รายงานสรุป

โครงการ : "กลไกการกระตุ้น Cell Macrophage โดยผ่าน CD 14 ด้วย Lipopolysaccharide (LPS) จากเชื้อ *Burkholderia Pseudomallei*"

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โครงการ "กลไกการกระตุ้น Cell Macrophage โดยผ่าน CD14 ด้วย Lipopolysaccharide (LPS) จากเชื้อ *Burkholderia Pseudomallei*"

บทคัดย่อ

เมลิออยโดซิส เป็นโรคที่เกิดจากเชื้อแบคทีเรียชนิด *Burkholderia pseudomallei* โรคนี้พบมากในเขตอากาศร้อนชื้น เช่น ประเทศไทย แบคทีเรียชนิดนี้จะมี lipopolysaccharide (LPS) ซึ่งเป็นส่วนประกอบของผิวเซลล์และมีบทบาทในการเกิดโรค ในการศึกษานี้ได้มีการแยก LPS ออกจากเชื้อและใช้กระตุ้น macrophage cell line ของหนู (RAW 264.7) พบว่า LPS จากเชื้อ *Burkholderia pseudomallei* (BP-LPS) จะกระตุ้นการหลั่ง nitric oxide (NO) และ TNF-α ได้น้อยกว่าเซลล์ที่ถูกกระตุ้นด้วย LPS จากเชื้อแบคทีเรียที่ติดสีแกรมลบอื่นๆ เช่น *E. coli*-LPS และพบว่าเซลล์ที่ถูกกระตุ้นด้วย BP-LPS ใช้เวลาในการกระตุ้นการหลั่ง NO และ TNF-α นานกว่าการกระตุ้นด้วย *E. coli*-LPS การศึกษาทาง kinetic พบว่าเซลล์ที่ถูกกระตุ้นด้วย BP-LPS ใช้เวลาในการสร้าง mRNA ของ inducible nitric oxide synthase (iNOS) และ TNF-α นานกว่าการกระตุ้นด้วย *E. coli*-LPS โดยเป็นผลมาจากการส่งสัญญาณที่ช้ากว่าเมื่อ BP-LPS จับกับ receptor บนผิวเซลล์ การศึกษาการส่งสัญญาณนี้ใช้เทคนิค Western blot โดยใช้ Monoclonal Ab ต่อ pp38 ซึ่งเป็นโปรตีนภายในเซลล์ที่ใช้ในการส่งสัญญาณ นอกจากนี้ยังพบว่า polymyxin B สามารถยับยั้งการสร้าง NO จากเซลล์ที่กระตุ้นด้วย *E. coli*-LPS และมีผลน้อยมากกับเซลล์ที่กระตุ้นด้วย BP-LPS การศึกษานี้เป็นการแสดงถึงความแตกต่างทางโครงสร้างของ BP-LPS เมื่อเทียบกับ LPS อื่นๆ เช่น *E. coli*-LPS อย่างไรก็ตามการวิเคราะห์ binding competition โดยใช้ LPS ติดฉลากด้วยสารเรืองแสง fluoroisothiocyanate (FITC-LPS) พบว่า binding affinity ของ *E. coli*-LPS และ BP-LPS ไม่มีความแตกต่างกัน การใช้ Monoclonal Ab ต่อ CD14 ก็สามารถยับยั้งการ binding ของ BP-LPS และ *E.*

coli-LPS ได้ ผลการศึกษานี้บอกได้ว่า BP-LPS และ *E. coli*-LPS มี binding กับ CD14 บนผิวเซลล์ และ polymyxin B ยังสามารถยับยั้งการ binding ของ FITC-*E. coli*-LPS กับเซลล์ได้เป็นอย่างดี และมีผลน้อยต่อการ binding ของ FITC-BP-LPS การศึกษาอัตราการ binding ของ FITC-BP-LPS และ FITC-*E. coli*-LPS บนผิวเซลล์พบว่าไม่แตกต่างกัน โดย FITC-BP-LPS จะ bind บนผิวเซลล์และเข้าสู่สมดุลภายใน 60 นาที

การกระตุ้นเซลล์ด้วย heat-killed *B. pseudomallei* พบว่าจะกระตุ้นเซลล์ให้หลั่ง NO และ TNF-α ได้ช้ากว่าเซลล์ที่กระตุ้นด้วย heat-killed *E. coli* นอกจากนี้การศึกษาการใช้ Monoclonal Ab ต่อเอนไซม์ iNOS แสดงให้เห็นว่าเซลล์ที่ถูกกระตุ้นด้วย heat-killed *B. pseudomallei* จะมีปริมาณ iNOS เพิ่มขึ้นช้ากว่าเซลล์ที่กระตุ้นด้วย heat-killed *E. coli* ผลการศึกษานี้สอดคล้องกับการกระตุ้นเซลล์ด้วย LPS

ผู้วิจัยได้ทำการศึกษาต่อว่าเซลล์ที่ถูกกระตุ้นด้วย *B. pseudomallei* ไม่มีการสร้าง iNOS แม้ใช้อัตราส่วนของเชื้อต่อเซลล์สูงถึง 10:1 ในขณะเดียวกัน iNOS จะถูกสร้างจากเซลล์ที่กระตุ้นด้วย *E. coli* แม้อัตราส่วนของเชื้อต่อเซลล์เป็น 0.1:1 นอกจากนี้พบว่าเซลล์ที่ถูกกระตุ้นด้วย *B. pseudomallei* สามารถหลั่งปริมาณ TNF-α ออกมาน้อยกว่าเซลล์ที่ถูกกระตุ้นด้วย *E. coli* ผลการศึกษานี้บอกได้ว่าเชื้อ *B. pseudomallei* เป็นเชื้อที่กระตุ้นเซลล์ได้ไม่ดีเมื่อเทียบกับเชื้อที่ติดสีแกรมลบอื่นๆ ด้วยเหตุนี้อาจเป็นส่วนที่ทำให้เชื้อสามารถเจริญและเพิ่มจำนวนในเซลล์ได้โดยไม่มีการกระตุ้นกลไกการป้องกันของเซลล์

FINAL REPORT

ON

Role of CD14 in *Burkholderia pseudomallei* Lipopolysaccharide (LPS) Induced Macrophages Response

ABSTRACT

Melioidosis, a bacteria disease causes by *Burkholderia pseudomallei*, is endemic in tropical area such as Thailand. Among the bacteria virulence factor, lipopolysaccharide (LPS) have been shown to play roles in pathogenesis of gram negative bacteria infection. In this study, LPS (BP-LPS) isolated from *Burkholderia pseudomallei* has been investigated. The mouse macrophage cell line (RAW 264.7) treated with BP-LPS produced significantly less NO and TNF-α than other gram negative bacteria LPS such as *E. coli*-LPS. The time required for the BP-LPS to trigger substantial NO and TNF-α release was at least 30 min comparing with less than 5 min in those activated with *E. coli*-LPS. The time course analysis of inducible nitric oxide synthase (iNOS) and TNF-α mRNA expression also significantly slower in the cells that activated with BP-LPS. These results indirectly suggest that the slower rate of mediators release and gene expression may be due to the slower rate of signal transduction initiated by the interaction of BP-LPS with macrophage cell surface. Using MoAb to phosphorylated p38 in the Western blot analysis provided data compatible with the notion that the cells activated with BP-LPS phosphorylated p38 with a slower rate. Polymyxin B completely inhibit

NO release from the cells activated with *E. coli*-LPS but only partially inhibited NO release from the cells stimulated with BP-LPS. This result suggests that the unique LPS structure of *B. pseudomallei* may influence the cell activation. However, binding competition assay, using LPS labelled with fluoroisothiocyanate (FITC-LPS) indicates no significantly different in binding affinity between *E. coli*-LPS and BP-LPS. MoAb to CD14 (MY4) completely abrogated the binding of both BP-LPS and *E. coli*-LPS. This result suggests that both *E.coli*-LPS and BP-LPS binding to CD14 on the macrophage surface. In the present of polymyxin B, this drug strongly inhibited FITC-*E. coli*-LPS binding to the cells. However, polymyxin B had little effect on FITC-BP-LPS binding to the cells. The rate of FITC-BP-LPS binding to the cell surface was also investigated. The results indicated that FITC-BP-LPS gradually interacted to the cells and reached the plateau within 60 min. Similar result was also observed on FITC-*E. coli*-LPS binding to the macrophages. These results indicated that the kinetics of BP-LPS and *E. coli*-LPS binding to the cells were not significantly different.

The kinetics of macrophage activation activity by heat-killed *B. pseudomallei* also had been investigated. The heat-killed *B. pseudomallei* gradually activated NO and TNF-α released and reached the plateau between 1-2 hours. On the other hand, the cells activated with heat-killed *E. coli* released NO and TNF-α with significantly faster kinetics rate. The NO and TNF-α release from the cells activated with heat-killed *E. coli* reached plateau within 30 min of activation. Using MoAb to iNOS enzyme also indicated that the cells activated with heat-killed *B. pseudomallei* upregulated iNOS enzyme expression with significantly slower rate than the cells activated with heat-killed *E. coli*. These results are consistent with the cells treated with LPS.

We have furthure demonstrated that the macrophages infected with *B. pseudomallei* produced almost nondetectable iNOS enzyme even when the cells infected with high MOI (10:1). However, expression of iNOS enzyme was observed following *E. coli* infection (MOI of 0.1:1). The macrophages infected with *B. pseudomallei* also released significantly lower amount of TNF-α comparing with the cells infected with *E. coli*. These results indicated that *B. pseudomallei* is not a good macrophage activation comparing with other gram negative bacteria. Being a poor macrophage activator may facilitate the bacteria to enter the cells without turning on the cell defense mechanism. This would lead to bacteria survival and multiplication inside macrophage.

INTRODUCTION

Melioidosis, a bacterial disease caused by *Burkholderia pseudomallei* , is endemic in tropical area such as southeast Asia, northern Australia and other temperate regions that border the equator (1-4). Acquisition of melioidosis occurs via inoculation of the damaged tissue surface with contaminated soil or water; or by inhalation and aspiration of contaminated dust particles (5). Infection by this gram-negative bacteria may cause acute septicemic melioidosis which affects various organs throughout the body, particularly the lungs, liver, spleen and lymph nodes (4). In acute septicemia, there are fever, chill, muscle pain and other signs and symptoms resulting from localized abscess. Systemic infection is associated with high mortality rate, slow response to antimicrobial therapy and high rate of relapse despite prolonged treatment (6). Subclinical or asymptomatic infection is the most common form of melioidosis (3). In some cases the bacteria remain latent and cause clinical manifestations after a long period

of latency (7). These observations suggest the possible presence of a unique microbial virulence factor(s) which permit evasion from humoral and cell-mediated immunity. Among the many virulence factors of gram-negative bacteria, lipopolysaccharide (LPS) is a major contributing factor in systemic sepsis and tissue injury (8). It is well documented that the LPS activates macrophages and induces a number of molecules including reactive nitrogen intermediates and cytokines (e.g., TNF-α, IL1 and IL6) both in vivo and in vitro (9). The macrophage response to LPS is initiated by the binding of LPS and LBP (lipopolysaccharide binding protein) complex with CD14, which is a GPI-anchored cell surface glycoprotein. Within 1 min of exposure, the LPS is able to bind to the CD14 on the cell surface (10). The time required to trigger the mediator production (such as TNF-α) from the monocytes activated by LPS is only 5 to 15 min (10). The features of melioidosis suggest that *B. pseudomallei* is a facultative intracellular bacteria. Several investigation have demonstrated that this bacteria survive and multiply inside the cells such as phagocytic cells (11, 12). The mechamism by which this organism survives within the phagocytic cells is not fully known.

Generally, host defense against infection with facultative intracellular bacteria is predominantly mediated by cellular immune mechanism (13). Several microbicidal mechanism are activated once the organism entering the cells. Among the antimicrobial mechanisms of macrophages, nitric oxide (NO), produced by inducible nitric oxide synthase (iNOS) plays a major role as bactericidal. In cell lines resistance to bacteria growth often associated with expression of iNOS (14). Inhibition of this enzyme with the inhibitors has been shown to worsen the course of disease (15). In this study, we have demonstrated that *B. pseudomallei* invades and multiplies inside mouse macrophage cell

line (RAW 264.7) without activating substantial macrophages response. The infected cells did not express iNOS at the detectable level and the release significantly low amount of cytokine such as TNF-α. However, IFN-γ enhances both expression of iNOS enzyme and TNF-α release on the cells infected with *B. pseudomallei*. The strong inhibition of intracellular multiplication was also observed when the cells were pretreated with IFN-γ.

LPS isolated from *B. pseudomallei* (BP-LPS) has been reported to exhibit weaker macrophage activation activity than enterobacterial LPS by at least one order of magnitude (16). On the other hand, the BP-LPS appears to have stronger mitogenic activity for murine splenocytes than the enterobacterial LPS. The BP-LPS also has an unusual acid stable structure in the inner core region attached to the lipid A moiety (17). Whether this unique structure can influence its biological activity or how this unique LPS which has a weaker macrophage activation potential plays a role in pathogenesis of *B. pseudomallei* remains to be investigated. In the present study, we investigated the kinetics of NO and TNF-α release and the kinetics of iNOS and TNF-αgene expression from mouse macrophage cells (RAW 264.7) activated with BP-LPS.

MATERIALS AND METHODS

Cell line and culture condition

Mouse macrophage cell line (RAW 264.7) was obtained from American Type Culture Collection (ATCC, Rockville, MD). If not indicated otherwise, the cells were cultured in DMEM (GIBCO Labs, Grand Island, NY) supplemented with 10% FBS

(HyClone, Logan, UT) and grown at 37 °C under a 5% CO_2 atmosphere.

Bacterial isolation

B. pseudomallei strain 844 (arabinose-negative strain) was isolated from patients admitted to Srinakarind hospital in the melioidosis endemic Khon Kaen province of Thailand. The bacterium was originally identified as *B. pseudomallei* based on its biochemical characteristics, colonial morphology on selective media, antibiotic sensitivity profiles and reaction with polyclonal antibody (18). The *E. coli* used for comparison throughout these experiments was maintained at Ramathibodi Hospital (Mahidol University, Bangkok, Thailand) and kept as stock culture in our laboratory.

Preparation of B. pseudomallei LPS (BP-LPS)

LPS was extracted from individual *B. pseudomallei* and *E. coli* isolates by the modified phenol-chloroform-petroleum ether method (17) and characterized by SDS-PAGE and immunoblotting as previously reported (19). The LPS from *E. coli* strain 0111:B4 (Sigma, St Louis, MO) was also used for comparison. The LPS carbohydrate content was determined by the orcinol-sulfuric acid method using glucose as standard (20).

Treatment of mouse macrophage cell line (RAW 264.7) with BP-LPS

Mouse macrophage cells (1×10^6) were exposed to various concentrations of BP-LPS and *E. coli*-LPS. Eighteen hours after exposure, the supernatant was analyzed for NO (21) and TNF-α release.

Kinetics of NO and TNF-α release from mouse macrophage cell line (RAW 264.7) with BP-LPS

Mouse macrophage cells (1×10^6) were exposed to BP-LPS (100 ng/ml), *E. coli*-LPS (10 ng/ml) for 5, 15, 30 min, 1, 2 and 4 h at 37 °C. The cells were washed 3 times with PBS before incubating in the medium. At 18 hours after the cells exposed to LPS, the supernatant was analyzed for NO and TNF-α release. The cells were lyzed before subjecting to western blot analysis for iNOS.

Nitrite assay

The presence of nitrite in cell culture supernatants was determined by Griess reaction (21). One hundred microliters aliquots of culture supernatants were added to 96 well tissue culture plates (Costar, Cambridge, MA) and followed by 100 µl of Griess reagent (equal quantities of 1% sulfanilamide in 5% H3PO4 and 0.1% naphthylethylene in water). The plates were read at 550 nm.

TNF-α assay

TNF-α activity was measured by a cytotoxic assay against L-929 (22). Treated cells were stained with crystal violet after 18 h. Change in absorbance at 540 nm was measured and converted to unit per milliliter of TNF based on a standard curve using murine TNF-α (Genzyme, Cambridge, MA) as standard.

Western blot analysis for iNOS

Mouse macrophage cell line (RAW 264.7) were lyzed in buffer containing 20 mM Tris, 100 mM NaCl, 1% NP40. Lysates containing 30 μg of protein were electrophoresed on 8% SDS-polyacrylamide gel before transferred to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 5% milk for 1 h before reacted overnight with polyclonal antibody to iNOS (Santa Cruz, Santa Cruz, CA). Blots were then reacted with horse radish peroxidase-conjugated swine anti-rabbit IgG (Dako, Glostrup, Denmark). Proteins were detected by enhance chemiluminescence as recommended by the manufacturer (Pierce, Rockford, IL).

FITC-LPS

LPS from both *B. pseudomallei* or *E. coli* were labeled with fluorescein isothiocyanate (FITC) (23). Briefly, two milligrams of *E.coli*-LPS or BP-LPS was incubated with 8 mg of FITC (Sigma, St Louis, MO) in 1 ml of 0.1 M sodium borate, pH 10.5 at room temperature. After 3 hours of incubation, the mixture was dialyzed against 0.15 M NaCl. The labeled LPS was kept in small aliquots at –20 °C.

Binding of FITC-labeled BP-LPS to mouse macrophage cell line (RAW 264.7), human PBMC

The binding kinetics was performed as previously described (10). Briefly, the macrophage cells (5×10^5) were incubated at 37 °C with 0.5 μg/ml of FITC-labeled LPS obtained from *B. pseudomallei* or *E. coli*. At various time intervals, the cells were washed twice with cold PBS containing 0.1% sodium azide and 0.1% gelatin before

being analyzed by flow cytometer using LYSYSY II software performed on FACStarplus (Becton Dickinson, Mountain View, CA). The analysis was restricted to macrophage cells population according to their light scatter characters.

For the competition binding of polymyxin B with FITC-LPS, 1 μg/ml of polymyxin B sulfate (Sigma) was preincubated with 0.5 μg/ml of FITC-labeled LPS from *B. pseudomallei* or *E. coli* at 37 °C for 30 min. The mixture was incubated with mouse macrophage cells (5×10^5) at 37 °C for 0, 5, 15, 30, 45, 60 and 120 min, washed twice before being analyzed by flow cytometer. At each time point, binding percentage was calculated as follows: % binding =

$$\left[1 - \frac{\text{(Median Fluorescence Intensity from polymyxin B treated cells – Autofluorescence)}}{\text{(Median Fluorescence Intensity from polymyxin B untreated cells – Autofluorescence}}\right] \times 100$$

To determine the rate of binding of FITC-LPS to human PBMC, the cells ($1x10^6$) were incubated with 10 ng/ml of FITC-*E. coli*-LPS or FITC-BP-LPS for 5, 15, 30, 60 and 120 minutes. The samples were washed twice with cold PBS containing 0.1% sodium azide and 0.1% gelatin before being analyzed by flow cyotmeter.

Preparation of heat-killed bacteria

Briefly, living bacteria was heated at 80 °C for 1 hour. The viability of bacteria was determined by colony counting.

Treatment of mouse macrophage cell line (RAW 264.7) with heat-killed bacteria

Mouse macrophage cells (1×10^6) were exposed to various ratio of heat-killed *B. pseudomallei* and heat-killed *E. coli* to macrophage cell. Eighteen hours after exposure, the supernatant was analyzed for NO and TNF-α release.

Kinetics of NO and TNF-α release from mouse macrophage cell line (RAW 264.7) with heat-killed bacteria

Mouse macrophage cells (1×10^6) were treated with heat-killed *B. pseudomallei* (bacteria : macrophage cell ratio, 100 :1), heat-killed *E. coli* (bacteria : macrophage cell ratio, 10:1) for 5, 15, 30 min, 1, 2 and 18 h at 37 °C. The cells were washed 3 times with PBS before incubating in the medium. At 18 h after the cells treated with heat-killed bacteria, the supernatant was analyzed for NO and TNF-α release. The cells were lyzed before subjecting to western blot analysis for iNOS.

Western blot analysis for phosphorylated p38

Mouse macrophage cells (1×10^7) were activated with BP-LPS (100 ng/ml) or *E. coli*-LPS (10 ng/ml) for 5, 15, 30 and 60 min at 37 °C. After stimulation, the cells were washed twice with ice-cold PBS containing 1 mM Na_3VO_4. The cells were lyzed with 400 µl of ice-cold lysis buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% triton x-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml leupeptin) (24). The lysates (35 µg) were subjected to electrophoresis in 10% SDS-PAGE before transfered to PVDF membrane (Bio-Rad, Hercules, CA). The membrane was blocked in 5 % skim milk for 1 h followed by reacted with MoAb specific to pp38 (Santa Cruz, Santa Cruz, CA) at 4 °C overnight. The membrane was then reacted with horseradish peroxidase-conjugated rabbit anti-mouse IgG (Dako, Glostrup, Denmark) for 1 h. The reaction was detected by enhanced chemiluminescence as recommended by the manufacturer (Pierce, Rockford, IL).

Infection of mouse macrophage cell line (RAW 264.7) with bacteria

Mouse macrophage cells (1×10^6) were infected with bacteria at various MOI (multiplicity of infection) for 1 hour. To remove extracellular bacteria, the cells were washed with 2 ml of PBS 3 times before incubating in DMEM containing 250 μg/ml kanamycin (GIBCO Labs). After 2 hours of incubation, the medium was replaced with DMEM containing 20 μg/ml kanamycin and incubated for an additional 5 hours. In order to inhibit phagocytosis, macrophage cells were preincubated with 2.5 μg/ml cytochalasin D (Sigma) for 1 hour before the bacteria were added. The same concentration of cytochalasin D was present in the medium throughout the experiment. The cells were lyzed before subjecting to immunoblotting while the supernatant was used for TNF-α determination.

RT-PCR

Mouse macrophage cells (3×10^6) were stimulated with BP-LPS (100 ng/ml) and *E. coli*-LPS (10 ng/ml) for 15, 30, 60 and 120 min at 37 °C before replaced with media containing only 10% FBS. After 9 h, the cells were extracted with trizol reagent (GIBCO Labs) for total RNA isolation. The extracted RNA was subsequently treated with DNase (Promega, Madison, WI) according to the manufacturer's instructions before used for cDNA synthesis by AMV reverse transcriptase (Promega) (25).

The PCR reaction was conducted by using cDNA as template for iNOS and TNF-α amplification by a GeneAmp PCR System 2400 (Perkin Elmer, Norwalk, CT). The primers for iNOS were: sense 5′CCG AAG TTT CTT GTG GCA GCA GCG-3′, anti-sense 5′GAG CCT CGT GGC TTT GGG CTC CTC-3′ and for TNF-α were: sense

5 AGC CCA CGT CGT AGC AAA CCA CCA A-3', anti-sense 5'ACA CCC ATT CCC TTC ACA GAG CAA T-3'. The amplified products were electrophoresed on 1.8% agarose gel before transferred to Hybond-N+ membrane (Amersham, UK). The membranes were prehybridized in buffer containing 1% BSA, 7% SDS, 1mM EDTA, 0.5 M phosphate buffer at 60 °C for 2 h prior to hybridizing at 60 °C overnight with radiolabeling ^{32}P-ATP oligonucleotide probes of iNOS (5'ACG TTC AGG ACA TCC TGA AAA AGC AGC TGG-3') or TNF-α (5'CTG GAA GAC TCC TCC CAG GTA TAT GGG-3'). Thereafter, the membranes were washed and subjected to autoradiography (25).

RESULTS

1. Activation of mouse macrophage cell line (RAW 264.7) with LPS

1.1 BP-LPS stimulates NO and TNF-α release

To determine if the LPS isolated from *B. pseudomallei* could induce NO and TNF-α release, the macrophage cells were first exposed to various concentrations of BP-LPS at 37 °C for 18 h and the supernatant was analyzed for NO and TNF-α. When these cells were stimulated with the BP-LPS, the production of these mediators was detectable from a dose as low as 1 ng/ml, reaching a plateau level at 100 ng/ml, while those of the cells stimulated with the *E. coli*-LPS or *S. typhi*-LPS reached the plateau level at 10 ng/ml (Fig. 1). Judging from these results, it appears that macrophage activating activity of BP-LPS was one order magnitude weaker than those of the *E. coli*-LPS or *S. typhi*-LPS. Based upon these findings, subsequent experiments were carried out using 100 ng/ml of

1A

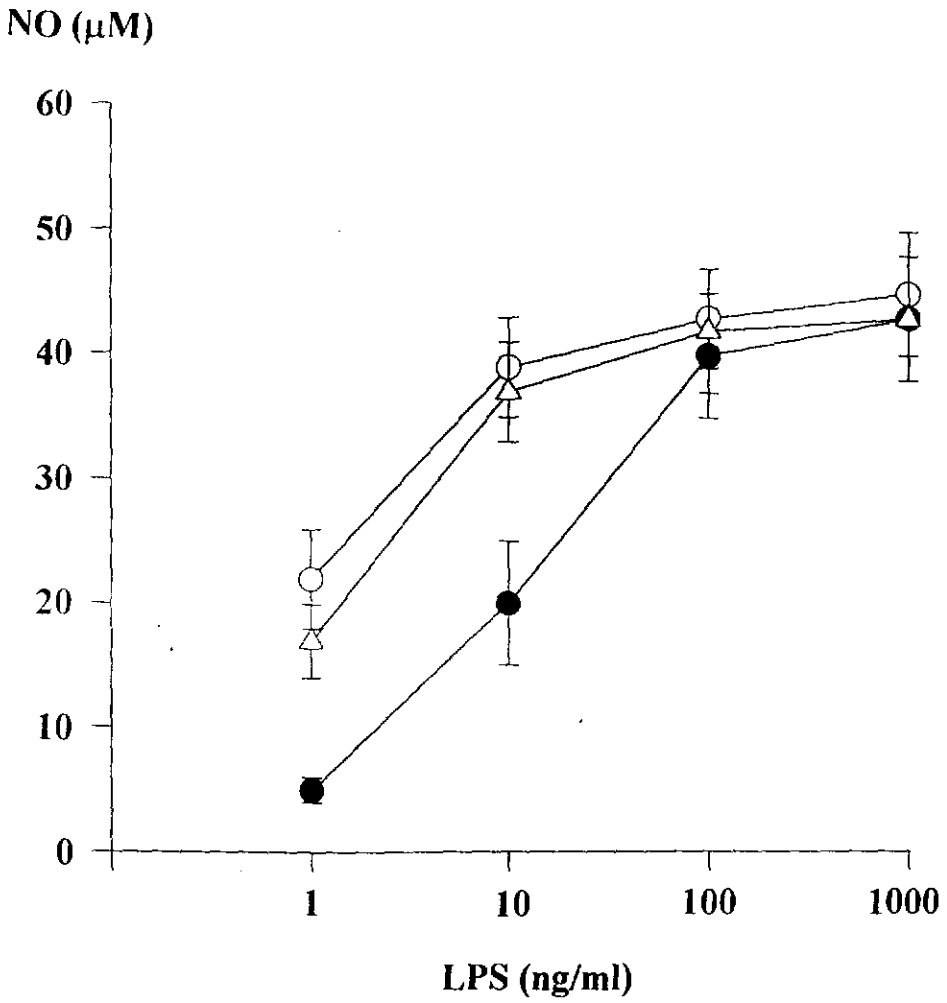


Fig. 1 NO and TNF-α release from mouse macrophage cells (RAW 264.7) activated by LPS. The macrophage cells were treated with various concentrations of BP-LPS (•), *E. coli*-LPS (o) or *S. typhi*-LPS (Δ). After 18 h of activation, the supernatant was analyzed for NO (A) and TNF-α release (B).

1B

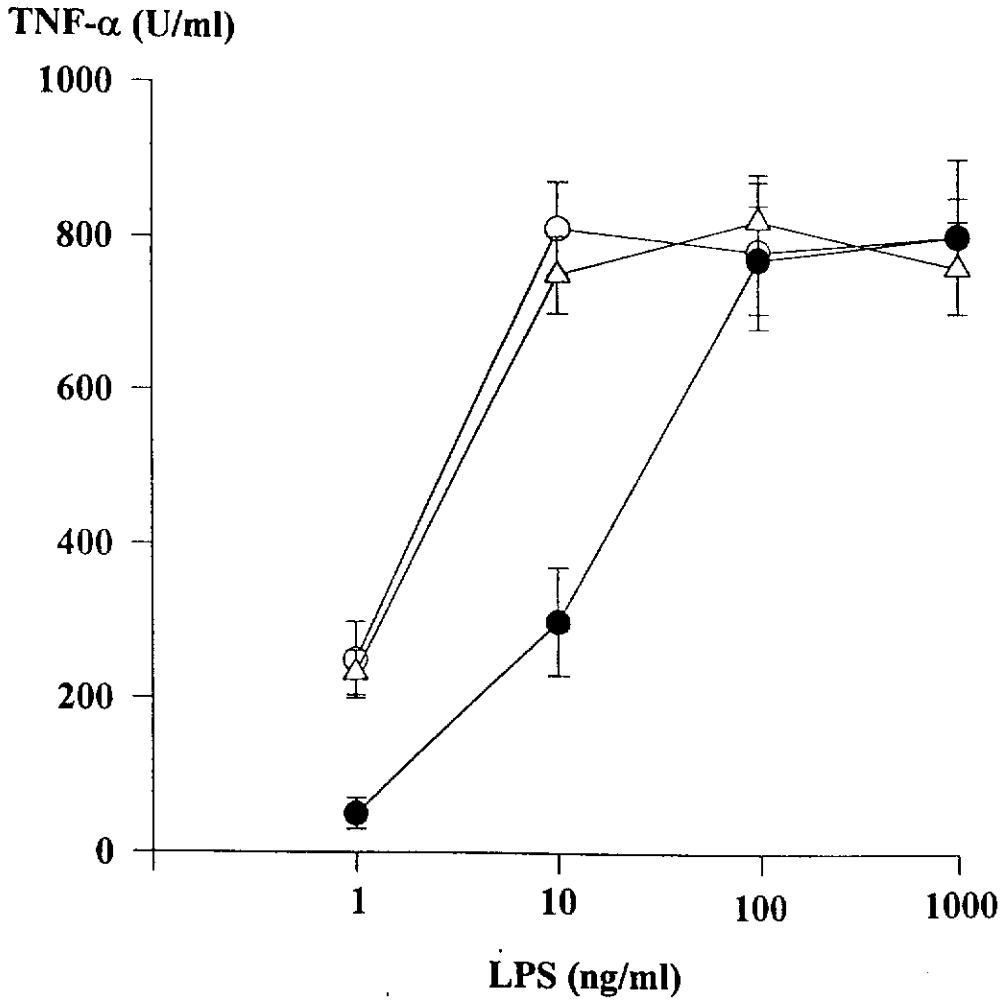


Fig. 1 NO and TNF-α release from mouse macrophage cells (RAW 264.7) activated by LPS. The macrophage cells were treated with various concentrations of BP-LPS (•), *E. coli*-LPS (o) or *S. typhi*-LPS (Δ). After 18 h of activation, the supernatant was analyzed for NO (A) and TNF-α release (B).

BP-LPS and 10 ng/ml of *E. coli*-LPS.

1.2 Kinetics of NO and TNF-α release by mouse macrophage cell line (RAW 264.7) activated with BP-LPS

Mouse macrophages were stimulated with either BP-LPS (100 ng/ml) or *E. coli*-LPS (10 ng/ml) for 5, 15, 30 min, 1, 2 and 4 h. The supernatants were at times indicated, analyzed for NO and TNF-α. The results showed that the BP-LPS stimulated NO or TNF-α release at a significantly slower rate (Fig. 2). The time required to activate the NO or TNF-α release was between 30-60 min before reached the plateau after 2 h. Unlike BP-LPS, the mouse macrophage cells activated with *E. coli*-LPS exhibited a faster kinetic rate. The time required to stimulate NO or TNF-α release was less than 5 min and reached a plateau level within 30-60 min. Although the BP-LPS concentration used was 10 times higher than that of the *E. coli*-LPS, the maximum concentrations of NO, TNF-α release from macrophages activated by these two LPS were not significantly different from one another.

1.3 Kinetics of iNOS production from mouse macrophage cell line (RAW 264.7) activated by BP-LPS

In order to analyze the kinetics of iNOS production, macrophage cells were activated by BP-LPS (100 ng/ml) or *E. coli*-LPS (10 ng/ml) at 37 °C for 15, 30, 60, 120 min and 18 h. At the end of each incubation period the cells were washed 3 times with PBS and incubated further for a total of 18 h before lysis buffer was added as described under Materials and Methods. The cell extracts were electrophoresed on a 8% SDS

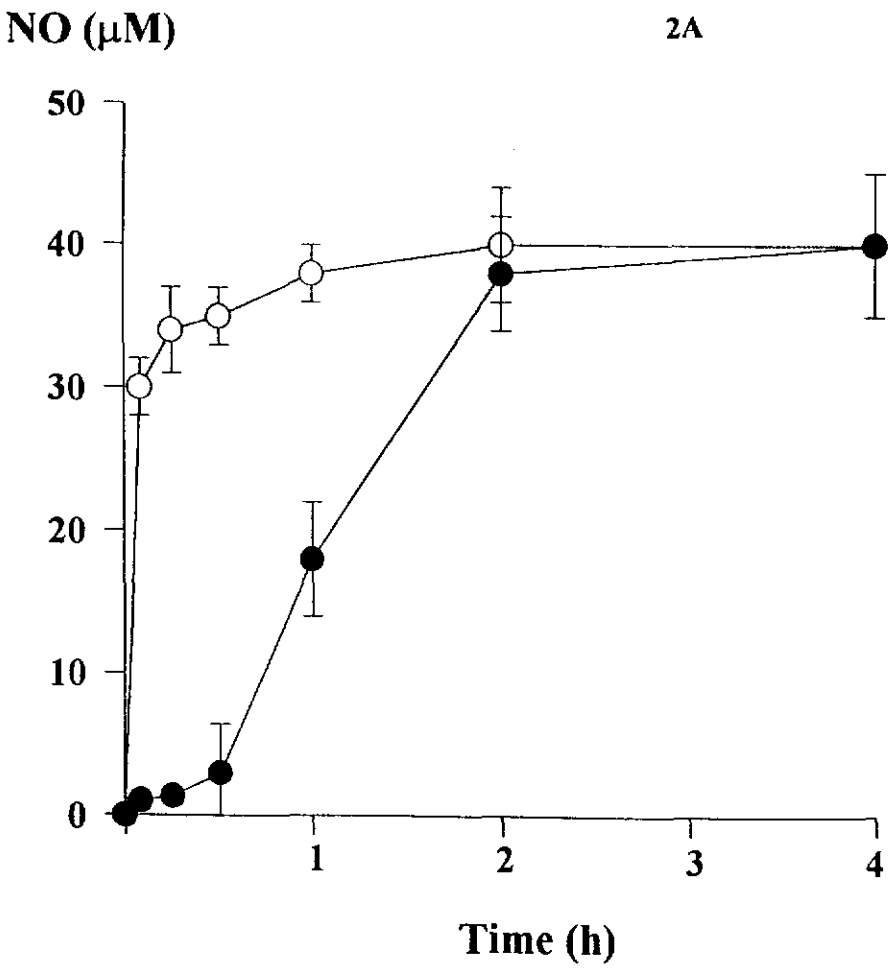


Fig. 2 Kinetics of NO and TNF-α release from mouse macrophage cells (RAW 264.7) activated by LPS. Macrophage cells were preincubated shortly with BP-LPS (100 ng/ml) (•) or *E. coli*-LPS (10 ng/ml) (o) for 5, 15, 30 min, 1, 2 and 4 h. To remove LPS the cells were washed with PBS 3 times before replaced with medium containing 10% FBS. After 18 h of activation, the supernatant was analyzed for NO (A) or TNF-α release (B).

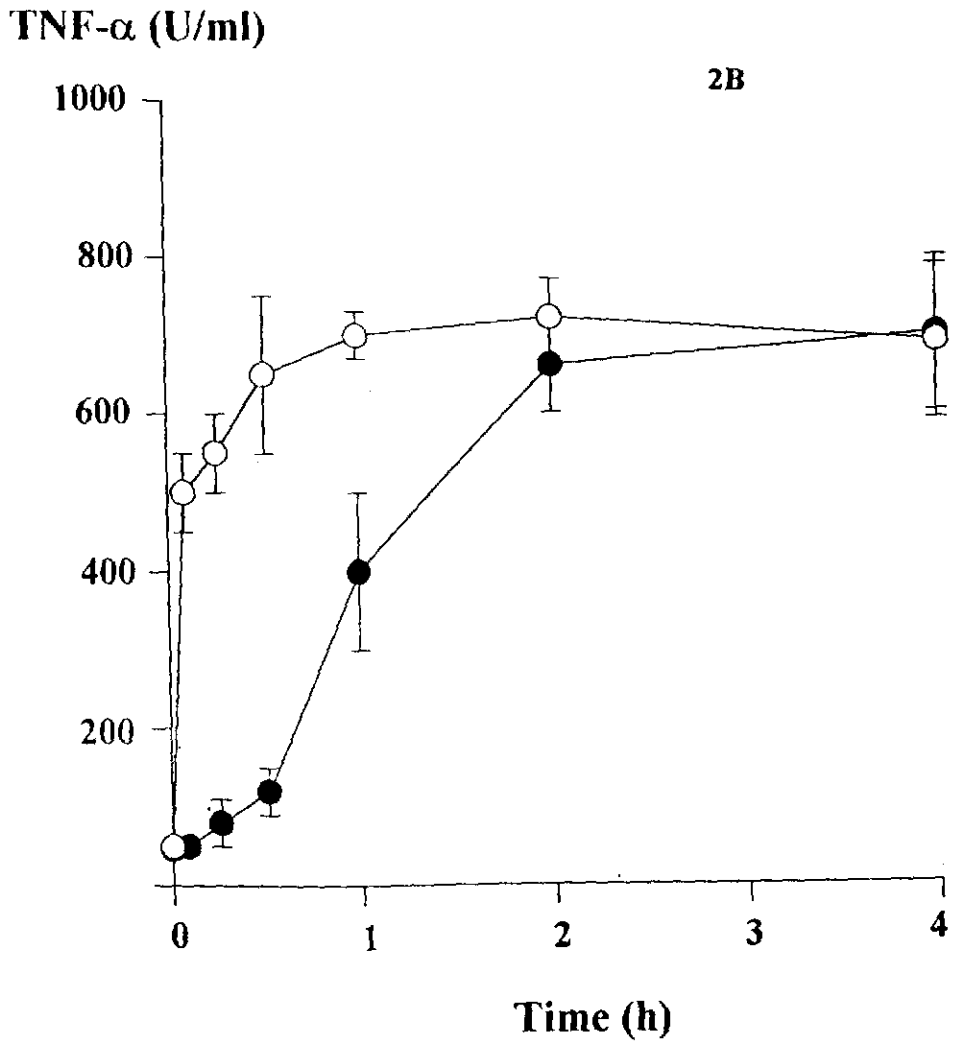


Fig. 2 Kinetics of NO and TNF-α release from mouse macrophage cells (RAW 264.7) activated by LPS. Macrophage cells were preincubated shortly with BP-LPS (100 ng/ml) (•) or *E. coli*-LPS (10 ng/ml) (o) for 5, 15, 30 min, 1, 2 and 4 h. To remove LPS the cells were washed with PBS 3 times before replaced with medium containing 10% FBS. After 18 h of activation, the supernatant was analyzed for NO (A) or TNF-α release (B).

polyacrylamide gel before transferred to PVDF membrane. The samples then were probed with anti iNOS antibody as described. Fig. 3 shows that BP-LPS required at least 1 h to stimulate detectable level of iNOS while those activated with *E. coli*-LPS produced iNOS enzyme within 15 min of activation.

1.4 Kinetics of iNOS and TNF-α gene expression from mouse macrophage cell line (RAW 264.7) activated with BP-LPS

The slower rate of mediators released from BP-LPS activated macrophages indirectly implied that the BP-LPS may also upregulate the mRNA of iNOS and TNF-α with a slower rate. The results in Fig. 4 show that, at 37 °C, the cells activated with the BP-LPS (100 ng/ml) only slowly upregulated the mRNA of both iNOS and TNF-α, while those treated with the *E. coli*-LPS expressed almost a maximum level of iNOS and TNF-α mRNA within 15 min. The level of β-actin, served as an internal control, was not influenced by LPS.

1.5 Time course studies of p38 phosphorylation from macrophages activated with BP-LPS

The kinetics difference in the production of NO and TNF-α and the level of iNOS and TNF-α mRNA by the macrophage activated with BP-LPS or *E. coli*-LPS may have been caused by the interference at a signal transduction rate. This possibility was investigated by comparing the rate of p38 phosphorylation. To determine the kinetics of signal transduction, the macrophage cells were activated with BP-LPS (100 ng/ml) or *E. coli*-LPS (10 ng/ml) for 5, 15, 30 and 60 min. Immediately, the cells were lyzed in lysis

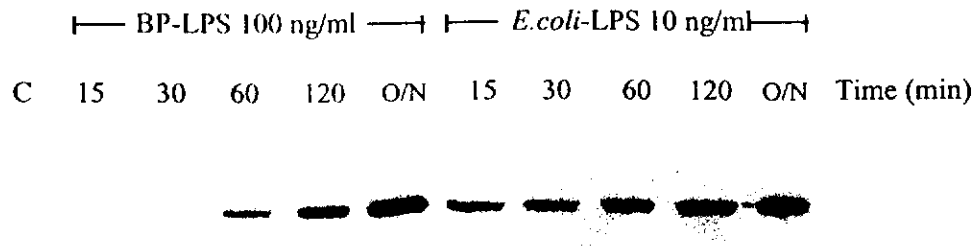


Fig. 3 The kinetics of iNOS production from mouse macrophage cells (RAW 264.7) activated by BP-LPS or *E. coli*-LPS. Mouse macrophage cells were activated by BP-LPS (100 ng/ml) or *E. coli*-LPS (10 ng/ml) for 15, 30, 60, 120 minutes and 18 hours. The cells were replaced with medium containing 10% FBS. At 18 hours after activation, the cells were lyzed and subjected to 8% SDS-PAGE, and the proteins were analyzed by anti-iNOS immunoblotting. Control (C) was the untreated cells with LPS.

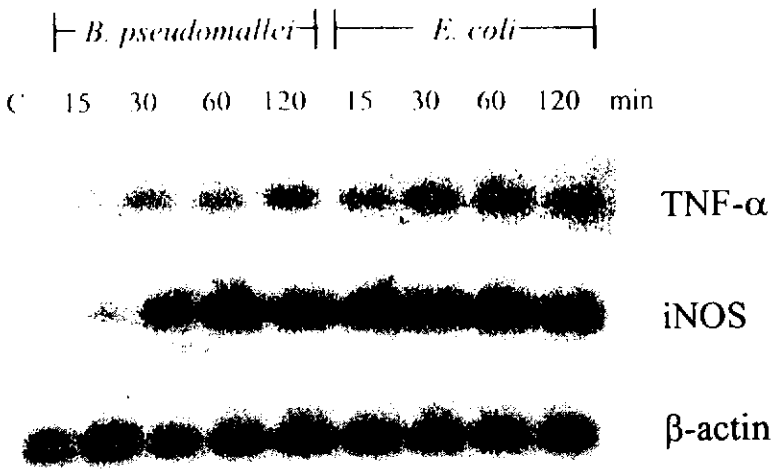


Fig. 4 Kinetics of iNOS and TNF-α mRNA expression from the mouse macrophage cells (RAW 264.7) activated by LPS. The macrophage cells were activated with BP-LPS (100 ng/ml) or *E. coli*-LPS (10 ng/ml) for 15, 30, 60 and 120 min. After 9 h of incubation, the cDNA was synthesized from macrophage cells RNA before subjected to PCR following by hybridized with appropriate radiolabeled probes. The β-actin served as an internal control.

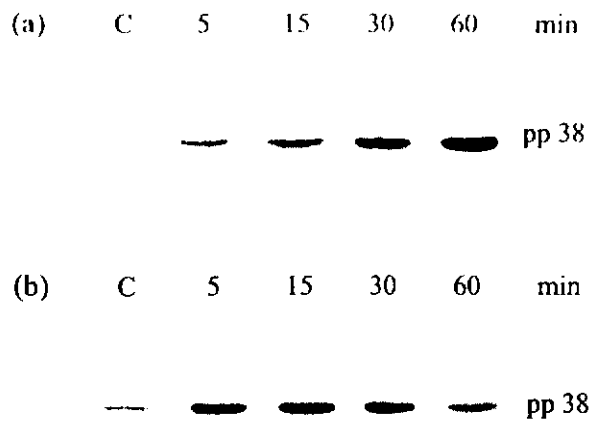


Fig. 5 Phosphorylation of p38 from mouse macrophage cell (RAW 264.7) activated by LPS. The macrophage cells were treated with BP-LPS (100 ng/ml) (a) or *E. coli*-LPS (10 ng/ml) (b) for 5, 15, 30 and 60 min. Phosphorylation of p38 was analyzed by immunobloting using MoAb to pp38.

buffer as described. The samples were immunoblotted using MoAb to pp38 and the results show that the pp38 was gradually phosphorylated and reached a maximum after 60 min following activation by BP-LPS (Fig. 5a). On the other hand, the phosphorylation of p38 from the cells activated with *E. coli*-LPS reached the maximum only after 5-15 min of activation (5b Fig.). These results suggested that the activation of cells by BP-LPS had a time lag delay of signal transduction longer comparing with the cells activated with *E. coli*-LPS.

1.6 Effect of polymyxin B on NO release from mouse macrophage cell line (RAW 264.7) activated by BP-LPS

To investigate the effect of polymyxin B on LPS activating macrophage cells, various concentrations of polymyxin B were mixed with BP-LPS (100 ng/ml) or *E. coli*-LPS (100 ng/ml) and allowed to incubate at 37°C for 1 h before adding to the cell suspension. After 18 h of incubation, the supernatant was analyzed for NO release. At 10 μg/ml of polymyxin B, macrophage cells stimulated with BP-LPS was able to release NO at 60% of control (without polymyxin B) (Fig. 6). However, at the same polymyxin B concentration, the cells activated by *E. coli*-LPS was able to release NO only 10% of control. This result indicates that polymyxin B was a less potent inhibitor for moacrophage response by BP-LPS than *E. coli*-LPS.

1.7 BP-LPS stimulated TNF-α release from human PBMC

Human PBMC were incubated with various concentrations of LPS at 37°C for 18 h. The supernatant was determined for TNF-α as described under Materials and Methods.

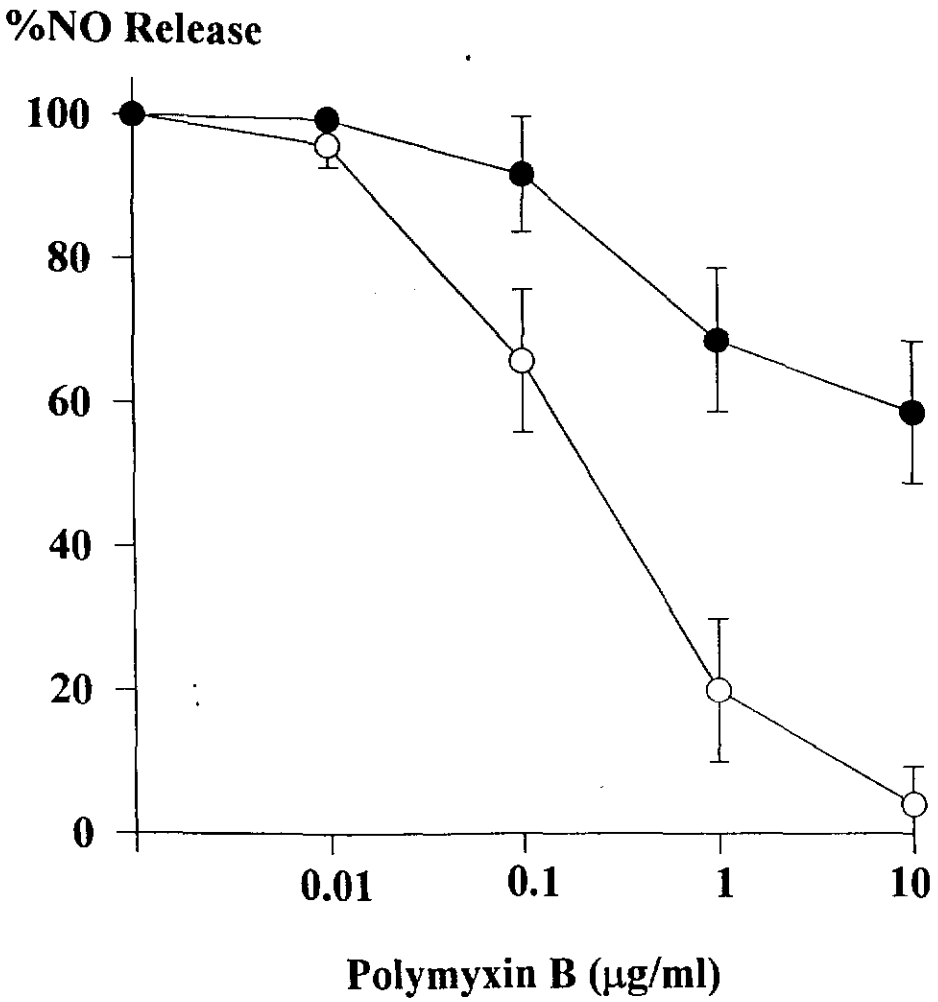


Fig. 6 Effect of polymyxin B on NO release from mouse macrophage cells (RAW 264.7) activated by LPS. BP-LPS (•)(100 ng/ml) or *E. coli*-LPS (o)(100 ng/ml) were premixed with various concentraion of polymyxin B at 37°C for 1 hour. The mixtures were added to macrophage cells and incubated for 18 hours. The supernatants were analyzed for NO release.

The TNF-α released from the cells activated with BP-LPS was detected at BP-LPS concentration of 1 ng/ml and reached the plateau level at 100 ng/ml (Fig. 7). This result suggests that macrophage activation activity of BP-LPS was one order magnitude weaker than those of *E. coli*-LPS. These data also consistant with mouse macrophage cell line activated with BP-LPS.

2. Binding of FITC-BP-LPS to mouse macrophage cell line (RAW 264.7)

2.1 Kinetics of FITC-BP-LPS binding to mouse macrophage cell line (RAW 264.7)

Mouse macrophage cells were incubated with 0.5 μg/ml of FITC-BP-LPS or FITC-*E. coli*-LPS in the medium containing 10% serum as a LBP source. The cells were washed 3 times with PBS before analyzing the binding with flow cytometer. The binding kinetics of FITC-BP-LPS and FITC-*E. coli*-LPS were not significantly different. They both started slowly and reached the equilibrium within 45-60 min (Fig. 8).

2.2 Effect of polymyxin B on FITC-LPS binding to mouse macrophage cell line (RAW 264.7)

To investigate the effect of polymyxin B on LPS binding to macrophage cells, FITC-BP-LPS or FITC-*E. coli*-LPS (0.5 μg/ml) was mixed with polymyxin B (1 μg/ml) at 37°C for 30 min before adding to the mouse macrophage cell line. After incubation for 5, 15, 30, 45, 60 and 120 min, the reaction was terminated with ice cold azide-PBS, cells were analyzed for associated FITC. The percentage of FITC-LPS binding was calculated as described in Materials and Methods. Fig. 9 shows that polymyxin B was less potent to inhibit FITC-BP-LPS binding to the macrophage cells than FITC-*E. coli*-

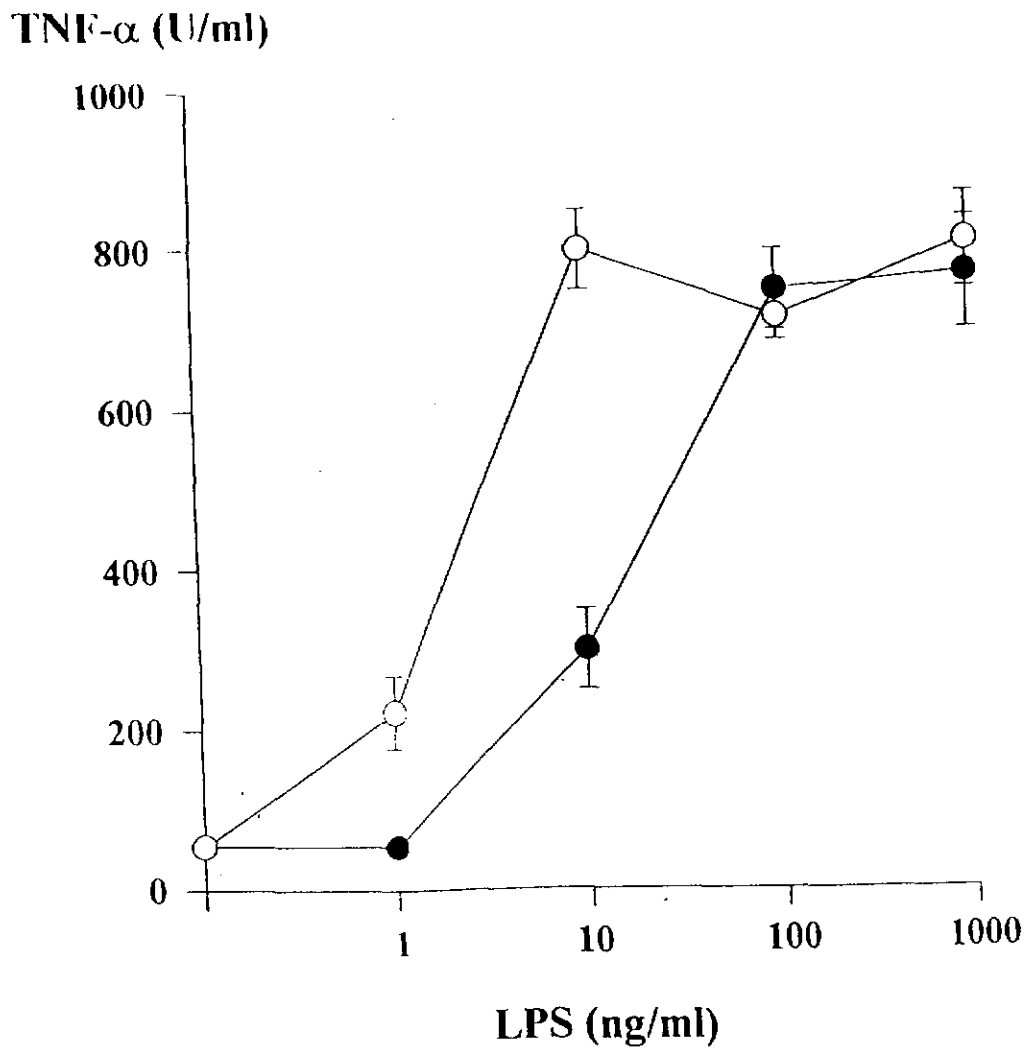


Fig. 7 TNF-α release from human PBMC activated by LPS. Human PBMC were treated with various concentrations of BP-LPS (•), *E. coli*-LPS (o). After 18 hours, the supernatant was analyzed for TNF-α release.

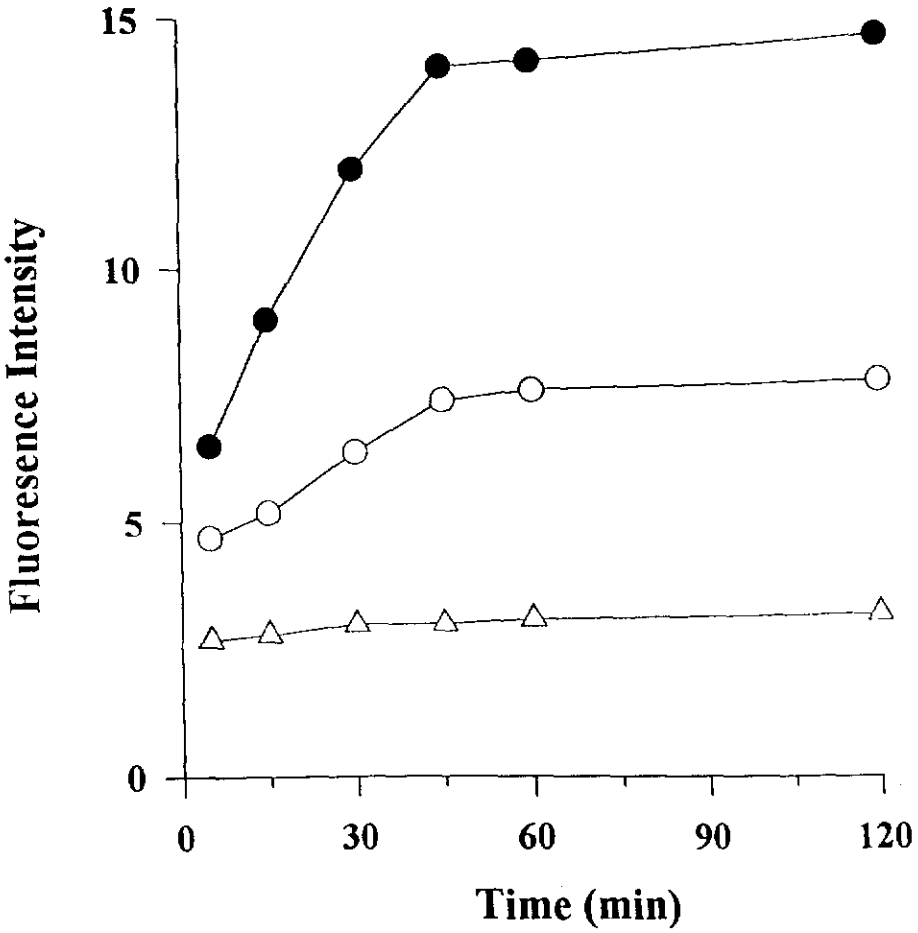


Fig. 8 Kinetics of FITC-LPS binding to mouse macrophage cells (RAW 264.7). Mouse macrophage cells (5×10^5) were incubated for 5, 15, 30, 45, 60 and 120 minutes with 0.5 μg/ml FITC-BP-LPS (•), FITC-*E. coli*-LPS (o) in DMEM supplemented with 10% FBS. Binding was measured by flow cytometry and results are expressed as mean fluorescence units of mouse macrophage cells. (Δ) represents autofluorescence. This experiment is representative of 5 separate experiments.

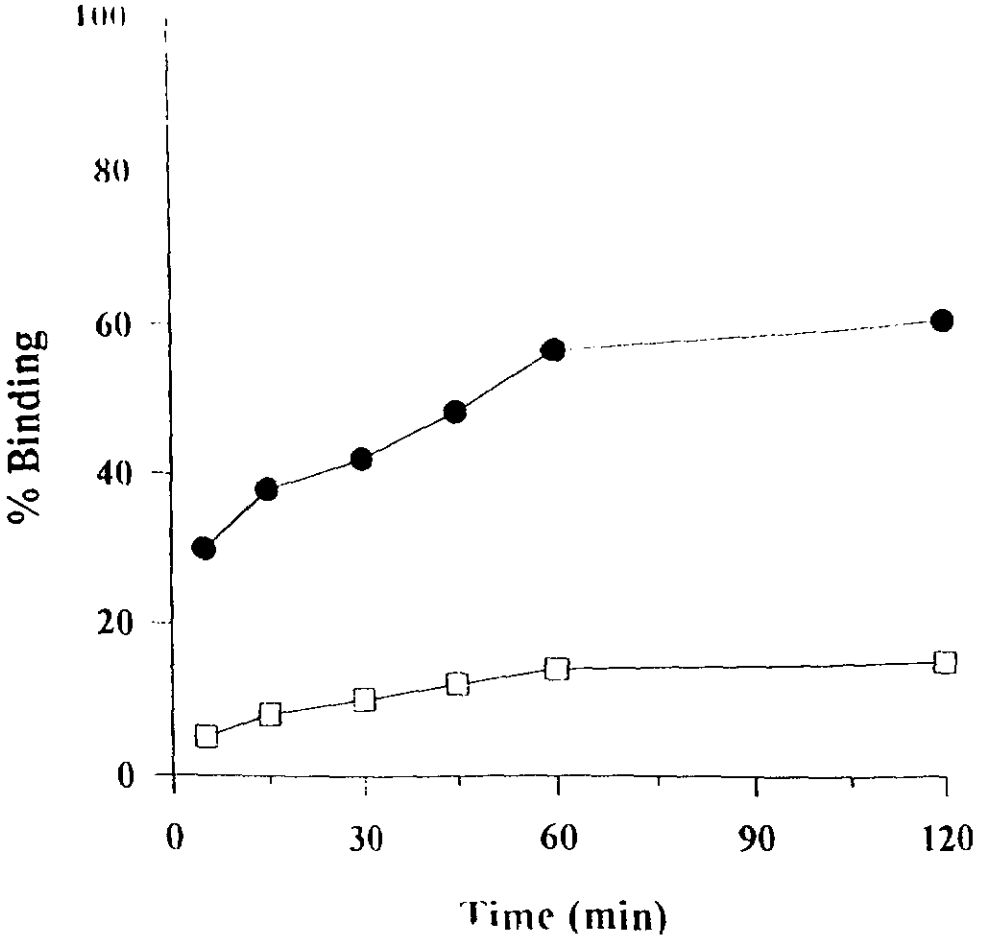


Fig. 9 Effect of polymyxin B on FITC-BP-LPS or FITC-*E. coli*-LPS binding to mouse macrophage cells (RAW 264.7). FITC-BP-LPS (•) or FITC-*E. coli*-LPS (o) was preincubated with 1 μg/ml polymyxin B for 30 minutes at 37 °C before being added to mouse macrophage cells (5×10^5). The cells were incubated for 5, 15, 30, 45, 60 and 120 minutes, then washed with ice-cold azide-PBS before analyzed by flow cytometer. The binding percentage was calculated as described in Materials and Methods. This experiment is representative of 3 separate experiments.

LPS. At 1 μg/ml of polymyxin B, this drug almost completely inhibit FITC-*E. coli*-LPS binding, while it has significantly less effect on FITC-BP-LPS binding. This result indicates that polymyxin B has less inhibitory effect on FITC-BP-LPS binding to mouse macrophage cell lines than FITC-*E. coli*-LPS.

2.3 Kinetics binding of FITC-BP-LPS to human PBMC

Human PBMC were incubated with 10 ng/ml of FITC-BP-LPS or FITC-*E. coli*-LPS in the medium containing 10% human serum. The cells were washed 3 times with PBS before analyzing the FITC-LPS binding with flow cytometer. Both FITC-LPS slowly bound to the cells and reached the equilibrium within 60 min of incubation (Fig. 10). This result suggests that the rate of BP-LPS binding was not significantly different from the binding rate of FITC-*E. coli*-LPS.

2.4 Competitive binding of FITC-BP-LPS with anti-CD14, E. coli-LPS

To determine the competitive binding of FITC-BP-LPS and FITC-*E. coli*-LPS with anti-CD14 antibody (MY4), human PBMC were incubated with FITC-BP-LPS (10 μ g/ml) or FITC-*E. coli*-LPS (1 μg/ml) in the present of various concentrations of CD14 Ab. The cells were washed with PBS before analyzing by flow cytometer. Fig. 11A, B show that CD14 Ab was able to inhibit the binding of both FITC-BP-LPS and FITC-*E. coli*-LPS with the IC_{50} (50% inhibition) of 0.7 μg/ml and 0.5 μg/ml of CD14 Ab respectively. This result indicated that BP-LPS, similar to other gram negative bacteria LPS, use CD14 on the cell surface as their receptors.

To determine the relative binding affinity of BP-LPS comparing with *E. coli*-LPS,

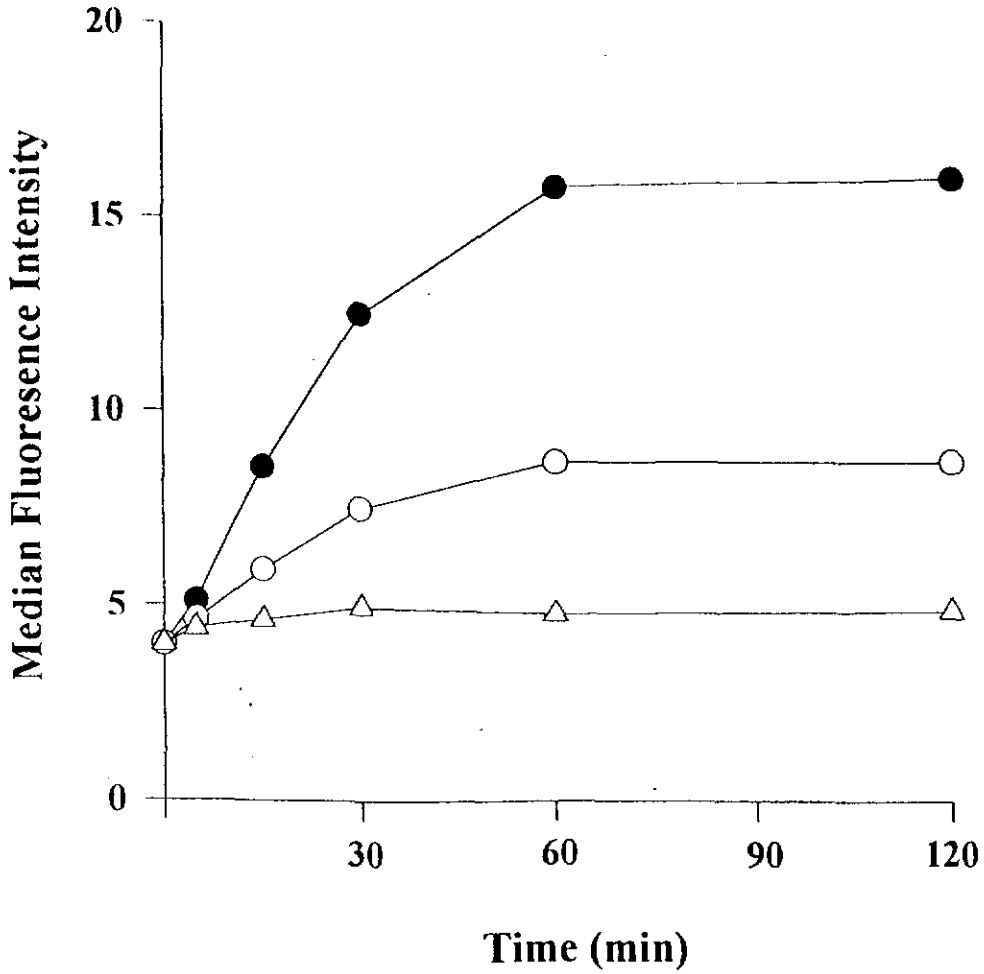


Fig. 10 Kinetics of FITC-LPS binding to human PBMC. Human PBMC ($1x10^6$) were incubated for 5, 15, 30, 60 and 120 minutes with 0.5 µg/ml FITC-BP-LPS (•), FITC-*E. coli*-LPS (o) om DMEM supplemented with 10% human serum. Binding was measured by flow cytometry and results are expressed as mean fluorescence units of cells. (Δ)j represents autofluorescence.

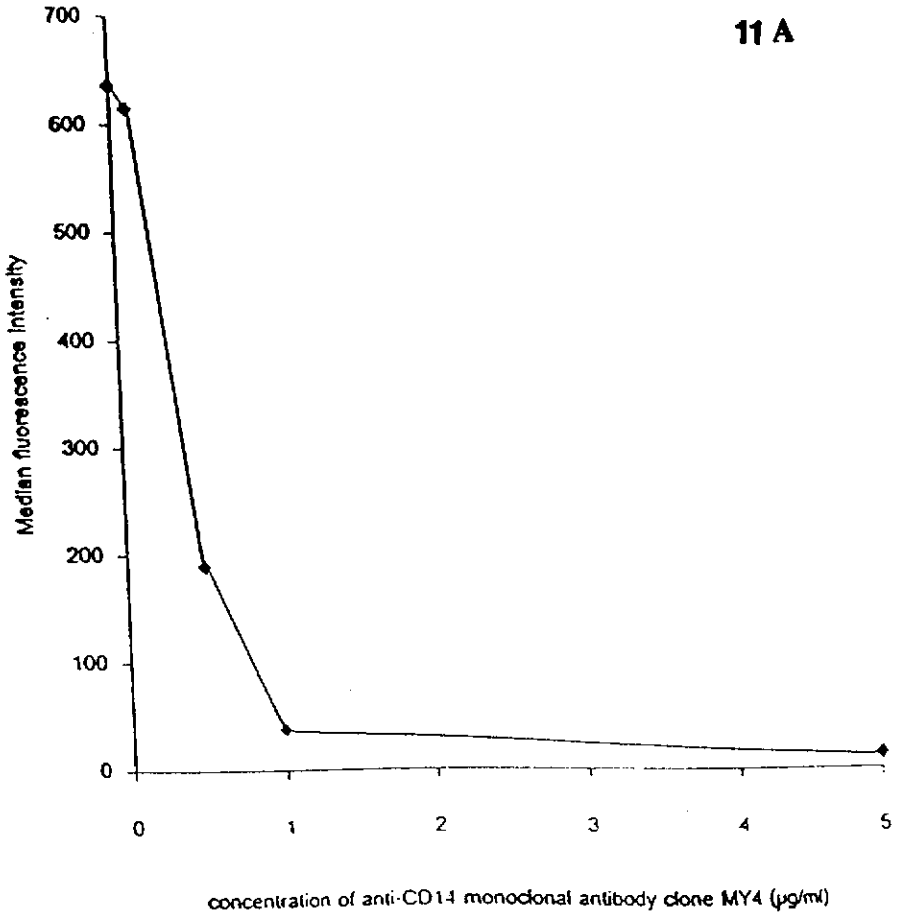


Fig. 11 Competitive binding assay between unlabelled anti-CD14 antibody and FITC-*E. coli*-LPS, FITC-BP-LPS. Human PBMC($1x10^6$) were incubated with FITC-*E. coli*-LPS (1 µg/ml)(A) or FITC-BP-LPS (10µg/ml)(B) in the present of various concentrations of unlabelled anti-CD14 antibody for 1 hour. The cells were washed 3 times with PBS before analyzing by flow cytometry.

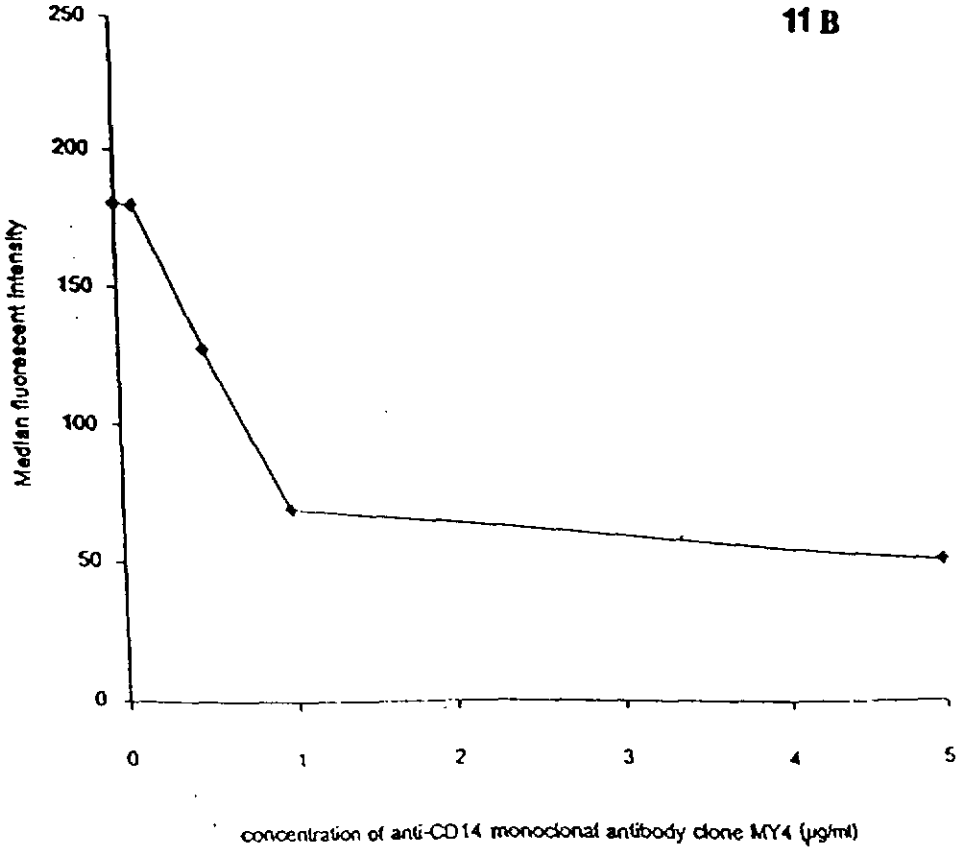


Fig. 11 Competitive binding assay between FITC-*E. coli*-LPS and unlabelled *E. coli*-LPS, unlabelled BP-LPS. Human PBMC ($1x10^6$) were incubated with FITC-*E. coli*-LPS (1µg/ml) in the present of various concentration of unlabelled *E. coli*-LPS or unlabelled BP-LPS for 1 hour. The cells were washed 3 times with PBS before analyzing by flow cytometry.

the human PBMC were incubated with FITC-*E. coli*-LPS (1 μg/ml) in the present of various concentrations of unlabelled *E. coli*-LPS or BP-LPS. After 1 h of incubation, the cells were washed 3 times with PBS before analyzing by flow cytometer. Fig. 12 shows that both unlabelled *E. coli*-LPS and BP-LPS was able to inhibit the binding of FITC-*E. coli*-LPS with IC_{50} of 1.3 μg/ml and 0.8 μg/ml of unlabelled *E. coli*-LPS and BP-LPS respectively. This result indicates that BP-LPS interact to the same binding epitope on the CD14 as *E. coli*-LPS. Moreover, the IC_{50} of BP-LPS was not significantly different comparing with *E. coli*-LPS which indirectly suggests that BP-LPS interact to the cells with similar binding affinity as *E. coli*-LPS.

To investigate the effect of polymyxin B on the binding of FITC-LPS, the human PBMC were incubated with FITC-BP-LPS (1 μg/ml) or FITC-*E. coli*-LPS (1 μg/ml) in the present of various concentrations of polymyxin B. The cells were washed 3 times with PBS before analyzing by flow cytometer. The result, shown in Fig. 13, indicated that Polymyxin B (10 μg/ml) almost completely inhibited the binding of FITC-*E. coli*-LPS. On the other hand, this drug had little effects on the binding of FITC-BP-LPS to the cells. At 50 μg/ml of Polymyxin B, the binding of FITC-BP-LPS to the cells was as high as 60% of control. This result indirectly suggets the differences in the structure of BP-LPS and *E. coli*-LPS.

3. Activation of mouse macrophage cell line (RAW 264.7) with heat-killed bacteria

3.1 Kinetics of NO and TNF-α release by mouse macrophage cell line (RAW 264.7) activated by heat-killed bacteria

To determine the kinetics of macrophage activation by heat-killed bacteria,

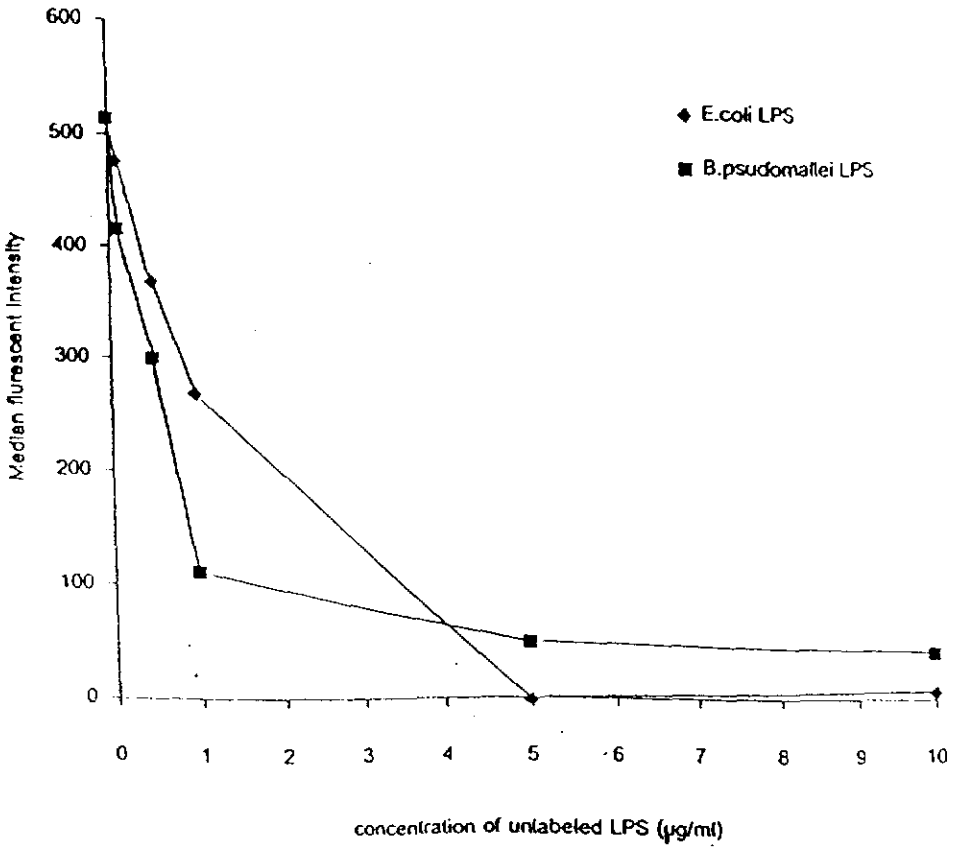


Fig. 12 Competitive binding assay between FITC-*E. coli*-LPS and unlabelled *E. coli*-LPS, unlabelled BP-LPS. Human PBMC ($1x10^6$) were incubated with FITC-*E. coli*-LPS (1μg/ml) in the present of various concentration of unlabelled *E. coli*-LPS or unlabelled BP-LPS for 1 hour. The cells were washed 3 times with PBS before analyzing by flow cytometry.

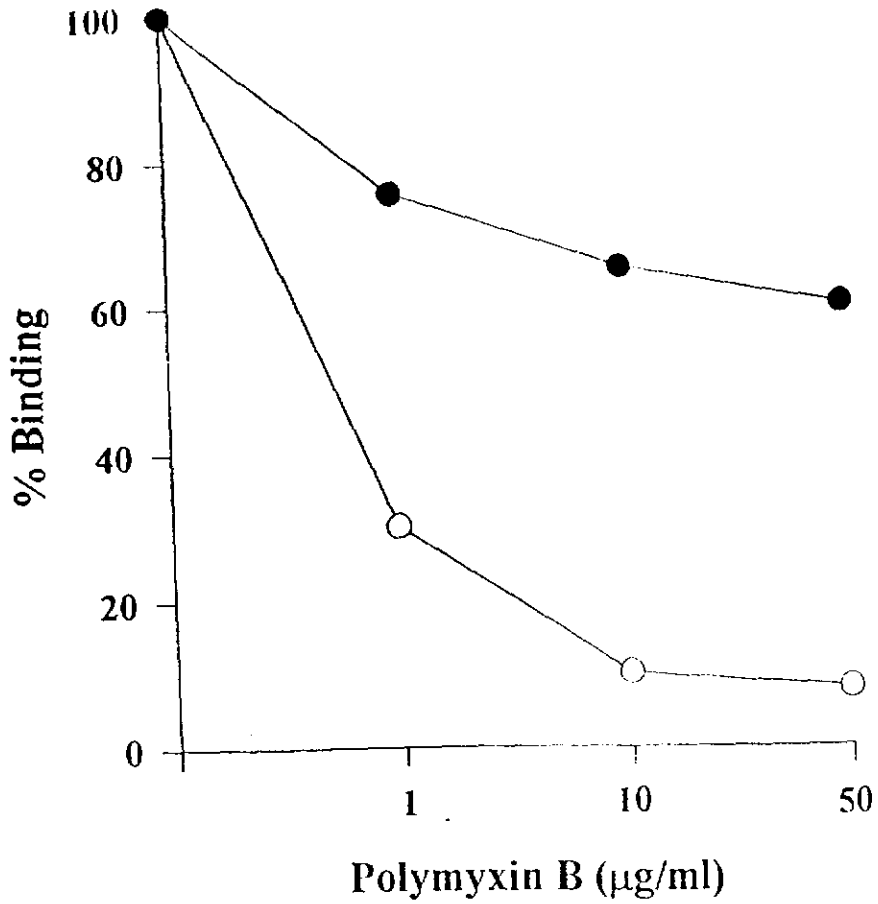


Fig. 13 Effect of polymyxin B on FITC-BP-LPS or FITC-*E. coli*-LPS binding to human PBMC. FITC-BP-LPS (1μg/ml)(•) and FITC-*E. coli*-LPS (1μg/ml)(o) .was preincubated with various concentrations of polymyxin B for 1 hour before being added to the cells. After 1 hour of incubation, the samples were washed 3 times with PBS before analyzing by flow cyotmetry.

macrophage cells were incubated with heat-killed *B. pseudomallei* (bacteria : macrophage cell ratio, 100 :1), heat-killed *E. coli* (bacteria : macrophage cell ratio, 10:1) for 5, 15, 30, min, 1, 2 and 18 h. The cells were washed 3 times with PBS and incubated for 18 h. The supernatant was then analyzed for NO and TNF-α release. Results presented in Fig. 14 showed that the heat-killed *B. pseudomallei* was not only less potent in stimulating NO or TNF-α release by one magnitude order but also the kinetics of the meidators release were significantly slower than the cells activated by heat-killed *E. coli*. The time required for heat-killed *E. coli* to activate NO and TNF-α release was within 5 min reaching a plateau between 30-60 min. Unlike the *E. coli*, heat-killed *B. pseudomallei* required at least 30 min to stimulate detectable NO and TNF-α release before reached the plateau at 2 h. When the cells were activated for 18 h with heat-killed *B. pseudomallei* at a bacteria to cell ratio of 100:1, the concentration of NO and TNF-α release was similar to those activated with heat-killed *E.coli* at a bacteria to cell ratio of 10:1. Activity for macrophage cells activation by heat-killed *B. pseudomallei* was weaker than heat-killed *E. coli* by one magnitude order.

3.2 Kinetics of iNOS production from mouse macrophage cell line (RAW 264.7) activated by heat-killed bacteria

In order to analyze the kinetics of iNOS production, mouse macrophage cells (1×10^6) were treated with heat-killed *B. pseudomallei* (bacteria : macrophage cell ratio, 100 :1), heat-killed *E. coli* (bacteria : macrophage cell ratio, 10:1) for 15, 30 min, 1, 2 and 18 h. At 18 h after the cells treated with heat-killed bacteria, the cells were lyzed before subjecting to western blot analysis for iNOS. Fig. 15 shows that BP-LPS required at least 1 h to stimulate detectable level of iNOS while those activated with *E. coli*-LPS produced

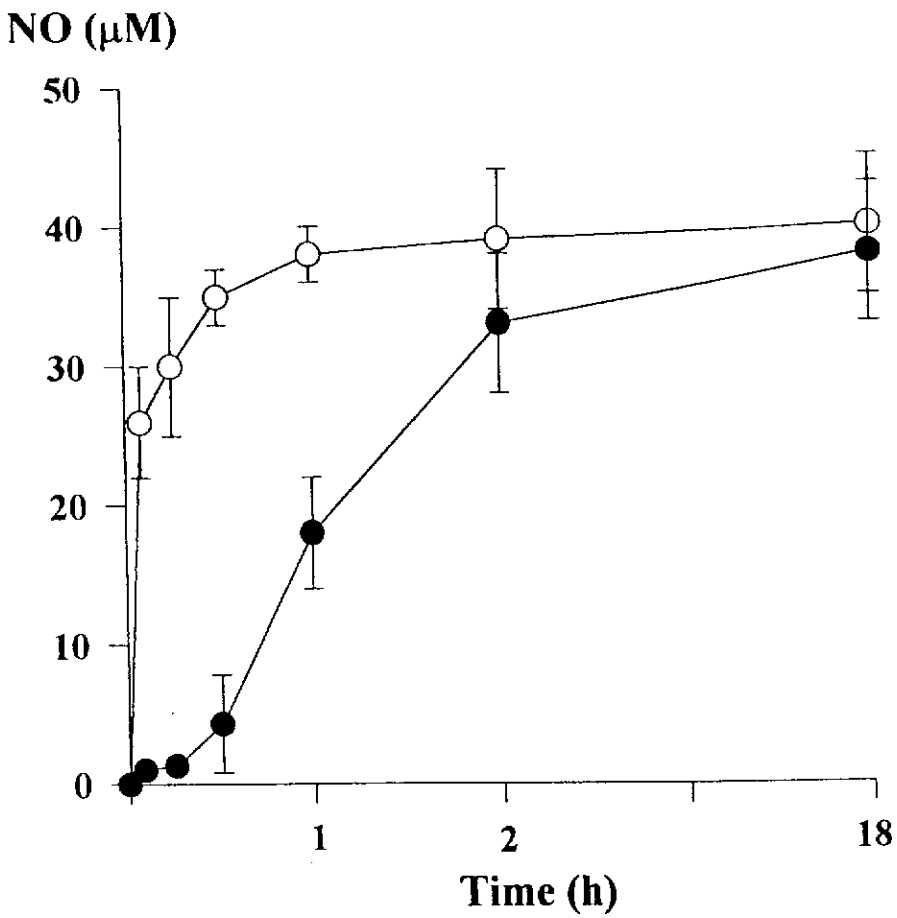


Fig. 14 The kinetics of NO, TNF-α release from mouse macrophage cells (RAW 264.7) activated by heat-killed *B. pseudomallei* (•) or heat-killed *E. coli* (o). Macrophage cells were incubated for 5, 15, 30 min, 1, 2 and 18 h with heat-killed *B. pseudomallei* or heat-killed *E. coli* at bacterial to cells ratio of 100:1 and 10:1 respectively. The cells were replaced with medium containing 10% FBS. At 18 h after infection, the supernatants were analyzed for NO (A) and TNF-α (B) release.

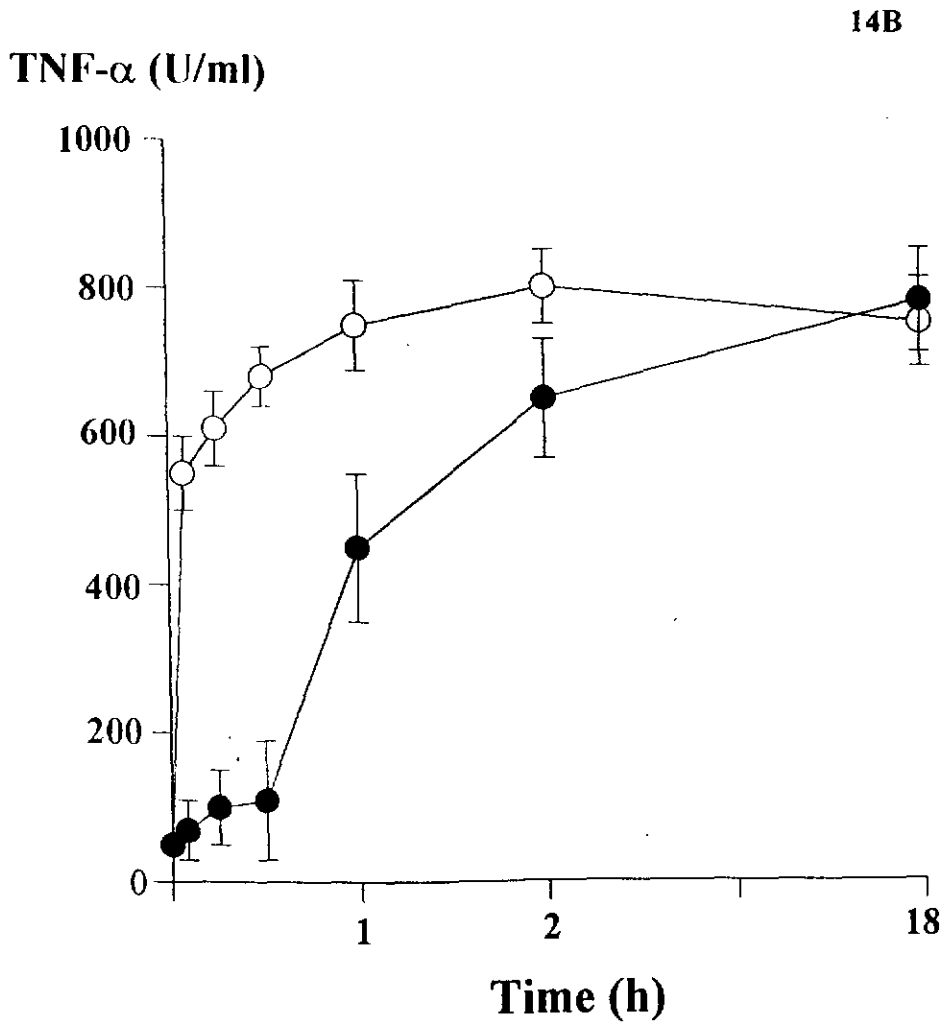
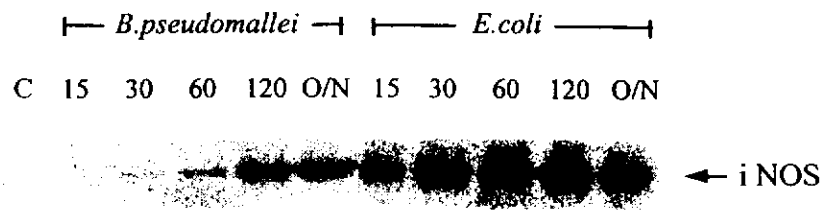


Fig. 14 The kinetics of NO, TNF-α release from mouse macrophage cells (RAW 264.7) activated by heat-killed *B. pseudomallei* (•) or heat-killed *E. coli* (o). Macrophage cells were incubated for 5, 15, 30 min, 1, 2 and 18 h with heat-killed *B. pseudomallei* or heat-killed *E. coli* at bacterial to cells ratio of 100:1 and 10:1 respectively. The cells were replaced with medium containing 10% FBS. At 18 h after infection, the supernatants were analyzed for NO (A) and TNF-α (B) release.


B.pseudomallei
E.coli
C 15 30 60 120 O/N 15 30 60 120 O/N
i NOS

iNOS enzyme within 15 min of activation.

4. Activation of mouse macrophage cell line (RAW 264.7) with bacteria

To determine if *B. pseudomallei* induces iNOS expression, the macrophage cells were first exposed to *B. pseudomallei* at 37 °C for 1 h and after washing 3 times with PBS, the cells were incubated in the medium containing 250 μg/ml of kanamycin for 2 h to kill extracellular bacteria. Subsequently, the supernatant was removed and fresh culture medium containing 20 μg/ml kanamycin was added and the cells were incubated for 5 more h before iNOS and TNF-α were analyzed. The expression of iNOS enzyme from macrophage cells infected with bacteria was determined by immunoblotting. The results presenteded in Fig. 16A showed that iNOS enzyme was not detected from macrophage cells infected with *B. pseudomallei* at MOI of 0.1:1, 1:1 or 10:1. On the other hand the macrophage cells infected with *E. coli* expressed detectable iNOS enzyme even at MOI of 0.1:1. It should be mentioned that during these time intervals, more than 90% of cells infected with both bacteria still survived, judged from trypan blue dye staining (data not shown). Inhibiting phagocytosis by cytochalasin D prior to infection with *B. pseudomallei*, the macrophage cells produced iNOS at MOI of 1:1 and 10:1 (Fig. 16B). In contrast, cytochalasin D did not have any effect on the iNOS expression on the cells infected with *E. coli*. The macrophage cells infected with *B. pseudomallei* expressed significantly less iNOS than the cells infected with *E. coli* at the same MOI. These results indicate that signalling iNOS expression was initiated upon the contact between bacteria and macrophages. It should be noted that although the iNOS expression was observed during this time interval the macrophage did not produce sufficient NO

16A

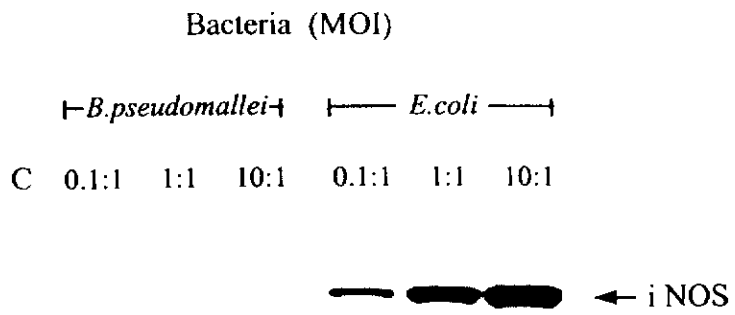


Fig. 16 Expression of iNOS from mouse macrophage cells (RAW. 264.7) infected by *B. pseudomallei* and *E. coli* in the absence or presence of cytochalasin D. The macrophage cells were pretreated without (A) or with (B) cytochalasin D (2.5 μg/ml) for 1 hour before infected with bacteria at MOI of 0.1:1, 1:1 and 10:1. To remove extracellular bacteria, the macrophages were incubated with medium containing kanamycin as described in Materials and Methods. Production of iNOS enzyme from macrophages infected cell was determined by immunoblotting. Control (C) was the cells without bacteria infection.

16B

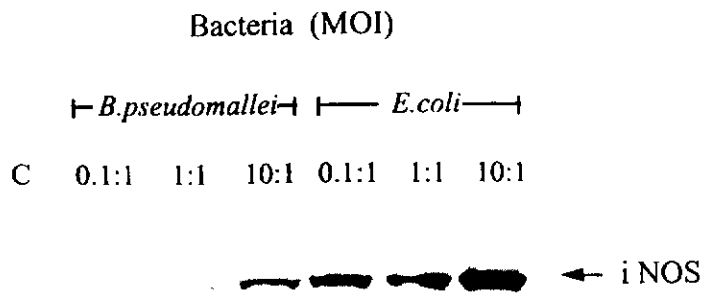


Fig. 16 Expression of iNOS from mouse macrophage cells (RAW. 264.7) infected by *B. pseudomallei* and *E. coli* in the absence or presence of cytochalasin D. The macrophage cells were pretreated without (A) or with (B) cytochalasin D (2.5 μg/ml) for 1 hour before infected with bacteria at MOI of 0.1:1, 1:1 and 10:1. To remove extracellular bacteria, the macrophages were incubated with medium containing kanamycin as described in Materials and Methods. Production of iNOS enzyme from macrophages infected cell was determined by immunoblotting. Control (C) was the cells without bacteria infection.

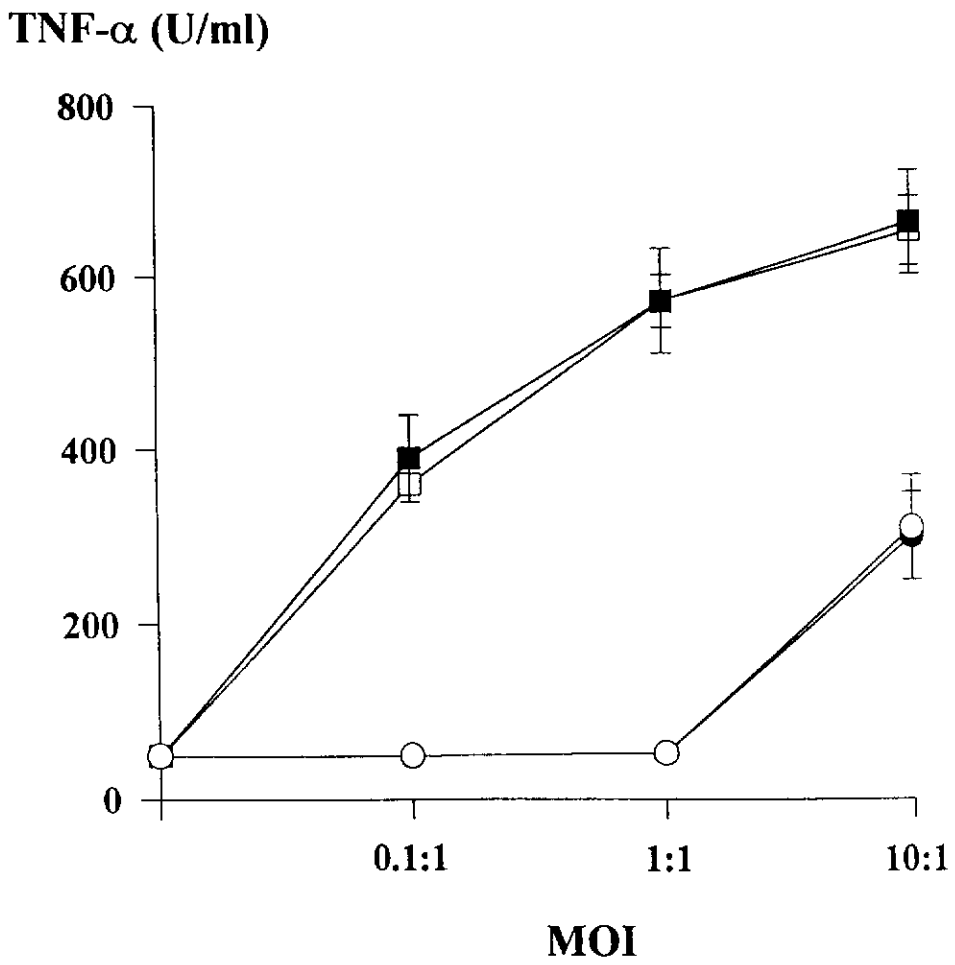


Fig. 17 TNF-α release from mouse macrophage cells (RAW 264.7) infected with *B. pseudomallei* (circle) or *E. coli* (square). The macrophage cells were pretreated with (filled) or without (open) cytochalasin D before infected with bacteria at MOI of 0.1:1, 1:1 and 10:1. To remove extracellular bacteria, the macrophages were incubated with medium containing kanamycin as described in Materials and Methods. The supernatant was used for the quantitation TNF-α release.

concentraion to be detected by Griess reaction (data not shown)

The supernatant from the macrophage cells infected with bacteria was analyzed for TNF-α release (Fig. 17). The cells infected with *B. pseudomallei* released a significant amount of TNF-α only at MOI of 1:1. In contrast, the TNF-α release was observed from the cells infected with *E. coli* even MOI of 0.1:1. Inhibiting phagocytosis with cytochalasin D did not have any effect on the TNF-α release from the cells infected either type of bacteria.

DISCUSSION

Lipopolysaccharide is a component of the outer cell membrane of gram-negative bacteria that is an important factor mediating the production and release of mediators (such as NO) and cytokines (such as TNF-α) from macrophages. Comparing with the LPS from other gram-negative bacteria the data presented in this study showed that the BP-LPS exhibits macrophage activating activity one order of magnitude weaker than that of the *E. coli*. This result is consistent with the data previously reported by Matsuura *et al* (16). In our comparative studies described herein, the BP-LPS was therefore used at a concentration ten times higher than that of the *E. coli*-LPS (100 ng/ml and 10 ng/ml respectively). Under this condition, we demonstrated that the kinetics of NO and TNF-α release from macrophage activated by BP-LPS was slower than those of the macrophages activated by *E. coli*-LPS (Fig. 2). The macrophage cells treated with the BP-LPS not only released the mediators with a rate slower than the cells activated with *E. coli*, but also the rate of gene expression of these mediators (iNOS, TNF-α) was also significantly slower. These results

indirectly suggest that the signals initiated by interaction of the BP-LPS to the cell surface may also be transduced with the slower rate than the one activated by the *E. coli*-LPS.

Mammalian cells have at least three major MAP kinase pathways that act as relays between extracellular signals and transcription factor (26). Among them, p38 is known to play an important role in the signal transduction by LPS such as TNF-α expression in LPS-stimulated macrophage (27,28). Phosphorylation of p38 occurred rapidly after the cells were activated with LPS (29). The time course study of p38 phosphorylation from the cells activated with BP-LPS shown in the present study indicates the slower of p38 phosphorylation comparing with the cells activated with *E. coli*-LPS (Fig. 4). This result is consistent with the upregulation of mRNA and the release of NO and TNF-α. Such a delay of signaling transduction may have contributed to the delay of the mediators release. The slower of macrophages activated with BP-LPS may have been affected by the unique structure of BP-LPS. Our data on the inhibitory effect of polymyxin B on NO production are consistent with this notion. The results presented in Fig. 6 showed that polymyxin B almost completely inhibited NO release by macrophages activated with *E. coli*-LPS while having considerably less inhibitory effect on the cells induced by BP-LPS. Moreover, polymyxin B completely abrogated the binding of FITC-*E. coli*-LPS to the cells but only partially inhibit the binding of FITC-BP-LPS to the receptor (Fig. 13). This drug has been shown to interact to the lipid A moiety of the LPS, thus it may interfere with the binding of LPS to the cell surface receptor and inhibit macrophage activation (30). It has been reported that the BP-LPS exhibits an unusual chemical structure in the acid stable inner core region attached to the lipid A moiety (17). The difference of the inhibitory effect of polymyxin B on NO release may be related to the uncommon structure of the BP-LPS. This unique

structure may have differential effects on the rate of LPS binding which may result in the delay of macrophage activation.

The slower of the mediators production on macrophages activated with BP-LPS could due to the slower of the binding rate of LPS to the cell surface. However, the kinetics of FITC-BP-LPS binding to the cells was not significantly different from FITC-*E. coli*-LPS. Moreover, competitive binding assay indicated that BP-LPS indicated that BP-LPS bind to CD14 on the macrophages with similar binding affinity comparing with *E. coli*-LPS. These results suggest that the delay of signal transduction of the macrophages activated with BP-LPS occurred after the binding of LPS to the receptors. Since CD14 is an GPI anchor protein lacking the cytoplasmic domain which responsible for transducing the signal across the membrane. This receptor requires other membrane proteins which contains the cytoplasmic domain to facilitate the signal transduction. A few proteins such as Toll Like Receptor 3 and 4 (TLR3, TLR4) have been reported to play a significantly roles for transducing the signal from CD14 across the membrane (31-35). After binding to CD14, the receptor-LPS required to form the complex with TLR in order to sent the signal. The delay of signal transduction from the cells activated with BP-LPS may due to the slower rate of complex forming between CD14-BP-LPS to TLR receptor.

To eliminate the impact of physical state in which LPS was presented to the cell, heat-killed bacteria were used to study for NO and TNF-α released from mouse macrophage cell line. The kinetic of the mediators released from the cells activated by heat killed *B. pseudomallei* showed similar result as the cells activated by BP-LPS. These results indicated that *B. pseudomallei* was not only less efficient in stimulating NO

and TNF-α release from macrophages, but the time lag was also longer than other gram negative bacteria such as *E. coli*. The slower release of NO by BP-LPS was probably due to the slower rate of signal transduction from the surface receptor into the cell. The data on iNOS are consistent with this contention.

Nitric oxide produced by mammalian macrophages is known to have antimicrobial activity. In cell lines, resistance to microbial killing is often associated with expression of iNOS (14). *Burkholderia pseudomallei*, like other gram-negative bacteria, have been reported to be susceptible to the killing effects of reactive NO in the mouse macrophage cell line (36). In our studies, we have demonstrated that mouse macrophages infected with *B. pseudomallei* did not express detectable iNOS enzyme at low MOI (0.1:1, 1:1) while the cells similarly infected with *E. coli* expressed a significantly higher amount of iNOS enzyme at the same MOI (Fig. 1A). The expression of iNOS from the cells infected with other intracellular bacteria such as *S. typhi* was similar to the cells infected with *E. coli* (data not shown). However, it was interesting to note that in the present of cytochalasin D, iNOS enzyme was detected when the cells were infected with *B. pseudomallei* at MOI of 1:1 and 10:1(Fig 1B). This result may indirectly indicate that unlike other gram-negative bacteria such as *E. coli* or *S. typhi*, *B. pseudomallei* requires more time after interacting to the macrophage surface to trigger the signal transduction before the bacteria are phagocytosed. Moreover, the macrophage cells infected with *B. pseudomallei* expressed significantly less iNOS than the cells infected with *E. coli* at the same MOI. The TNF-α released from macrophages infected with *E. coli* was significantly higher than the cells infected with *B. pseudomallei* at the same MOI. The cells infected with *B. pseudomallei* released TNF-α only when infected

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with MOI of 10:1. These results suggest that *B. pseudomallei* was not a good macrophage activator compared with other gram negative bacteria. This property may contribute to their intracellular survival within the macrophages.

Burkholderia pseudomallei has been demonstrated to survive and multiply in a member of phagocytic and non-phagocytic cells (37-39). However, this gram-negative bacterium was highly susceptible to reactive nitrogen intermediates produced by macrophage cells such as NO (36). In the present study, we have demonstrated that the BP-LPS stimulates not only NO and TNF-α release with a lesser quantity comparing with the *E. coli*-LPS, but also activates these mediators release with a slower kinetic rate. The delay of mediators release could have been caused by the slower rate of signal transduction which may in turn influence the rate of mRNA synthesis. Having weaker and slower macrophage activation activities may facilitate the *B. pseudomallei* to survive in the macrophages, thus helping it to evade the host defensive system and allowing it to survive for a long time after initial exposure.

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