis* in the atheromatous plaque[34,35,36], the bacteria can induce monocyte chemotactic protein-1 in human endothelial cells culture[72]. This periodontal pathogen may stimulate monocytes in the atherosclerosis lesion to secrete both pro-inflammatory cytokines and growth factors that resulting in progression of artherosclerosis . In animal model, Li *et al* found that the long term systemic challenge with *P gingivalis* can accelerate atherogenic plaque progression[37]. In addition, there are many epidemiologic studies reported the positive association between periodontal disease and cardiovascular disease. Beck and colleages reported that the high levels of alveolar bone loss were significantly correlated to the predictation of coronary heart disease incidence and stroke[5]. These finings were independent of other cadiovascular risk factors. Morrison and colleagues found that the periodontal disease was significantly associated to the risk of coronary heart disease[73]. These may conclude that periodontal pathogens may relation in development and progression of atherosclerosis lesion leading to coronary vascular disease.

CHAPTER IV
MATERIALS AND METHODS

Cell culture

The monocytic cell line THP-1 (ATCC, Rockville, MD, USA) was maintained in RPMI 1640 (GIBCO, Grand Island, NY, USA) containing 10% heat inactivated fetal bovine serum (GIBCO) and incubated at 37° C in 95% humidity and 5% CO_2. Cells were fed with fresh growth media every 3 days. The cell density was determined using a hematocytometer under a phase contrast microscope and maintained between 5×10^4 to 8×10^5 cells/ml. Fresh media was replaced 24 h before each study.

Bacterial culture

P. gingivalis strain A7436 was a gift from Professor S. Offenbacher. It was originally isolated from a patient with refractory periodontitis and was characterized in the Department of Oral Biology, University of Buffalo, Buffalo, NY, USA. The bacteria were grown on anaerobic blood agar (Oxoid, Hamphire, UK), supplemented with 5% human blood (Ramathibodi Hospital, Mahidol University, Thailand) in anaerobic chamber under anaerobic conditions (AnaeroPack™ Anaero, Japan) with an atmosphere of 10% CO_2, 5% H_2 and 85% N_2 at 37° C for 7 days. Before each study, the bacteria were suspended in RPMI 1640 and the density was determined using a spectrophotometer (Milton Roy, Ivyland, PA, USA) at 660 nm. An optical density of 1.0 is equal to 10^9 CFU/ml and confirmed by colony counting on anaerobic blood agar.

E. coli ATCC 25922 (ATCC) was grown on Brain Heart Infusion agar (BHI agar) (Becton Dickinson, Sparks, MD, USA) and incubated aerobically at 37° C for 24 h. Prior to the experiments, the bacteria were suspended in RPMI 1640 and the density was determined using a spectrophotometer at 590 nm. An optical density of 0.1 is equal to 10^8 CFU/ml and confirmed by colony counting on BHI agar.

Effect of gentamycin and metronidazole on viability of THP-1

Fresh media was replaced and THP-1 (10^6 cells) grown in RPMI 1640 containing 10% heat inactivated fetal bovine serum for 24 h, then centrifuged at 137 × g for 10 min and the media was removed. The cell pellet was resuspended in 1 ml of RPMI 1640 containing 300 μg/ml gentamycin (Sigma, St.Louis, MO, USA) and 200 μg/ml metronidazole (Sigma). THP-1 cells were incubated at 37° C in 95% humidity and 5% CO_2 for 0, 10, 30, 60 and 90 min. Then, cells were stained with 0.4% trypan blue solution (Sigma). The viable cells excluded the dye while the non-viable cells absorbed trypan blue and appeared blue. The viable and non-viable cells were counted in a hemacytometer under a phase contrast microscope. Each condition was performed 5 times independently. Cells incubated in RPMI without the drugs were used as control.

Effect of gentamycin and metronidazole on viability of *P. gingivalis*

P. gingivalis strain A7436 was grown on anaerobic blood agar as previously described. Then, the bacteria was suspended in RPMI 1640. The optical density was measured at 660 nm and adjusted to 1.0. Then, 1 ml of cell suspension was centrifuged at 570 × g for 10 min and the media was removed. The bacteria pellet was resuspended in 1 ml of RPMI 1640 containing 300 μg/ml gentamycin and 200 μg/ml metronidazole and incubated at 37° C in 95% humidity and 5% CO_2 for 0, 30, 60 and 90 min. After incubation, the cell suspension was centrifuged. The pellet was then resuspended in 1 ml of RPMI 1640 and diluted 10^6 folds. Cells were then plated on anaerobic blood agar under anaerobic conditions for 7 days and colonies were counted. Each condition was performed twice independently. The bacterial culture without the antibiotics was used as a control.

Effect of ddH$_2$O on viability of *P. gingivalis*

P. gingivalis strain A7436 was grown on anaerobic blood agar as previously described. Then, the bacteria were suspended in RPMI 1640. The optical density was measured at 660 nm and adjusted to 1. Then, 1 ml of cell suspension was centrifuged at 570 × g for 10 min and the media was removed. The bacterial pellet was

resuspended in either 1 ml of phosphate buffer saline (PBS) (GIBCO) or 1 ml of sterile ddH_2O. The bacterial suspension was then incubated at 37° C in 5% CO_2 for 10, 20 and 30 min and diluted 10^6 folds. The bacterial suspension in PBS or sterile ddH_2O was plated on anaerobic blood agar. Bacteria were grown under anaerobic condition for 7 days and colonies on anaerobic blood agar were counted. Each treatment was performed twice independently.

Effect of ddH_2O on THP-1 cells lysis

THP-1 (10^6 cells) in RPMI 1640 containing 10% heat inactivated fetal bovine serum were centrifuged at 137 × g for 10 min and the media was removed. Cells were washed once with 1 ml of PBS. Then resuspended in either 1 ml of RPMI 1640 (control group) or 1 ml of sterile ddH_2O water (experimental group). The cells were incubated at 37°C in 95% humidity and 5% CO_2 for 0, 5, 10, 20, 25 and 30 min. Cell lysis was observed under the phase contrast microscope. Each condition was performed 5 times independently.

Multiplicity of infection (MOI) of *P. gingivalis* and incubation time

P. gingivalis A7436 were inoculated to THP-1 cell suspension (10^6 cells/ml) at multiplicity of infection (MOI) of 1:10, 1:100 and 1:1000 and incubated at 37° C in 95% humidity and 5% CO_2 for 60, 90 and 120 min. After incubation, cells were centrifuged at 137 × g for 10 min at 4° C, resuspended in RPMI 1640 containing 300 μg/ml gentamycin and 200 μg/ml metronidazole and incubated for 1 h. THP-1 cells were collected by centrifugation and washed once as stated above. Then, 1 ml of sterile ddH_2O was added and left incubated for 20 min at room temperature (RT) in order to allow cell lysis. The lysate was diluted with PBS 100 folds, then plated on anaerobic blood agar under anaerobic condition for 7 days. The colonies forming unit (CFU) of bacteria were counted. The cell supernatant containing bacteria was also cultured on anaerobic blood agar under anaerobic condition to ensure the killing ability of antibiotics. Each condition was performed 4 times independently.

Bacterial localization in THP-1 cell

P. gingivalis A7436 were inoculated to THP-1 cell suspension (10^6 cells/ml) at MOI 1:100 and incubated at 37° C in 95% humidity and 5% CO_2 for 90 min. After incubation, cells were centrifuged at 137 × g for 10 min at 4° C. Cells were resuspended and incubated for 1 h in RPMI 1640 containing 300 μg/ml gentamycin and 200 μg/ml metronidazole. Cells were then centrifuged and resuspended in 1 ml of RPMI 1640 containing 10% heat inactivated fetal bovine serum and further incubated at 37° C in 95% humidity and 5% CO_2 for 0, 4 and 8 h. At the end of each time point, cells were harvested and washed once in PBS as stated above. THP-1 cells were fixed in 1 ml of phosphate buffered (PB) containing 4% glutaraldehyde pH 7.4 for 30 min at RT. After fixation, cells were centrifuged and washed 3 times with PB at RT. Then, the cells were fixed in 1 ml of PB containing 2% osmium teroxide for 45 min, washed 3 times with distilled water, stained with 0.5 ml of 2% uranyl acetate for 30 min at RT and dehydrated in a graded series of ethanol (70%-100%), 10 min each step, followed by propylene oxide twice, 20 min each time. Then, the cells were infiltrated with a mixture of propylene oxide and epoxy resin at the ratio of 1: 1 at 37° C for 30 min and later changed to the resin at 37° C for 2 h. The resin was polymerized in an oven at 70° C overnight. After polymerization, ultrathin sections (60-90 nm) were obtained using ultramicrotome. The thin sections were collected and mounted on the copper grids and stained with 5% uranyl acetate in 70% methanol following by lead citrate for 30 min each. Finally, the specimens were examined under a transmission electron microscope (JEOL JEM 1230, Japan). For studying the extracellular bacterial attachment, the infected THP-1 cells without antibiotics treatment were used.

Survival *of P. gingivalis* in THP-1

THP-1 cells and bacteria were prepared as previously described for bacterial localization in THP-1 cell experiments. After incubation in RPMI 1640, THP-1 cells were collected by centrifugation at 137 × g for 10 min at 4° C and washed once with 1 ml of PBS. Then, 1 ml of sterile ddH_2O was added and left incubated for 20 min at room temperature (RT) in order to allow cell lysis. The lysate was diluted with PBS 100 folds. The viability of intracellular bacteria was observed by plating the bacteria

on anaerobic blood agar under anaerobic condition for 7 days after cell lysis with sterile ddH_2O. The colonies of bacteria on anaerobic blood agar were counted. Each condition was performed 9 times independently. Infection of *E. coli* ATCC 25922 to THP-1 cell was performed similarly to infection of *P. gingivalis* and used as a control for survival ability of internal bacteria in THP-1 cells.

IL-1β and TNF-α production after invasion THP-1 cells by *P. gingivalis*.

THP-1 cell preparation

P. gingivalis A7436 were inoculated to THP-1 cell suspension (10^6 cells/ml) at MOI 1:100 and incubated at 37° C in 95% humidity and 5% CO_2 for 90 min. After incubation, cells were centrifuged at 137 × g for 10 min at 4° C, resuspended and incubated for 1 h in RPMI 1640 containing 300 μg/ml gentamycin and 200 μg/ml metronidazole. After exposing to antibiotics, media and pellet were separated by centrifugation and stored at -80° C until used or the cells were resuspended in 1 ml of RPMI 1640 containing 10% heat inactivated fetal bovine serum and further incubated at 37° C in 95 % humidity and 5 % CO_2 for 24 h. After incubation, THP-1 cells were centrifuged at 137 × g for 10 min at 4° C and the media and pellet were separated and stored at -80° C until used. The media was used for protein detection using ELISA assay kit and pellet was used for mRNA expression by RT-PCR. Each treatment was performed 10 times. The supernate and pellet of uninfected cells were used as negative controls. Positive control was performed similar to the experimental group but without adding antibiotics.

RT-PCR for IL-1 β and TNF-α mRNA detection

The extraction of IL-1 β and TNF-α mRNA was performed using Trizol reagent (Invitrogen, Carlsbad, CA, USA) at RT. Briefly, the pellets of treated THP-1 cells were mixed with Trizol reagent and left 5 min at RT. After incubation, 0.2 ml of chloroform was added to the mixture then shaken for 15 sec then left 2-15 min at RT. The sample was centrifuged at 12000 × g for 15 min at 4° C. The aqueous phase was transferred to the new tube. Isopropanol, 0.5 ml, was added and the sample was left

for 5-10 min at RT, then sample was centrifuged at 12000 × g for 10 min at RT. The supernatant was removed and 1 ml of 75% ethanol was added to RNA pellet and vortexed for 1 min. The sample was again centrifuged at 7500 × g 5 min at 4° C. Ethanol was removed as much as possible and the RNA pellet was air dried for 5-10 min. The Rnase and Dnase free water (Sigma, St.Louis, MO, USA) was added and mixed by pipeting and incubated at 55-60° C for 10 min. The sample was cooled on ice and spined for 10 sec. The RNA sample was quantitated spectrophotometrically at 260/280 nm. In reverse transcription process, 2 μg of RNA was made up to 8 μl in Rnase and Dnase free water and heated at 65° C for 10 min, then chilled on ice. The RNA solution was mixed with first-strand cDNA synthesis kit (5 μl Bulk first strand mix, 1 μl DTT and 1 μl random primer) (Amersham Biosciences, Piscataway, NJ, USA). The sample was incubated for 1 h at 37° C and then was heated at 90° C for 5 min and stored at -20° C.

For determining the linear range for polymerase chain reaction (PCR) cycle, 1 μl of cDNA was added to master mix of PCR kit (10X buffer 2.5 μl + 5X q-solution 5 μl + 10mM dNTP 0.5 μl + Hot star Taq DNA Polymerase 0.25 μl and Rnase and Dnase free water 13.75 μl) (Invitrogen, Carlsbad, CL, USA), containing 1μl of specific primer (IL-1β or TNF-α) (Ambion, Austin, TX, USA) and programmed the PCR machine for 36 cycles (1 cycle was heat at 94° C for 30 s, 57° C for 1 min and 72° C for 1 min). The sample was removed at 20, 22, 24, 26, 28, 30, 32, 34 and 36 cycles and placed on ice. The products were electrophoresed through 2% agarose gel, stained with 0.2 μg/ml ethidium bromide in Tris-borate/EDTA buffer and visualized by ultraviolet illumination. Pictures were taken and analyzed with Lumi-Imager Analyst machine and Lumi-Imager software version 3.1 (Roche, Indianapolis, IN, USA). The band intensity was plotted on the y-axis and the cycle number on the x-axis. PCR condition at 30 cycles was in the middle of detectable linear range in both target groups and was selected for further studies.

To determine the optimal ratio of 18s primer: competimer, the 1 μl of cDNA was added to master mix with specific primer (IL-1β or TNF-α) 1 μl +18s primer:competimer (1:9, 2:8 and 3:7) 1 μl. PCR cycle was operated for 30 cycles. PCR products were analyzed as stated above. The lane of the ratio 3:7 of 18S

primer:competimer of IL-1β group and 2:8 of TNF-α group were most similar to the level of gene specific product.

For the PCR step, DNA synthesis performed by using primer and competimer of gene specific relative RT-PCR for human IL-1β, TNF-α kit (Ambion, Austin, TX, USA) and master mix of PCR kit (Invitrogen, Carlsbad, CL, USA) and PCR cycle was operated for 30 cycles. PCR products were analyzed as stated above. The band intensity of IL-1β and TNF-α were compared to 18s rRNA band and the amount of IL-1β and TNF-α mRNA was estimated.

IL-1β and TNF-α by ELISA

The supernatant of treated THP-1 cells were assayed for IL-1β and TNF-α by using an enzyme-linked immunosorbent assay (ELISA) kits (R&D systems, Minneapolis, Minn, USA). The principle of this assay was the quantitative sandwich enzyme immunoassay technique in which a monoclonal antibody specific for IL-1β and TNF-α have been pre-coated onto a microplate. The 200μl of standard or sample was pipetted into the wells and incubated for 2 h at RT in order to let IL-1β and TNF-α bound to the immobilized antibody. After washing 3 times with washing buffer to wash away unbound substances, 200 μl of enzyme-linked polyclonal antibody specific for either IL-1β or TNF-α were added to the wells and incubated for 1 h at RT. Following by washing 3 times with washing buffer to remove the unbound antibody-enzyme reagent, then 200 μl of substrate solution was added to the wells for 20 min at RT and color was developed in proportion to the amount of IL-1β and TNF-α bound in the initial step. The color development was stopped by 50 μl of stop solution and the intensity of the color was measured at 450 nm in a spectrophotometer (Milton Roy, Ivyland, PA, USA). Duplicate reading for each standard, control and samples were taken and averaged. The average absorbance for each duplicate set of standard, control and samples was calculated. Results expressed as picrograms of IL-1β or TNF-α per milliliter of supernatant fluid.

Monocytes migration

Evaluation of MCP-1 concentration and migration time

THP-1 cells and bacteria were prepared as previously described for the invasion and survival of *P. gingivalis* experiments. After antibiotics exposure, media was removed and THP-1 cells were resuspended in RPMI 1640 containing 10% heat inactivated fetal bovine serum. The THP-1 cells were adjusted to 10^6 cells/ml. The 2.6 ml of RPMI 1640 containing 10% heat inactivated fetal bovine serum and monocyte chemotactic protein-1 (MCP-1) (Sigma) at concentration 10, 20 and 30 ng/ml was added to the lower compartment of transwell (polycarbonate membrane, 6 well size, pore size 8 μm, Costar Cooperation, Cambridge, MA, USA). Then the 1.5 ml cell suspension was added to the upper compartment of transwell and incubated at 37°C in 95% humidity and 5% CO_2 for 1, 2 and 3 h. At the end of each time point, THP-1 cells in the lower compartment of transwell were counted in hemacytometer under phase contrast microscope. Each condition was performed 4 times independently. The THP-1 without infected with *P. gingivalis* was used as a control.

Survival of P. gingivalis in monocytes after migration

THP-1 cells and bacteria were prepared as previously described for the invasion and survival of *P. gingivalis* experiments. After antibiotics exposure, media was removed by centrifugation at 137 × g for 10 min at 4° C and THP-1 cells were resuspended in RPMI 1640 containing 10% heat inactivated fetal bovine serum. The cells were adjusted to the concentration of 10^6 cells/ml. The 2.6 ml of RPMI 1640 containing 10% heat inactivated fetal bovine serum and MCP-1 at the concentration of 10 ng/ml was added to the lower compartment of transwell. Then, 1.5 ml of THP-1 cell suspension was plated into the upper compartment of transwell and incubated at 37° C in 95% humidity and 5% CO_2 for 2 h. At the end of incubation time, THP-1 cells in lower compartment of transwell were collected and counted in hematocytometer under phase contrast microscope. The media was removed by centrifugation at 137 × g for 10 min at 4° C. THP-1 cells were washed once with PBS. Then cells were lysed by incubation in sterile double distilled water at 37° C in 95 % humidity and 5% CO_2 for 20 min, diluted 10 folds with PBS and plated on anaerobic blood agar. Bacteria were grown under anaerobic condition for 7 days and bacterial colonies were counted. Each condition was performed 9 times independently. The

media without MCP-1 was considered as the negative control. THP-1 cells without incubation with bacteria were used as the positive control.

CHAPTER V
RESULTS

Effects of gentamycin and metronidazole on viability of THP-1

The effects of gentamycin and metronidazole on THP-1 were tested. Approximately 10^6 THP-1 cells were suspended in RPMI 1640 with or without 300 μg/ml gentamycin and 200 μg/ml metronidazole. The viability of THP-1 cells culture in RPMI 1640 containing 300 μg/ml gentamycin and 200 μg/ml metronidazole was determined. The percentage of viable THP-1 cells in control groups without antibiotics at 0, 10, 30, 60 and 90 min were 100%, 99.98%, 99.77%, 100% and 99.64%, respectively and those in experimental groups with antibiotics were 100%, 100%, 99.95%, 99.77% and 100%, respectively (Table 5.1). At each time point the percentage of THP-1 viability in both groups were not statistically different. In addition the morphology changing of THP-1 cells under the phase contrast microscope was not observed in both groups. This result showed that these antibiotics had no effect on viability of THP-1 cell (Figure 5.1).

Table 5.1 Effects of gentamycin and metronidazole on viability of THP-1 (n=5).

Incubation time (min)	% viability (Mean±SD)	
	In RPMI 1640	RPMI1640 with antibiotics
0	100±1.53	100±5.50
0	99.98±4.75	100±2.75
30	99.77±3.70	99.95±3.45
60	100±5.27	99.77±5.75
90	99.64±7.45	100±4.52

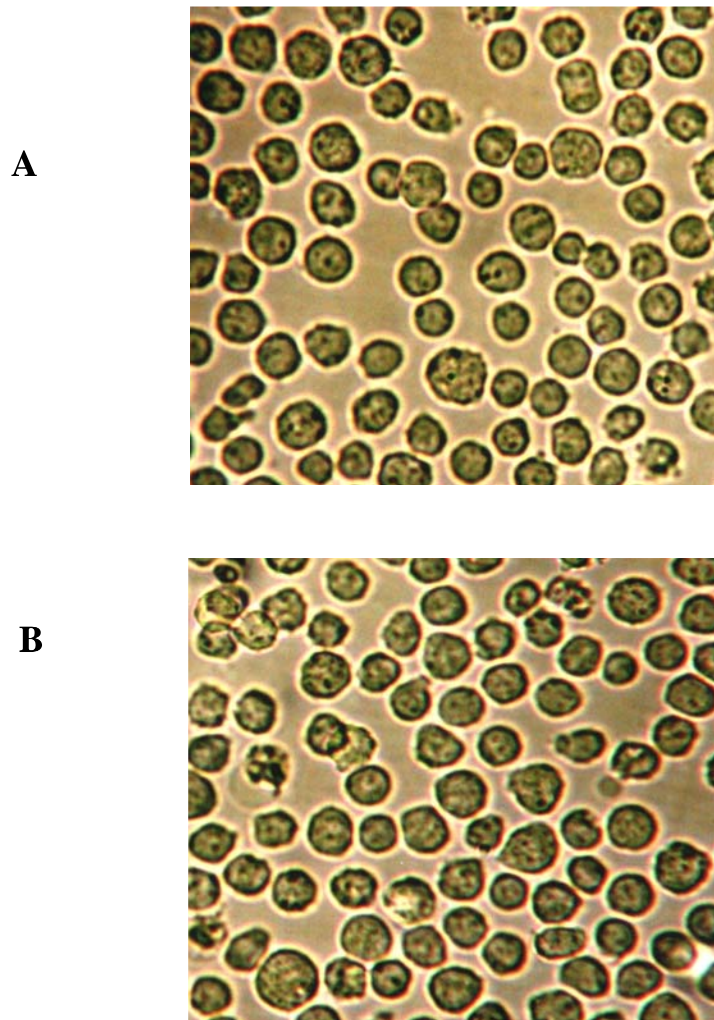

Figure 5.1 The morphology of THP-1 cells under the phase contrast microscope (X40), (A) cultured in RPMI 1640 with out the antibiotics, (B) after cultured in RPMI 1640 containing 300 μg/ml gentamycin and 200 μg/ml metronidazole for 90 min.

Effects of gentamycin and metronidazole on viability of *P. gingivalis*

The effective concentration of both gentamycin and metronidazole were initially investigated. The concentrations of 300 μg/ml gentamycin and 200 μg/ml metronidazole in RPMI 1640 were previously reported to be able to completely kill *P. gingivalis* 33277. Therefore, such conditions were selected for this experiment. After incubation of 10^9 cells of *P. gingivalis* in RPMI 1640 with or without the combination of antibiotics, for 0, 30, 60, and 90 min, bacterial cells were plated onto the anaerobic blood agar to determine viability of bacteria. At each time point the percentage of CFU was compared to the inoculum. Table 5.2 showed that in the present of antibiotics, no bacterial colony was found after incubation for 30 min. Therefore, the 300 μg/ml gentamycin and 200 μg/ml metronidazole in RPMI 1640 were able to completely kill 10^9 cells of *P. gingivalis* cells after 30 min incubation.

Table 5.2 Effect of gentamycin and metronidazole on viability of *P. gingivalis* (n=2).

Incubation time (min)	% CFU	
	RPMI 1640 with antibiotics	RPMI 1640
0	100	100
30	0	98.89
60	0	99.71
90	0	98.13

Effect of ddH_2O on viability of *P. gingivalis*

The effect of ddH_2O on viability of *P. gingivalis* was tested. The bacterial pellet was resuspension in either PBS or ddH_2O and incubated for 0, 10, 20 and 30 min. Then, the bacterial suspension was diluted and plated on anaerobic blood agar under anaerobic condition of 7 day. The number of bacterial colonies were determined. At each time point the percentage of CFU was compared to the inoculum. The percentages of CFU in PBS groups at 0, 10, 20 and 30 min were 100%, 98.64%, 99.90% and 98.85, respectively and those in ddH2O groups were 100%, 95.59%, 100% and 95.20%, respectively (Table 5.3). At each time point the percentage of CFU in each group were not statistically different. Therefore, ddH_2O did not affect on *P. gingivalis* viability.

Table 5.3 Effect of ddH_2O on viablility of *P. gingivalis* (n=2).

Incubation time (min)	% CFU	
	PBS	ddH_2O
0	100	100
10	98.64	95.59
20	99.90	100
30	98.85	95.20

Effect of ddH_2O on THP-1 cells lysis

The suitable lysis time of THP-1 cells in sterile ddH_2O was also determined. Approximately 10^6 cells of THP-1 were diluted in 1 ml of either sterile ddH_2O or RPMI 1640 as control. At indicated time, intact cells were counted using hematocytometer. As shown in Table 5.4, almost THP-1 cells were lyzed in sterile ddH_2O after 20 min incubation whereas the number of intact THP-1 cells in RPMI 1640 remains constant throughout the experiment. The acceptable cell lysis was 98%. Therefore, the proper time for lysis of the THP-1 cells in sterile ddH_2O was 20 min.

Table 5.4 The number of THP-1 cells after incubation in RPMI 1640 and ddH_2O (n=5).

Incubation time (min)	% of intact THP-1 cell (Mean±SD)	
	In RPMI	ddH_2O
0	100±5.75	100±5.20
5	96.47±3.50	7.93±1.47
10	95.88±4.25	5.05±0.92
15	96.47±4.52	2.70±0.50
20	97.65±5.31	0.18±0.05
25	96.94±5.25	0.18±0.04
30	96.94±2.70	0.18±0.02

Multiplicity of infection (MOI) of *P. gingivalis* and incubation time

The survival of *P. gingivalis* in THP-1 with various MOIs (1:10, 1:100 and 1:1000) and incubation times (60, 90 and 120 min) was studied. After 60, 90 and 120 min of incubation, it was found that bacteria at MOI 1:100 were able to survive at all incubation times tested. Furthermore at the MOI 1:100, the bacteria showed the maximum survival efficiency which was highest at 90 min (Table 5.5). From the statistical analysis, the survival efficiency of MOI 1:100 and incubated for 90 min was significantly higher than the other groups. For the following studies, the MOI 1:100 and 90 min incubation time were used.

Table 5.5 The effect of MOI and incubation time on bacterial survival efficiency of *P. gingivalis* in THP-1 cells (n=4).

	% bacterial survival		
Incubation time (min)	MOI =1:10	MOI =1:100	MOI =1:1000
60	.0050±.0008	.0075±.0013	.0060±.0008
90	.0058±.0010	.0108±.0010*	.0078±.0010
120	.0063±.0010	.0098±.0017	.0075±.0058

Bacterial survival was calculated from CFU recovered intracellularly as a percentage of total bacteria inoculated with THP-1 cells.

* The highest bacterial survival efficiency

Bacterial localization in THP-1 cell

Transmission electron micrograph showed that after incubation THP-1 cells with *P. gingivalis* for 90 min, the infected THP-1 cells were found. Figure 5.2 showed the THP-1 cell with an irregular nucleus, euchromatin in the middle and heterochromatin along the periphery of the nucleus. The nucleolus was observed to be intact and eccentrically located in the nucleus. The cytoplasm contained mitochondria, rough endoplasmic reticulum and ribosomes. The cell surface of infected THP-1 cells also showed pseudopodia (Figure 5.2, 5.3 and 5.4). *P. gingivalis* appeared to be aggregated and adhered at the certain area of THP-1 cells (Figure 5.4) and at these adhesion area, the cell membrane of THP-1 presented with the electron dense (Figure 5.3).

After 90 min incubation and treating THP-1 cells with antibiotics, *P. gingivalis* was clearly visible within the cytoplasm of THP-1 cells, and *P. gingivalis* did not find outside the THP-1 cell. The bacteria seemed to survive in 2 forms in the cytoplasm: one of which the bacteria were closely surrounded by the thin membranous vacuole, cytoplasm, ground substance and rough endoplasmic reticulum (Figure 5.5), another form was presented in a large vacuole. (Figure 5.6)

After treating THP-1 cells with antibiotics and further incubated in RPMI1640 for 4 h, transmission electron microscopy of THP-1 cells showed *P. gingivalis*, which were surrounded with close and thin membrane, ground substances and rough endoplasmic reticulum in cytoplasm of THP-1 cells (Figure 5.7) moreover some bacteria in this location appeared to be under gone cell division (Figure 5.8). In addition, in cytoplasm of some THP-1 cells revealed numerous vacuoles that contained unidentified materials debris (Figure 5.9). The other organs of THP-1 cell, *i.e*. mitochondria, rough endoplasmic reticulum, ribosome and nucleus were also observed.

After 8 h of incubation, the internal bacteria were still present in THP-1 cytoplasm (Figure 5.10). At this stage, the vacuole containing unidentified materials debris was absent.

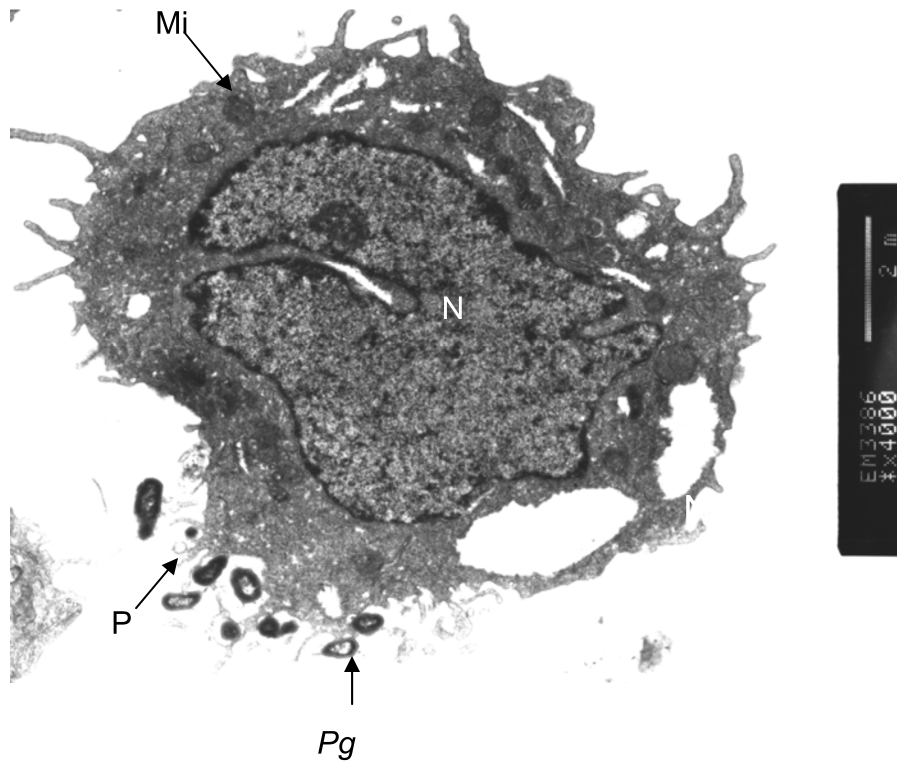

Figure 5.2 Transmission electron microscopy (TEM) of infected THP-1 cells. THP-1 cells presented with an irregular nucleus (N) with eccentric nucleolus, mitochondria (Mi) and pseudopodia (P) at the cell surface. *P. gingivalis* (*Pg*) attached to the cell surface of the THP-1 cell.

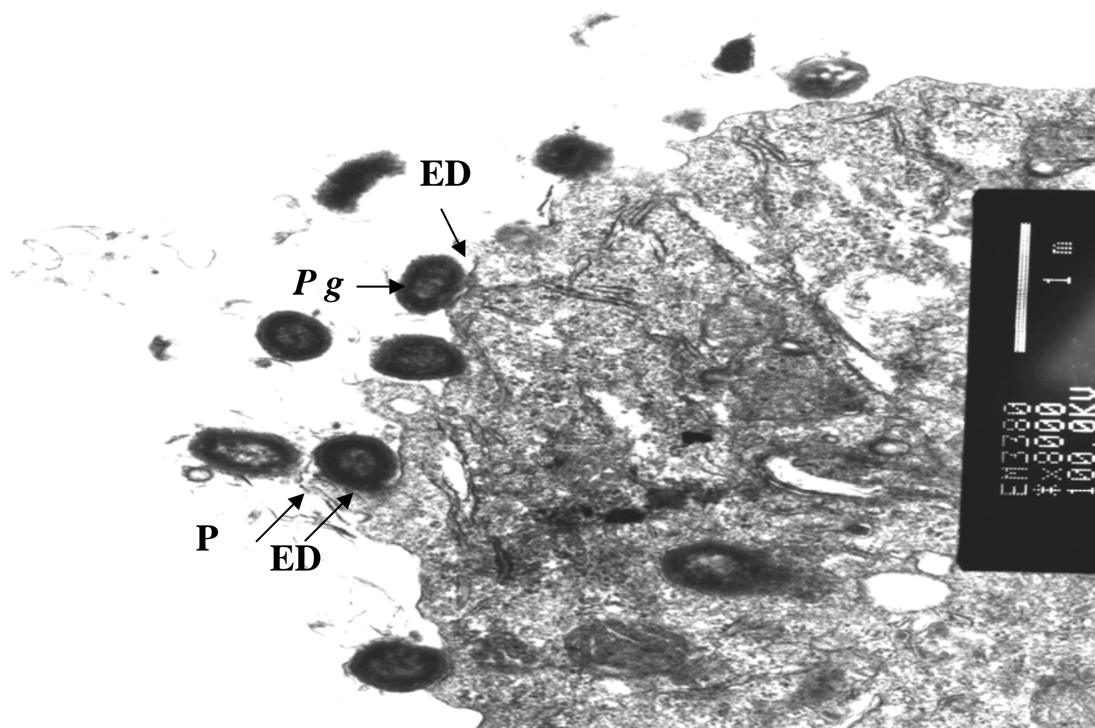

Figure 5.3 The cell surface of THP-1 presented with the electron dense (ED) and pseudopodia (P).

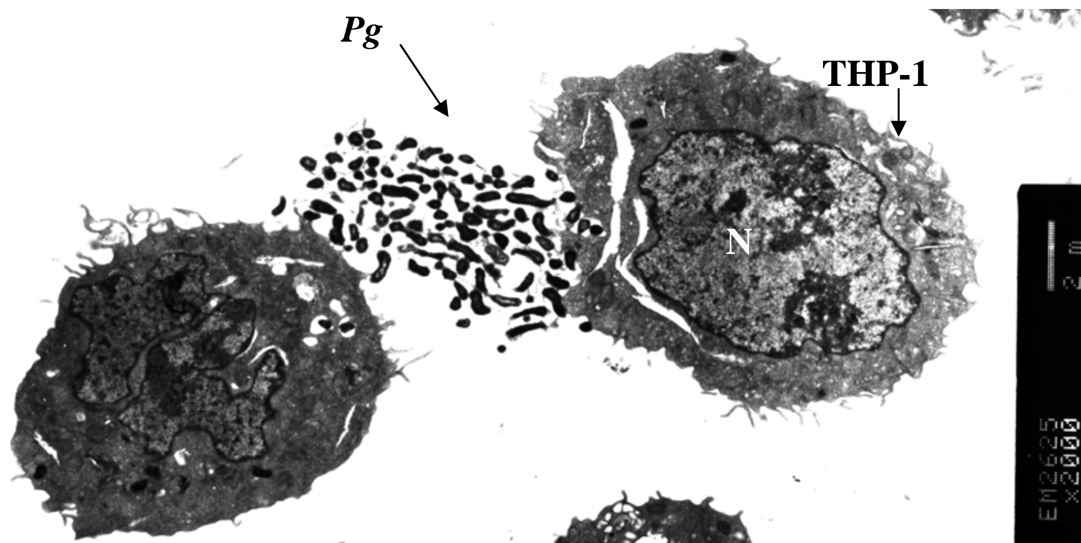

Figure 5.4 This TEM picture presented *P. gingivalis (Pg*) aggregated and contacted at certain area of THP-1 membrane.

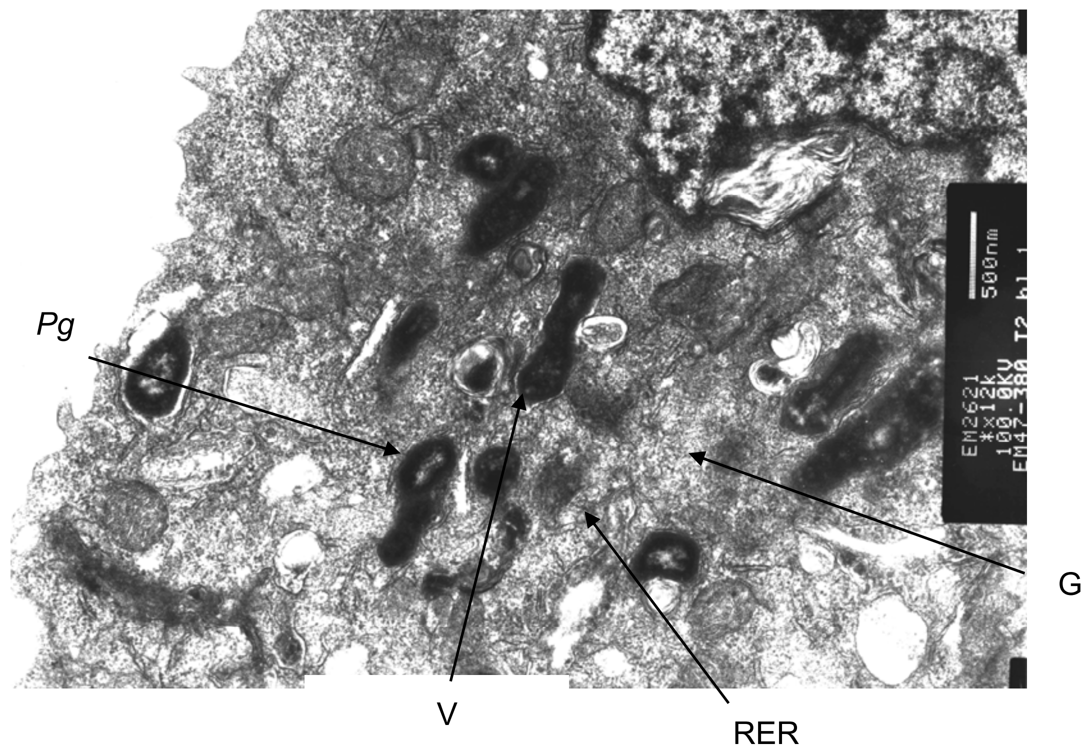

Figure 5.5 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics. Intracellular *P. gingivalis* (*Pg*) was inside the thin membranous vacuole (V), associated with cytoplasm ground substance (G) and rough endoplasmic reticulum (RER).

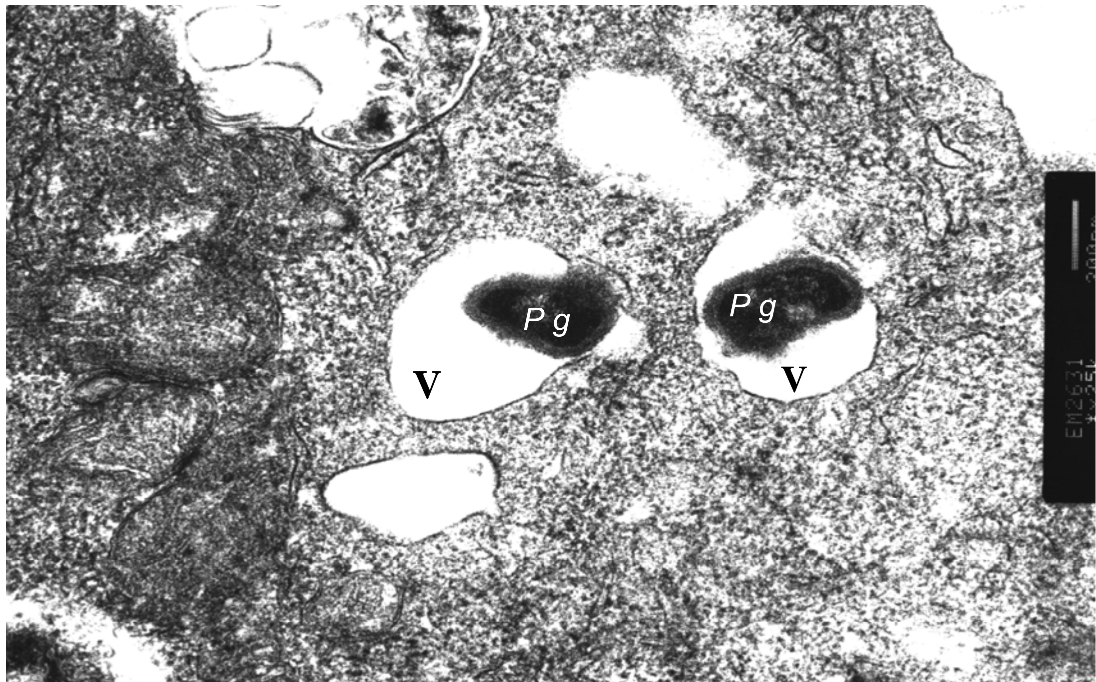

Figure 5.6 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics. Internal *P. gingivalis* (*Pg*) was found inside a large vacuole (V).

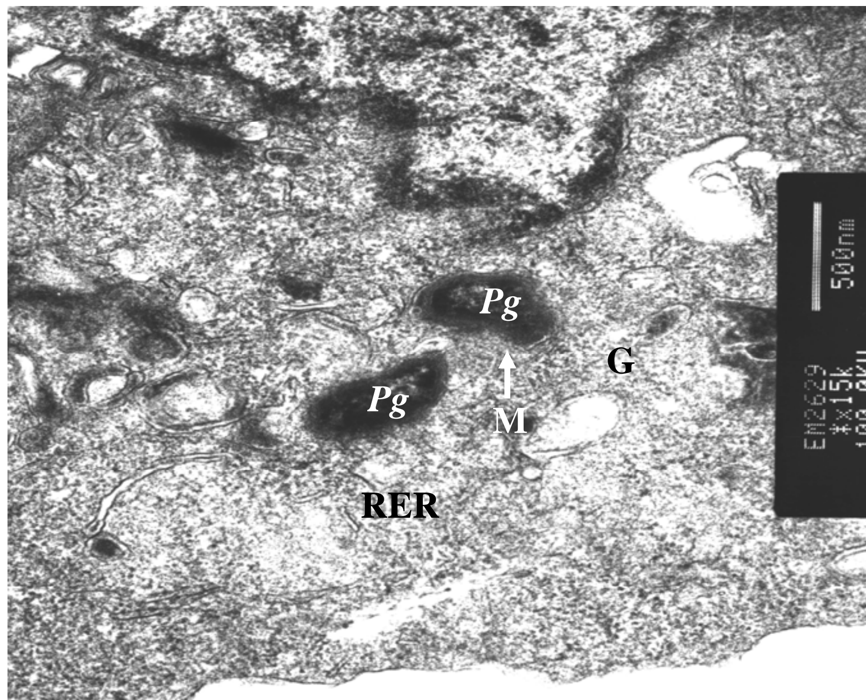

Figure 5.7 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics in RPMI 1640 for 4 h. Internal *P. gingivalis* (*Pg*) was surrounded by close and thin membrane (M), associated with cytoplasmic ground substance (G) and rough endoplasmic reticulum (RER).

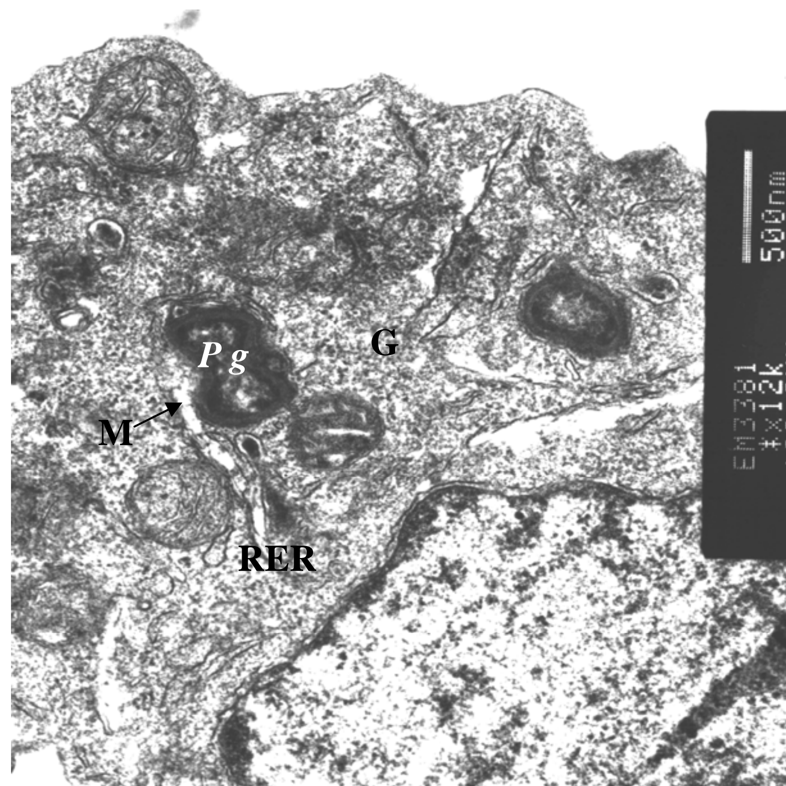

Figure 5.8 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics and incubation in RPMI 1640 for 4 h. The bacteria (*Pg*) that appeared to be under gone cell division were within close and thin membranous (M), cytoplasm ground substance (G) and rough endoplasmic reticulum (RER).

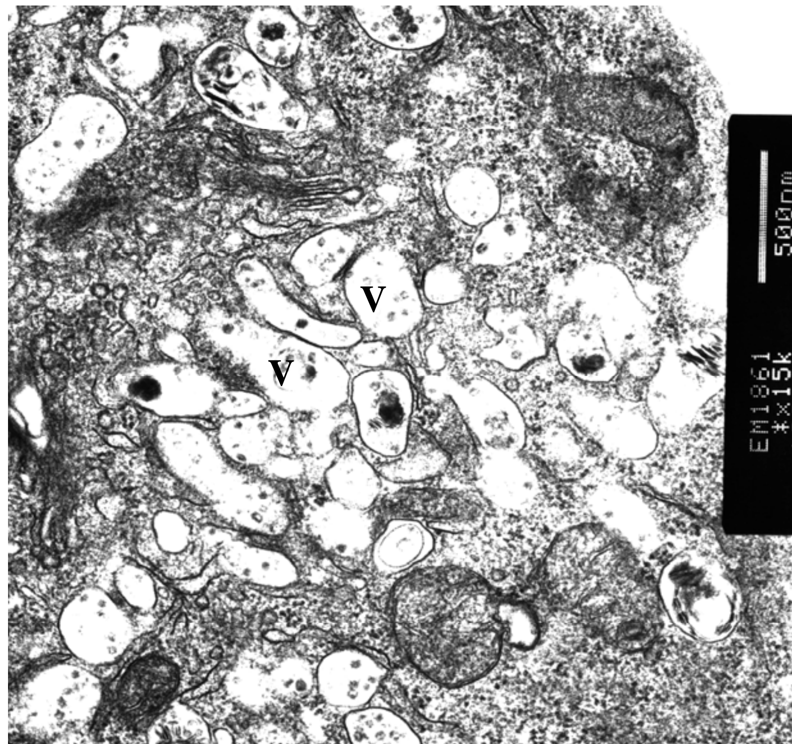

Figure 5.9 Transmission electron microscopy (TEM) of infected THP-1 cells after treating with antibiotics and incubation in RPMI 1640 for 4 h. The vacuole (V) contained unidentified materials debris.

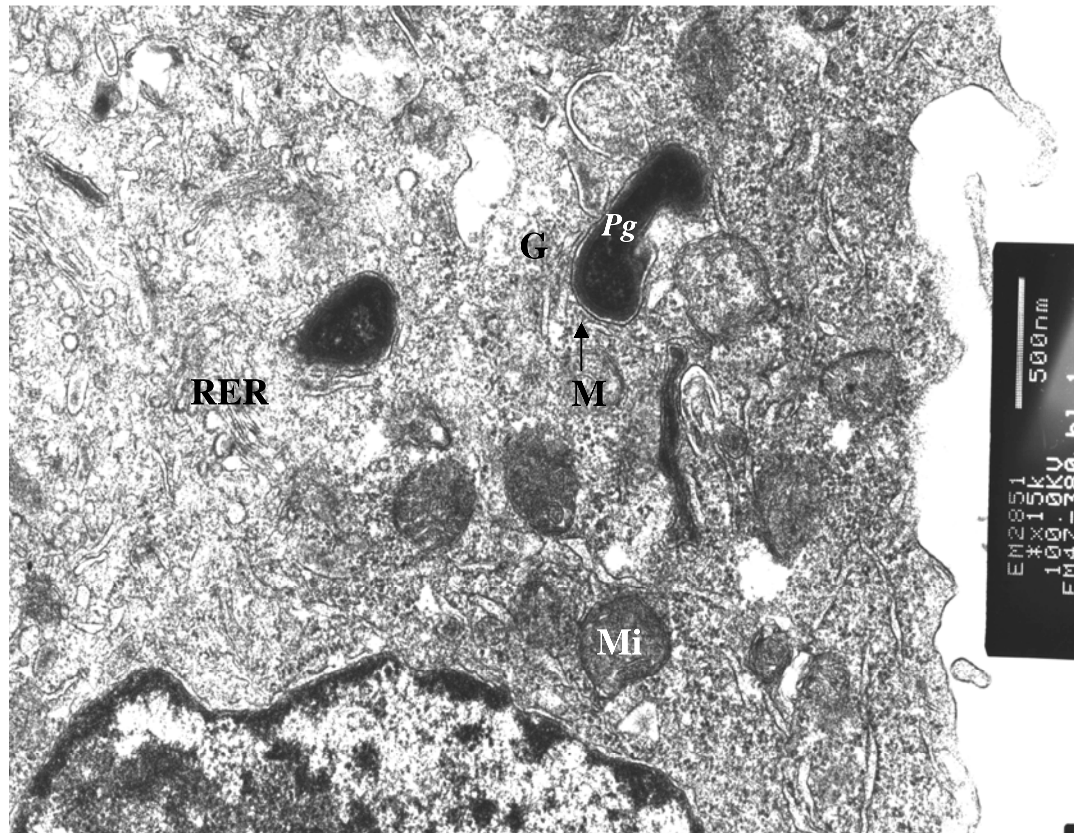

Figure 5.10 Transmission electron microscopy (TEM) of infected THP-1 cells after incubation in RPMI 1640 for 8 h. The internal bacteria (*Pg*) were presented in THP-1 cytoplasm. It was surrounded by close and thin membrane (M), associated with ground substance (G) and rough endoplasmic reticulum (RER).

Survival of *P. gingivalis* in THP-1

The viability of internal bacteria was observed by culturing bacteria on anaerobic blood agar under anaerobic condition after lysis of THP-1 with sterile ddH_2O. The bacterial colonies of *P. gingivalis* on blood agar were counted. The *P. gingivalis* survival efficiency after incubation for 0, 4 and 8 h were 0.0112%, 0.0122% and 0.0114%, respectively (Figure 5.11). These survival efficiency of *P. gingivalis* in THP-1 cells at various times showed no significantly different ($P<0.05$).

The survival efficiency of *E. coli* in THP-1 was 0.004%, 0.0003%, and 0% (Figure 5.12). The survival efficiency decreased with increased incubation time and was undetectable when incubated up to 8 h. In contrast with *P. gingivalis*, *E. coli* survival efficiency was significantly different when compared with differentiation incubation time ($P<0.05$). This suggested that THP-1 can eliminate *E. coli* on the other hand it can not kill *P. gingivalis* under this experimental condition.

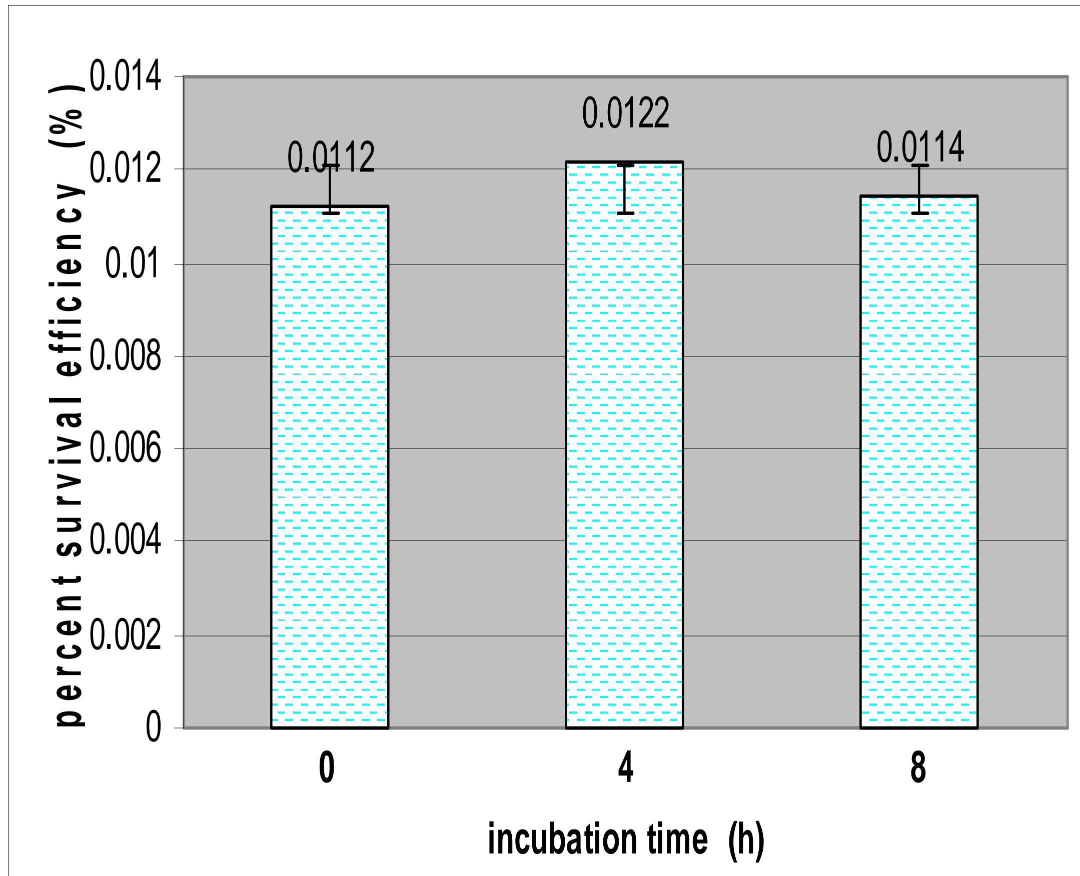

Figure 5.11 The survival efficiency of *P. gingivalis*

The survival efficiency was calculated from CFU of *P. gingivalis* recovered intracellular THP-1 cells as a percentage of total bacteria inoculated and incubated with THP-1 cells for 0, 4, 8 h. These survival efficiency of *P. gingivalis* in THP-1 cells were not significantly different when compared among each others incubation time group ($P<0.05$) (n=9).

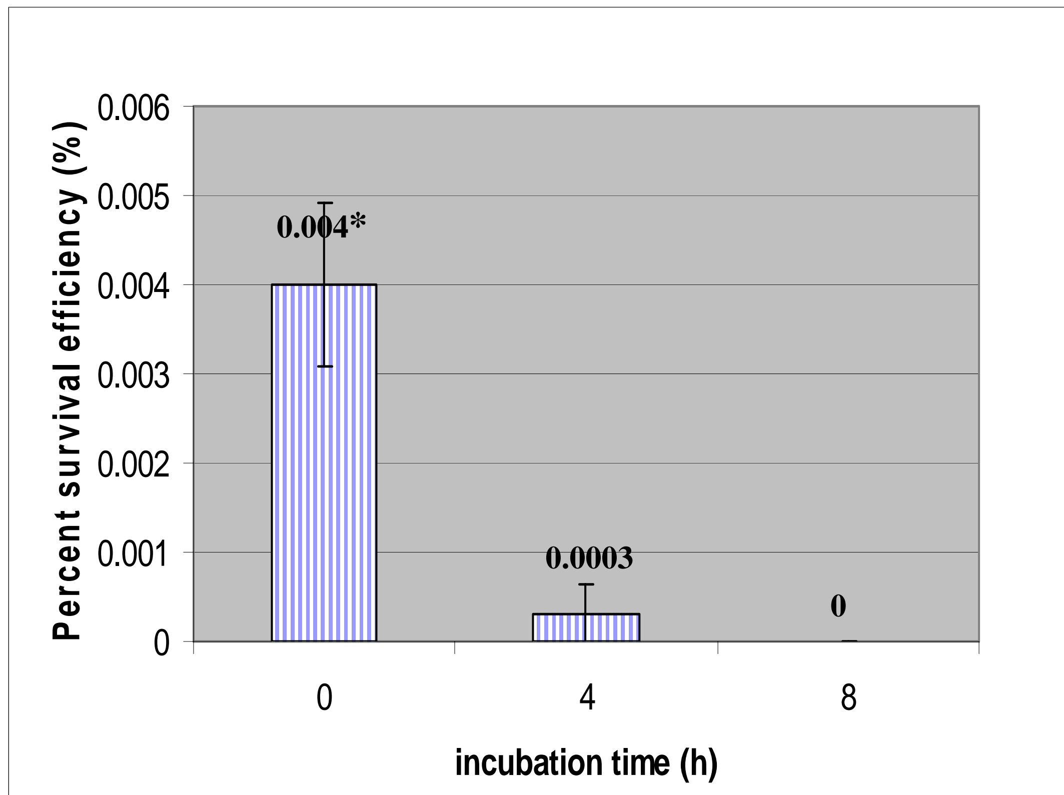

Figure 5.12 Percent survival efficiency of *E. coli* in THP-1 cell following antibiotic treatment and varied in incubation time.

* Significantly different when compared with 4 and 8 h incubation time ($p<0.05$).

IL-1β and TNF-α production after invasion THP-1 cells by *P. gingivalis*

IL-1β and TNF-α expression at both mRNA level and protein level were studied. Using RT-PCR, neither IL-1β nor TNF-α mRNA were detected in unstimulated THP-1 while after stimulation with *P. gingivalis*, it was found that the level of both IL-1β and TNF-α were significantly increased as shown in Figure 5.13 and 5.14. The ratio of band intensity between IL-1β to 18s rRNA was 0.92 in positive control group while in experimental group treated with antibiotics for 1 h and treated with antibiotics for 1 h then left grown in media for 24 h, the ratio of band intensity between IL-1β to 18s rRNA were 0.94 and 0.85 respectively. The result showed that the level of IL-1β mRNA expression was not significantly changed after 24 h of incubation in media. The ratio of band intensity between TNF-α to 18s rRNA in positive control group, experimental group treated with antibiotics for 1 h and treated with antibiotics for 1 h and left grown in media for 24 h were 0.93, 0.90 and 0.54 respectively. The result showed that when THP-1 cells were treated with antibiotics and left grown in media for 24 h, the level of TNF-α mRNA expression was significantly reduced.

The normal level of IL-1β protein synthesized from THP-1 cells was 2.85±2.77 pg/ml. THP-1 cells infected with *P. gingivalis* without antibiotics (300 μg/ml gentamycin, 200 μg/ml metronidazole) treatment showed significant stimulation of IL-1β production with the mean concentration of 186.43±6.58 pg/ml. The mean concentration of IL-1β produced from THP-1 cells after infected with *P. gingivalis* then treated with antibiotics was 189.47±6.02 pg/ml. When further incubated with RPMI 1640 containing 10% heat in activated FBS for 24 h it was found that mean concentration of IL-1β was significantly increased to 224.39±10.01 pg/ml as shown in Table 5.6 and Figure 5.15.

In the absence of *P. gingivalis*, the mean concentration of TNF-α produced by THP-1 was 2.65±2.63 pg/ml. The production level of this cytokines was found to be increased when monocytes were infected with *P. gingivalis*. The mean concentration was increased to 881.7±48.85 pg/ml. When THP-1 cells were infected with *P. gingivalis* then treated with antibiotics similar level of stimulation was found. The mean concentration of TNF-α was 884.1±35.29 pg/ml. When further incubated with

RPMI 1640 containing 10% heat inactivated FBS for 24 h, it was found that the mean concentration of TNF-α was significantly reduced to 215.90±34.13 pg/ml as shown in Table 5.6 and Figure 5.16.

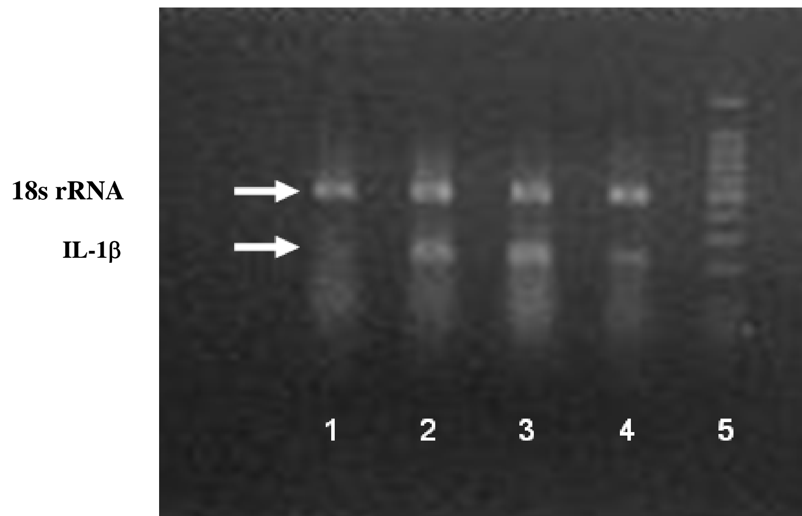

Figure 5.13 The expression of house keeping gene 18s rRNA and the IL-1β expression in THP-1 cells after *P. gingivalis* infection (n=4).

Lane 1 Negative control group: THP-1 in the absence of *P. gingivalis* and antibiotics.
Lane 2 Positive control group: THP-1 infected with *P. gingivalis* but did not treat with antibiotics.
Lane 3 Experimental group: THP-1 infected with *P. gingivalis* and treated with antibiotics.
Lane 4 Experimental group: THP-1 infected with *P. gingivalis*, treated with antibiotics and incubated in RPMI 1640 containing 10% heat inactivated FBS for 24 h.
Lane 5 Molecular DNA ladder.

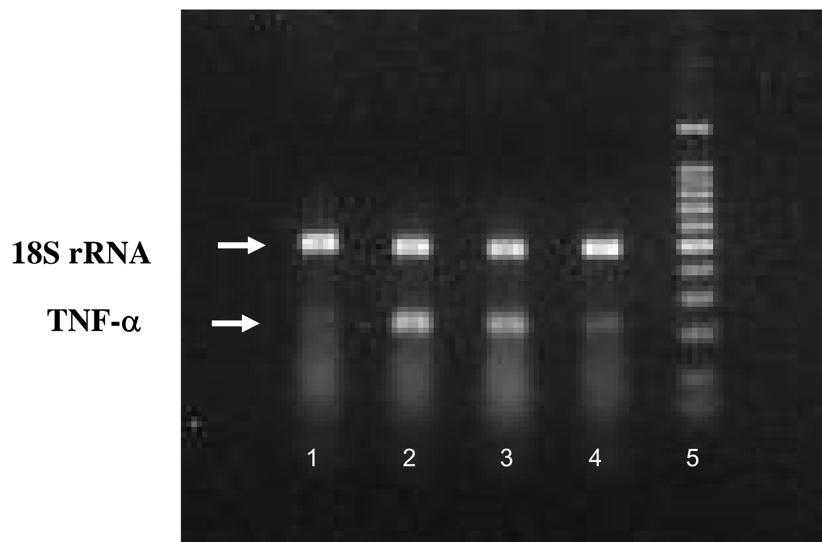

Figure 5.14 The expression of house keeping gene 18s rRNA and the TNF-α expression in THP-1 cells after *P. gingivalis* infection (n=4).

Lane 1 Negative control group: THP-1 in the absence of *P. gingivalis* and antibiotics.
Lane 2 Positive control group: THP-1 infected with *P. gingivalis* but did not treat with antibiotics.
Lane 3 Experimental group: THP-1 infected with *P. gingivalis* and treated with antibiotics.
Lane 4 Experimental group: THP-1 infected with *P. gingivalis*, treated with antibiotics and incubated in RPMI 1640 containing 10% heat inactivated FBS for 24 h.
Lane 5 Molecular DNA ladder.

Table 5.6 TNF-α and IL-1β production by THP-1 cells after treatment with *P. gingivalis* infection (n=10).

Treatment	Mean ± SD (pg/ml)	
	TNF-α	IL-1β
Non-infected cells	2.65±2.63	2.85±2.77
Infected cells	881.70±48.85	186.43±6.58
Infected cells and treated with antibiotics	884.10±35.29	189.47±6.02
Infected cells and treated with antibiotics and RPMI	215.90±34.13*	224.39±10.01**

* Significantly decrease when compared with infected cells and infected cells and treated with antibiotics ($P<0.05$).

** Significantly increase when compared with infected cells and infected cells and treated with antibiotics ($P<0.05$).

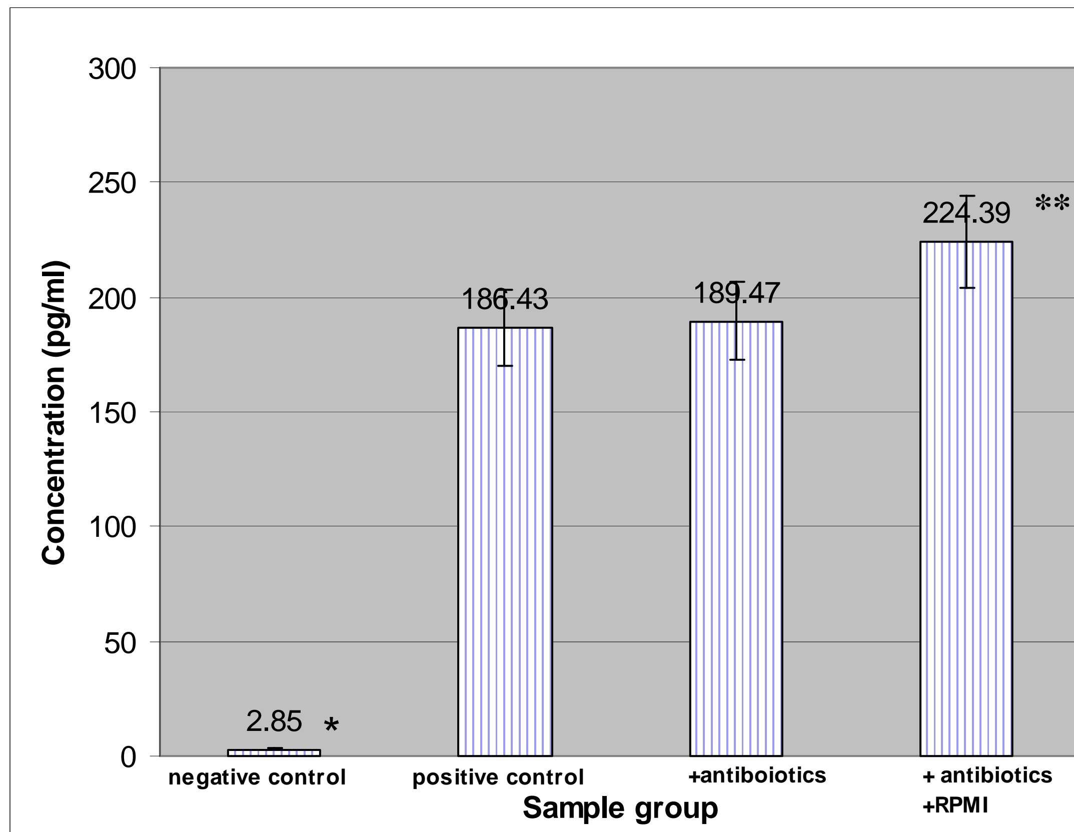

Figure 5.15 IL-1β production by THP-1 cells after *P. gingivalis* infection (n=10).

* Significantly different when compared with other groups (*P<0.05*).

** Significantly increase when compared with positive control and + antibiotics for 1 h (*P<0.05*).

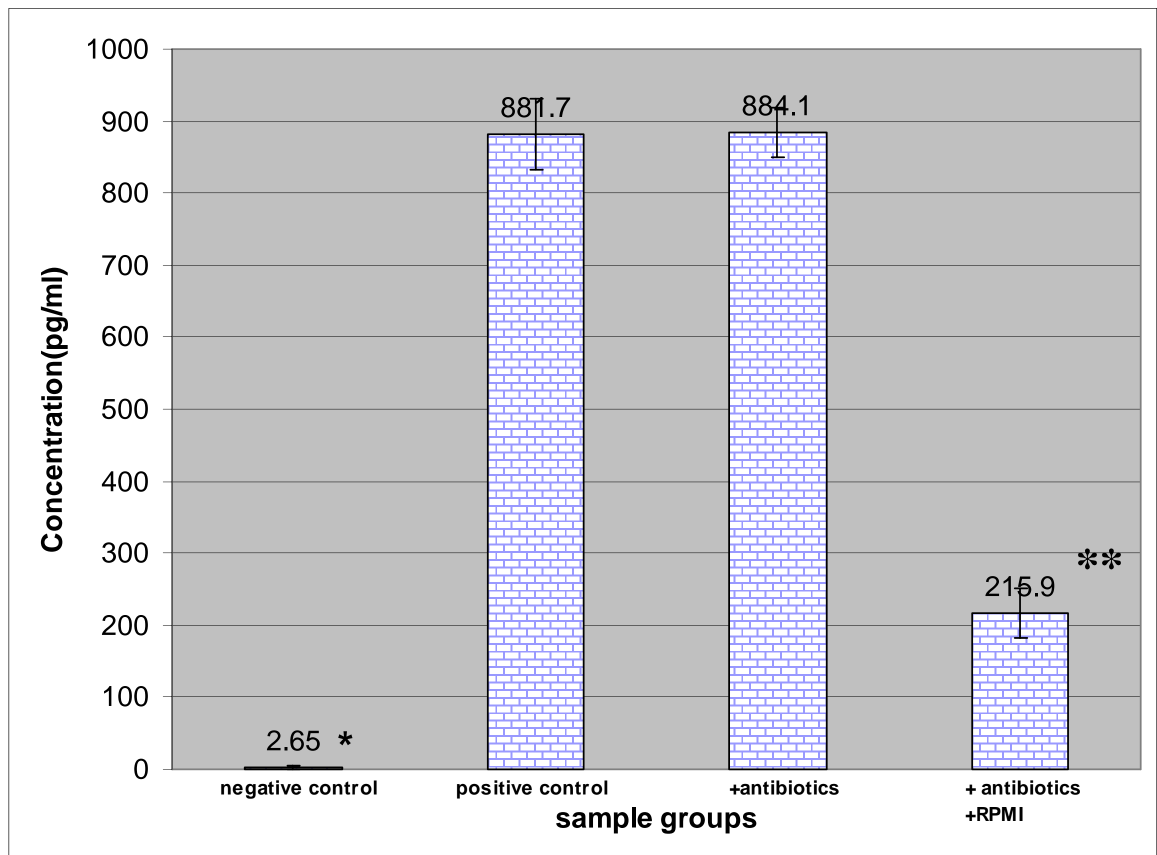

Figure 5.16 TNF-α production by THP-1 cells after *P. gingivalis* infection (n=10).

* Significantly different when compared with other groups (*P<0.05*).

** Significantly decrease when compared with positive control and + antibiotics for 1 h (*P<0.05*).

Monocytes migration

Evaluation of MCP-1 concentration and migration time

Infected THP-1 cells were able to migrate through the membrane as shown in Table 5.7. Using transwell plate, THP-1 cells could move through the membrane to the lower compartment. At each time point, various concentrations of MCP-1 (10 ng/ml, 20 ng/ml and 30 ng/ml) showed no significant effect on the number of migrated THP-1 cells. On the other hand, the number of THP-1 in the lower compartment at 2 and 3 h incubation was significantly higher than that at 1 h incubation. The number of migrated cells was found at the highest level after incubation for 3 h. However, no significant different was found when compared between incubation time at 2 h and 3 h ($P<0.05$) (n=4). Therefore, the concentration of MCP-1 at 10 ng/ml and incubation time for 2 h were selected for further study. Parallel experiments using non-infected cells stimulated with MCP-1 showed that the number of migrating THP-1 was range from 10.07-10.54 × 10^4 cells at 1 h incubation and 19.82-21.80 × 10^4 cells at 2 and 3 h incubation, regardless of MCP-1 concentrations.

Table 5.7 The number of THP-1 cells in lower compartment, when stimulated by different concentration of MCP-1 (n=4).

Time (h)	Number of THP-1 cells in lower compartment (×10^4) (Mean ±SD)		
	10ng/ml	20ng/ml	30ng/ml
1	10.88±1.75	10.56±1.56	11.56±1.09
2	20.75±1.71	21.00±2.07	21.44±2.85
3	21.75±1.88	22.31±1.77	21.00±1.74

Survival of P. gingivalis in monocytes after migration

The number of THP-1 cells in the lower compartment of transwell were counted and the living *P. gingivalis* in THP-1 cells were quantified by determining the CFU recovered on blood agar following THP-1 lysis (Table 5.8).

In the absence of incubation THP-1 with *P. gingivalis* strain A7436, mean number of THP-1 in lower compartment was $19.11 \pm 1.76 \times 10^4$ cells in positive control group. In negative control, which was treated with *P. gingivalis* but without MCP-1, mean number of THP-1 in lower compartment was $1.25 \pm 0.57 \times 10^4$ cells. In experimental group, monocyte cells was treated with *P. gingivalis* and MCP-1, mean number of THP-1 migrated was $18.64 \pm 2.33 \times 10^4$ cells. The number of THP-1 from negative control was significantly ($P<0.05$) less than positive control and experimental group. There are no significantly different between positive control and experimental group ($p<0.05$).

In positive control that did not incubation THP-1 with *P. gingivalis* strain A7436, CFU of *P. gingivalis* was undetectable. In negative control, which was treated with *P. gingivalis* but without MCP-1, mean CFU of *P. gingivalis* per cell was $10.34 \pm 7.56 \times 10^{-3}$ CFU/cell. In experimental group, monocyte cells were treated with *P. gingivalis* and MCP-1, mean CFU of *P. gingivalis* per cell was $5.83 \pm 1.45 \times 10^{-3}$ CFU/cell. The CFU of per cell from positive control was significantly ($P<0.05$) less than negative control and experimental group. There was no significantly difference between negative control and experimental group ($p<0.05$).

Table 5.8 Number of THP-1 in lower compartment and CFU of *P. gingivalis* per THP-1 cells following lyzed the THP-1 cells (n=9)

group	number of THP-1 cells ($\times 10^4$) (Mean± SD)	CFU per THP-1 cell ($\times 10^{-3}$) (Mean ±SD)
Positive control (Stimulated, non-infected THP-1)	19.11±1.76	0
Negative control (Un-stimulated, infected THP-1)	1.25±0.57 *	10.34±7.56
Experimental (Stimulated, infected THP-1)	18.64±2.33	5.83±1.45

* Significantly different when compared with positive control and experimental group ($P<0.05$).

CHAPTER VI
DISCUSSION

Porphyromonas gingivalis is considered to be a major pathogen of adult periodontitis. Interactions of *P. gingivalis* with host cells are believed to be the basic process for the destructive inflammatory response, which is the characteristic of periodontal disease. One of the possible pathogenesis of periodontal disease is bacterial invasion and survival in host cells. *P. gingivalis* may use this mechanism to pass through epithelial barrier and to protect them from host immune response. Several previous studies have demonstrated the invasion ability of *P. gingivalis in vitro*, such as invasion into human oral epithelium cells[22], buccal oral epithelium cells[21] and coronary artery cells[23]. *P. gingivalis* also has the capacity to pass through the epithelial barrier[43] and to replicate within gingival epithelium cells[59] and oral epithelium cells[60].

Invasion into host cells is a common mechanism of many bacteria including *P. gingivalis* to evade the host immune system. The intracellular location of *P. gingivalis* has been defined within a variety of host cells. Dorn *et al.* have demonstrated that *P. gingivalis* could reside within the vacuole that resembles autophagosome in human coronary artery cells[23]. It has been reported that free *P. gingivalis* could be found in cytoplasm of gingival epithelial cells[59]. Other studies also found evidences that *P. gingivalis* could be found as a free cell in the cytoplasm as well as within the endosome in oral epithelial cells[22] and human junctional epithelium cells[43]. Although many studies demonstrated that *P. gingivalis* could invade and survive in a variety of human nonphagocytic cells, up to now there is no report on these ability of *P. gingivalis* in phagocytic cells. In this study, the survival efficiency and location of *P. gingivalis* in human monocytic cell line (THP-1) have been examined in order to understand the invasion and survival mechanism of *P. gingivalis* in the phagocytic cell.

From this study, it was found that the quantity of bacterial inoculum and the incubation time between bacteria and host cells could affect the survival efficiency. The incubation time between *P. gingivalis* and THP-1 cell for at least 90 min was required for the bacteria to complete the process of invasion into the THP-1 cells. Incubation time up to 120 min did not significantly increase the number of *P. gingivalis* in THP-1 cells when compared with incubation time of 90 min. The maximum survival efficiency was observed at MOI 1:100 (1 THP-1cell:100 *P. gingivalis* cell) and the survival efficiency at MOI 1:10 or 1:1000 were less than at MOI 1:100. This result was similar to previous reports that studied in the gingival epithelium cell[59] and the human coronary cell[23]. This result may be due to the binding of bacteria to the receptors for bacteria on the surface of human cell before passing into the human cell. Once these receptors are fully occupied by the bacteria, the number of bacteria will not further increase[59].

As demonstrated by transmission electron micrographs (TEM) *P. gingivalis* was found to invade into the THP-1 cell and the bacteria still appeared intact within the autophagosome like structure of THP-1 cell. After incubation for 90 min, some bacteria were attached to the THP-1 cell surface. Pseudopodia were also observed surrounding the bacteria. Most extracellular bacteria appeared to be concentrated and adhere at the certain area along the cellular membrane. This could be due to bacterial aggregation resulting in the engulfing of aggregated bacteria, as a whole, by the THP-1 cell. It is also possible that the mTHP-1 cell may have the specific receptors for *P. gingivalis* at the certain area of cell membrane. This phenomenon was also observed and repeated in previous studies on the invasion of *P. gingivalis* into the human coronary artery cell[23] and oral epithelial cell[74].

After incubation for 90 min and treated THP-1 cells with antibiotics, *P. gingivalis* was clearly visible within the cytoplasm of THP-1 cells, and seemed to survive in the cytoplasm. Two forms of *P. gingivalis* was observed: one of which the bacteria were closely surrounded by the thin membranous vacuole, cytoplasm, ground substance and rough endoplasmic reticulum that Dun *et al.* indicated this to be autophagosome[75]; another form was presented in a large vacuole that Dorn *et al.* indicated this to be phagosome[23]. Thus, this study suggest that bacterial transportation inside the THP-1 could be passed through two pathways namely:

autophagosome and phagosome pathway. This phenomenon was also found in the previous study on *P. gingivalis* into the human coronary artery cell[23].

After treating THP-1 cells with antibiotics and further incubated in RPMI1640 for 4 h, transmission electron micrographs of THP-1 cells showed that *P. gingivalis* was located within the autophagosome like structure and some bacteria in this location appeared to be under gone cell division. In addition, in cytoplasm of some THP-1 cells revealed numerous vacuoles that contained unidentified materials debris. These transmission electron micrographs presented that *P. gingivalis* may survive and replicate within autophagosome only similar to the phenomenon that found in the study of the invasion of *P. gingivalis* into the human nonphagocytic cells[23].

After 8 h of incubation, the internal *P. gingivalis* were still present in autophagosome of THP-1 cytoplasm. However, no dividing bacteria were found in this period. The transmission electron micrographs showed that *P. gingivalis* may be survived in autophagosome but this bacteria can not replicate during this period. This result may due to the lack of cellular proteins for replication since the bacteria used all of the host cell proteins in autophagosome, so they did not have enough source of energy for replication.

Autophagocytic pathway is an important cellular process for the degradation of cellular organs and proteins. It is the essential lysosomal process for the selective removal of nonfunctional organelles and cellular proteins in order to maintain cellular amino acids by protein degradation[76,77]. The intracellular trafficking of *P. gingivalis* via autophagosomes may be similar to the trafficking of *Brucellar abortus*[78] and *Legionella pneumophila*[62]. Therefore, *P. gingivalis* species may use autophagosomes to escape from intracellular killing mechanism (phagolysosome pathway) and use this organelle as their replication site in the host cell, similar to that found in *B. abortus* and *L. pneumophila* [79,80].

In order to confirm the survival of *P. gingivalis* in such cells, THP-1 cells were lysed by sterile double distilled water then plated on blood agar and incubated under anaerobic condition. The survival efficiency of *P. gingivalis* showed no difference among different incubation times but the survival efficiency of *E. coli* was decreased with incubation time and disappeared after 4 h incubation.

From these results, the model of *P. gingivalis* trafficking within the THP-1 cell is proposed. *P. gingivalis* cells may enter the THP-1 cells and reside in the endosome like vacuoles. These vacuoles may traffic into two alternative pathways; 1) autophagosome pathway, which is an important process for the degradation of cell organelles and 2) phagosome pathway, which is an important process for the degradation of intracellular bacteria. *P. gingivalis* in the early stage may still vital in both pathways. Later, the degradation of *P. gingivalis* in vacuole may occur in the phagosome pathway. However, *P. gingivalis* in the autophagosome pathway may still survive and may be able to replicate. This is the reason why the percentage of the vital bacteria in THP-1 cell did not decrease and even after 4 h incubation later, the number of *P. gingivalis* in THP-1 cells was still the same. It is possible that the environment in this period may not be suitable for *P. gingivalis* to replicate.

The monocyte is a phagocytic cell that plays an important role in the regulation of host response. It performs many important functions in host response against bacterial infection. The functions of monocytes include phagocytosis and secretion of biologically active molecules, including TNF-α and IL-1β. These molecules are the proinflammatory cytokines that are essential for the initiation of inflammatory response and tissue destruction[81]. They can amplify the degree of inflammation[82], induce the connective tissue destruction[83] and reduce the capacity to repair the damaged tissue[84] by stimulate the production of other mediators. The ability of *P. gingivalis* or its products to stimulate IL-1β and TNF-α production from human monocytes may play an important role in progression of periodontal tissue destruction in periodontal disease[45]. In this study, the ELISA technique demonstrated that THP-1 cells produced both IL-1β and TNF-α after stimulation with *P. gingivalis*. This result agrees with the past study of Baqui *et al.*[85] who reported that IL-1β and TNF-α were produced by THP-1 cells when stimulated with LPS of *P. gingivalis*[85].

The untreated THP-1 cells also produced small amount of IL-1β and TNF-α. This result was contrast to the study of Baqui *et al.* [85], showing that untreated THP-1 cells did not produce both IL-1β and TNF-α in the culture media. The production of these cytokines may be due to the cell stimulation during the process of cell treatment, such as centrifugation and resuspension of cells. Nevertheless, the concentration of

IL-1β and TNF-α in the untreated cells was significantly less than the treated cells. This phenomenon was also reported in previous study of Robert *et al*. that studied the effects of *P. gingivalis* lipopolysaccharides on monocyte[86].

The combination of metronidazole and gentamycin were used in this study for killing *P. gingivalis*. These combination antibiotics were found to be able to completely killed 10^9 bacteria within 1h and vital bacteria were not found in media after incubation of these antibiotics for 1h. This result is similar to the result from the study of Richard *et al.*[59]. No significant difference of IL-1β and TNF-α production between positive controls and THP-1 infected with *P. gingivalis* and treated with antibiotics. It may be concluded that these antibiotics combination did not affect the IL-1β and TNF-α production ability of THP-1 cells after treated with *P. gingivalis.* The reason for these phenomena may be explained by either the different in exposure time or the ability of drugs penetration into cells. The penetration of eukaryotic cells by gentamycin is slow and can be detectable after 24 to 48 h after antibiotic exposure[87] and in this study only short time exposure was used. Moreover, the entry of metronidazole into cells must use electron transport proteins of anaerobic metabolic energy-yielding pathways before the drug diffusion into the cells could occur[88]. Therefore, metronidazole provides bactericidal effect on anaerobic bacteria without any effect on THP-1 cell. So these two antibiotics can not penetrate into THP-1 cells and did not affect the IL-β and TNF-α production.

It was found that the mean concentration of IL-1β was significantly increased after stimulating THP-1 cells with *P. gingivalis* then treated with antibiotics and further incubated for 24 h. On the other hand, the mean concentration of TNF-α produced by THP-1 cell after treated under the similar condition was found to be significantly decreaed.

This phenomenon was also reported by Baqui *et al.*[85] that TNF-α production reached a plateau by 8 h after monocyte stimulation and then slowly declined[85]. But, IL-1β production reached a plateau by 4 days after stimulation before declined[85]. This result may be due to the augmentation of IL-1β after IL-1β production while TNF-α can not undergo augmentation TNF-α production from the human cells[20]. In

other words, the IL-1β production in media stimulated IL-1β releasing from THP-1 cells and increased the concentration of IL-1β in media.

Using RT-PCR technique, IL-1β and TNF-α mRNA were also studied. Semi-quantitative method comparing the ratio of band intensity of IL-1β or TNF-α to 18s rRNA was done. From this study, IL-1β and TNF-α bands in the negative control group were not found. Therefore, in this group, the absence of *P. gingivalis* might not stimulate the THP-1 cells. Similar result was also reported by Gessani *et al.* [89].

The ratios of band intensity between IL-1β to 18s rRNA in the experimental groups, one treated with antibiotics for 1 h and another treated with antibiotics for 1 h, then left in media for 24 h were 0.94 and 0.85, respectively. The results showed that the level of IL-1β mRNA expression was not significantly changed after 24 h of incubation in media. The ratios of band intensity between TNF-α to 18s rRNA in those experimental groups, *i.e.*, treated with antibiotics for 1 h and treated with antibiotics for 1 h and left in media for 24 h, were 0.90 and 0.54 respectively. The results showed that when THP-1 cells were treated with antibiotics and left in media for 24 h, the level of TNF-α mRNA expression was significantly reduced.

These results may be due to the complexity of cytokine network. Since one cytokine can affect the synthesis of other cytokines[20], therefore the kinetics of cytokine production resulted from the stimulation of monocytes with bacteria may be differed for IL-1β and TNF-α[90]. These results supported that IL-1β is an important proinflammatory cytokine in human, which is not only more potent in promotion inflammation but also a long time production after stimulation.

Monocyte chemoattractant protein 1 (MCP-1) is an inflammatory chemotactic cytokines (chemokines) that target monocytes and other cells expressing the CC chemokine receptor (CCR_2)[32]. This chemokines is classified as CC chemokine because of the first two cysteines near the N-terminal of this protein[91]. MCP-1 was found at the site of chronic inflammation and it has been suggested to be a key player in the recruitment of monocytes from the blood into the atherosclerotic lesion[33]. These circulating blood monocytes were suggested to be the precursors of the foam cells at the atherosclerotic lesion[33], which were important in plaque formation. Many studies demonstrated the presence of *P. gingivalis* in atherosclerotic lesion by PCR

detection[34,35,36]. In animal model *P. gingivalis* promoted atherosclerotic progression by elevation the level of serum IL-1β[37]. The migration of infected monocyte to disease lesion may carry this bacteria to atherosclerotic lesion and promote atherosclerotic lesion formation. The migration of monocytes involves the cytoskeleton changing[92] and the inhibition of some cytoskeleton polymerization effected the survival ability of intracellular *P. gingivalis* in human epithelium cell[93]. Therefore, the survival ability of *P. gingivalis* in THP-1 cell after migration has been examined in this study.

From this study it showed that after 2 h incubation of monocyte with 10 ng/ml MCP-1 in RPMI 1640, the monocyte migration was detected. This phenomenon was also found in the previous study on monocyte migration after 2 h incubation with RPMI 1640 containing MCP-1 10 ng/ml. This result presented that RPMI 1640 containing MCP-1 10 ng/ml was enough to stimulate monocyte migration after 2h of incubation.

The mean number of THP-1 cells in the lower compartment of transwell of the positive control group, which THP-1 cells were treated with MCP-1 but without *P. gingivalis*, was significantly higher than the negative control group that THP-1 cells were treated with *P. gingivalis* but without MCP-1. However, the mean number of THP-1 cells in lower compartment of the experimental group that THP-1 was treated with *P. gingivalis* and MCP-1 showed no significantly different from the positive control. This result may suggest that the infected THP-1 with *P. gingivalis* did not affect the migration ability of THP-1 cell. On the other hand, Rao *et al.*[38] presented that the products of *Mycobacterium avium* promoted the monocyte migration[38].

Mean CFU of *P. gingivalis* per THP-1 cell in the negative control, which THP-1 cells were treated with *P. gingivalis* but without MCP-1, showed no significantly difference from the experimental group that THP-1 was treated with *P. gingivalis* and MCP-1. This result suggested that the monocyte migration after stimulation with MCP-1 did not affect the survival ability of intracellular *P. gingivalis*. This result may be due to the ineffective of THP-1 mobility and MCP-1 on autophagosome formation and development so that both THP-1 mobility and MCP-1 did not affect the vital number of *P. gingivalis* in THP-1 cell.

This data presented that the intracellular *P. gingivalis* did not affect the migration ability of monocyte. The stimulation with MCP-1 did not affect the survival ability of *P. gingivalis* in the THP-1 cells. The result suggested that *P. gingivalis* in atherosclerotic lesion that was detected by PCR[34,35,36] may be carried by infected monocytes to atherosclerotic lesion and the *P. gingivalis* may also promoted the progression of the atherosclerosis lesion[94].

CHAPTER VII
CONCLUSION

From this study, transmission electron micrographs showed that *P. gingivalis* was found located in autophagosome like structure of THP-1 cells. They still presented after further incubated in RPMI 1640 for 0, 4 and 8 h. The survival efficiency of *P. gingivalis* in THP-1 cells showed no difference among different incubation times while the survival efficiency of *E. coli* was decreased with incubation time. This finding indicated that *P. gingivalis* may survive in THP-1 cells by residing in autophagosome like structure.

The result from both ELISA and RT-PCR techniques demonstrated that THP-1 cells produced both IL-1β and TNF-α after stimulation with *P. gingivalis*. The combination of metronidazole and gentamycin did not affect the IL-1β and TNF-α production ability of THP-1 cells after treated with *P. gingivalis*. THP-1 cells after stimulation with vital *P. gingivalis* showed similar IL-1β and TNF-α production pattern similar to those stimulated with lipopolysaccharide of *P. gingivalis*.

The monocyte migration after stimulation with MCP-1 did not affect the survival ability of intracellular *P. gingivalis*. In addition, *P. gingivalis* did not affect the migration ability of THP-1 cells after stimulation with MCP-1. These results may be indicated that infected monocyte may be a vehicle for carrying *P. gingivalis* to atherosclerotic lesion. However, an animal model study is required to confirm these vitro studies.

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APPENDIX

APPENDIX

1. Media for cell culture

1.1 RPMI 1640 containing 10% heat inactivated fetal bovine serum

Heat inactivated fetal bovine serum	100	ml
RPMI 1640	900	ml

Preparation. Fetal bovine serum is inactivated by incubation at 56° C for 30 min. Then, 100 ml of heat inactivated fetal bovine serum is added to 900 ml of RPMI 1640 and mixed. This solution is sterilized by cellulose acetate membrane filter (pore size 0.2µm) and stored at 4° C.

1.2 RPMI 1640 containing 300µg/ml gentamycin and 200µg/ml metronidazole

Gentamycin	30	mg
Metronidazole	20	mg
RPMI 1640	100	ml

Preparation. RPMI 1640 is added to above antibiotics to make a total volume of 100 ml and mixed. The solution is sterilized by cellulose acetate membrane filter (pore size 0.2µm) and stored at 4° C.

2. Agar for bacterial culture

2.1 Brain heart infusion agar

Classical formula per liter purified water :

Brain heart, infusion from (solid)	8	g
Peptic digest of animal tissue	5	g
Sodium chloride	5	g
Dextrose	2	g
Pancreatic digest of casein	16	g
Disodium phosphate	2.5	g
Agar	13.5	g

pH 7.4±0.2

Preparation. Suspend the 52 g of dry material in 1l of distilled water (DW) by heating in a boiling water bath or a current of steam. Sterilize by autoclaving at 121° C for 15 min, cool in water bath at 50° C, and pour on plates.

2.2 Blood agar

Classical formula per liter purified water :

Peptone	16.1	g
Yeast extract	7	g
Sodium chloride	5	g
Starch	1	g
Dextrose	1	g
Sodium pyruvate	1	g
Arginine	1	g
Ferric pyrophosphate	0.5	g
L-cysteine hydrochloride	0.25	g
Dithiothreitol	0.25	g
Haemin	0.005	g
Vitamin K	0.0005	g
Agar	12	g

pH 6.8±0.2

Preparation. Suspend the 46 g of dry material in 1 l of DW by heating in a boiling water bath or a current of steam. Sterilize by autoclaving at 121° C for 15 min, cool in water bath at 50° C, then supplement with 5% sterile sheep whole blood and pour on plates.

3. Reagent for transmission electron microscope (TEM)

3.1 Phosphate buffer (PB)

Solution A	2.26% sodium dihydrogen phosphate in DW
Solution B	2.52% sodium hydroxide in DW
Solution C	5.4% glucose in DW
Solution D	Mix 41.5 ml of solution A and 8.5 ml of solution B check the pH and adjust to pH 7.4

Preparation. Mix 45 ml of solution D and 5 ml of solution C and store at 4°C.

3.2 4% glutaraldehyde in PB

Glutaraldehyde	4	ml
PB	96	ml

Preparation. PB is added to glutaraldehyde and mixed. The solution is stored at 4° C.

3.3 2% osmium teroxide in PB

Osmium	1	g
PB	50	ml

Preparation. PB is added to osmium to make a total volume of 50 ml, mixed and stored at 4° C.

3.4 2% uranyl acetate

Uranyl acetate	1	g
DW	50	ml

Preparation. PB is added to uranyl acetate to make a total volume of 50 ml, mixed and stored at 4° C.

3.5 Epoxy resin

Epon 812	8.5	ml
Dodecenyl succinic anhydride	4.0	ml
Nadic methyl anhydride	5.25	ml
2, 4, 6-tridimethylamino methyl phenol	0.27	ml

Preparation. All the reagents above are mixed and used.

3.6 5% uranyl acetate

Uranyl acetate	2.5	g
Methanol 70%	50	ml

Preparation. Methanol 70% is added to uranyl acetate to make a total volume of 50 ml, mixed and stored at 4° C.

3.7 Lead citrate

Lead nitrate	1.33	g
Sodium citrate	1.76	g
DW	30	ml

Preparation. DW is added to lead nitrate and sodium citrate to make a total volume of 30 ml, mixed and stored at 4° C.

4. Reagents for electrophoresis

4.1 Tris-borate/EDTA buffer (1X TBE/EDTA buffer)

Tris base	54	g
Boric acid	27.5	g
EDTA	3.72	g

Preparation. DW is added to reagents above to make a total volume of 5 l, mixed and stored at 4° C.

4.2 0.2 μg/ml ethidium bromide in TBE/EDTA buffer

Ethidium bromide	200	μg

Preparation. TBE/EDTA buffer is added to ethidium bromide to make a total volume of 1 l, mixed and stored at 4° C in dark place.

4.3 2% agarose gel

Agarose gel	2	g

Preparation. TBE/EDTA buffer is added to agarose gel to make a total volume of 100 ml, heated in microwave oven with high temperature for 3-4 min until the gel dissolve, mixed well together and used.

5. Reagents for ELISA

5.1 1x washing buffer

Washing buffer concentrate	20	ml

Preparation. DW is added to washing buffer concentrate to make a total volume of 500 ml, mixed and used.

6. Reagents for monocyte migration

6.1 Monocyte chemotactic protein-1 (MCP-1) in RPMI 1640 containing 10% heat inactivated FBS

MCP-1	10	μg

Preparation. MCP-1 is suspended in 10 ml of RPMI 1640 containing 10% heat inactivated FBS for stock solution and stored at -80° C. For 10% ng/ml MCP-1, RPMI 1640 containing 10% heat inactivated FBS is added to 100 μl of stock solution

to make a total volume of 10 ml and mixed. For 20% ng/ml MCP-1, RPMI 1640 containing 10% heat inactivated FBS is added to 200 µl of stock solution to make a total volume of 10 ml and mixed. For 30% ng/ml MCP-1, RPMI 1640 containing 10% heat inactivated FBS is added to 300 µl of stock solution to make a total volume of 10 ml and mixed.

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