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# Production of nitric oxide, interleukin-6 and tumor necrosis factor α from mouse peritoneal macrophages in response to *Bacillus anthracis* antigens

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**Abstract** : Anthrax caused by *Bacillus anthracis* is one of the most important zoonotic diseases. The bacterium produces several virulence factors. Of the factors, protective antigen (PA) of tripatite toxin has been identified as a central component in the pathogenesis of anthrax. However, precise roles of PA and other cellular components in the reaction with the target cells remain to be elucidated, especially in the initial stage of the disease. Three *B anthracis* antigens were prepared for investigation ; PA, sonicated cellular antigens (S-Ag) and formalin-inactivaed whole cell antigens (W-Ag). PA was purified from culture supernatant of the bacterium using FPLC system with MonoQ. S-Ag and W-Ag were prepared by sonication and formalin inactivation of the cultured cells, respectively. Purity of the antigens was confirmed by SDS-PAGE and Western blot analysis. The roles of these antigens in the production of inflammatory mediators such as NO, IL-6 and TNFα from mouse peritoneal macrophages were investigated. PA alone did not induce the production of the inflammatory mediators while the other antigens, S-Ag and W-Ag, did in a dose and time dependent manner. These results suggested that in addition to major virulence factors, other cellular antigens are also involved in the initial stage of the disease by the induction of inflammatory mediators.

**Key words** : NO, IL-6, TNFα, *B anthracis* antigens.

## Introduction

*Bacillus anthracis*, a Gram-positive, spore-forming bacterium, is the cause of anthrax, a disease recognized for centuries as an economically important zoonotic infection[1]. Virulent isolates of the organism carry a toxin-encoding plasmid, pXO1, and a capsule-associated plasmid, pXO2, both of which are required to produce disease[1~3]. Three bacterial secreted proteins, PA (protective antigen), LF (lethal

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factor), and EF (edema factor), encoded by the genes *pag*, *lef* and *cya*, respectively, combine pairwise to form the lethal (PA+LF) and edema (PA+EF) toxins[1]. Of the virulence factors, PA is a primary component and the most abundant of the three toxin components. These toxins are responsible for the major physiopathological effects observed during infection, i.e., edema and peracute infection leading to death[4]. Although PA had been recognized as a central component in the pathogenesis of the disease, the roles of PA alone in the early response to infection, especially in the production of inflammatory mediators, remain unknown. In addition, chromosomal factors and cellular components have been implicated as determinants for disease severity by the production of inflammatory mediators in the initial events of infection[4,5].

Macrophages play an important role in the first line defense against external stimuli including bacterial infections by producing inflammatory mediators such as cytokines, reactive nitrogen and oxygen intermediates[6~11]. These mediators are essential to eliminate or kill the invading pathogens. The responses to bacterial infections in the host are usually elicited by cellular constituents as well as bacterial products[12~14]. However, researches on the production and roles of proinflammatory mediators in the pathogenesis of anthrax are still unclear probably because anthrax is a peracute and fatal disease. This is compounded by the difficulties in the purification of the virulence factors to homogeneity for meaningful host-pathogen interaction studies[15]. Recently, induction of IL-1 and TNF$\alpha$ and production of reactive oxygen intermediates (ROI) with lethal toxin at sublethal concentration was reported[6,7]. However, immune responses by PA itself and other cellular components in the initial state of the disease in the target cells have not been documented.

Therefore, we investigated the initial cellular responses, measured by the production of nitric oxide and inflammatory cytokines, (IL-6 and TNF$\alpha$) with purified PA and other cellular antigens of *B anthracis* in mouse peritoneal macrophages.

## Materials and Methods

**Bacterial strains, media and reagents** : *Bacillus anthracis* Sterne 34F2 (pXO1$^+$, pXO2$^-$) was used for the purification of PA from culture supernatant of the bacterium. *B anthracis* Korean isolate (pXO1$^+$, pXO2$^+$) was used for the preparation of sonicated cellular antigen (S-Ag) and formalin-inactivated whole cell antigen (W-Ag). Brain heart infusion (BHI) broth and RM medium were used for preparation of S-Ag and W-Ag and purification of PA, respectively.

RPMI 1640 was used to culture mouse peritoneal macrophages. Reagents used in these experiments were purchased from Sigma Co. (St. Louis, MO, USA) ; lipopolysaccharide (LPS) from *Escherichia coli* O127 : B8, NG-monomethyl-L-arginine (L-NMMA) and polymyxin B. Mouse recombinant cytokines used in this experiment, interferon (INF$\gamma$), interleukin-6 (IL-6), and tumor necrosis factor (TNF$\alpha$), and ELISA MiniKit™ were purchased from Endogene Inc. (Cambridge, MA, USA).

**Antibodies** : Monoclonal antibody against purified PA of *B anthracis* was kindly gifted by Dr. John Ezzell (US Army Medical Research Institute of Infectious Disease, Fort Detrick, Maryland, USA) and polyclonal antibody produced with purified PA in rabbit[16] was also kindly provided by Dr. CS Choi (Department of Microbiology, College of Medicine, Chungang University, Seoul, Korea). ELISA titer of the polyclonal antibody was $>$ 1 : 81,920.

**Preparation of *B anthracis* antigens** :

1) Protective antigen (PA) of *B anthracis* : PA was purified from culture supernatant of *B anthracis* Sterne 34F2 using a fast protein liquid chromatography (FPLC, Pharmacia) system with MonoQ (QAF) HR, Superose gel filtration FPLC and hydrophobic interaction HPLC followed as described by previous reports[15,16]. Briefly, the bacterial strain was incubated in RM medium at 37℃ for 26~28 hrs with gentle shaking (60rpm). After addition of 0.05mM 1,10-phenanthroline, 2mM EDTA and 2mM 2-mercaptoethanol as proteinase inhibitors, into bacterial cultures, the cultures were centrifuged at 10,000 × g for 20 min. After centrifugation, supernatant was collected and filtered with 0.45μM Millipore filter. The culture supernatant was incubated overnight at 4℃ after addition of 100ml of DEAE CL-6B which was washed with 20mM Tris and 50% (W/V) ammonium sulfate into the supernatant. Then, sepharose was packed

and eluted with 500ml of Tris and collected in 50ml fractions. Each fraction was dialysed after 100-fold concentration and protein profile was examined by SDS-PAGE and Western blot with monoclonal or polyclonal antibodies against PA of *B anthracis*. A fraction containing PA was dialysed against 20mM triethanolamine (pH 8.0) for 5hrs and 2.0ml of the fraction was eluted through HiTrap desalting column. The samples were loaded on a prepacked Mono-Q (QAE) HR 5/5 column and eluted in a 20ml of 20mM triethanolamine/NaOH buffer, pH 8.0, with two-stages NaCl gradient from 0.1 M to 1.0M. All predominant peaks were collected and protein concentration was measured as optical density at 280nm. Protein profile of them was analysed by SDS-PAGE and Western blot analysis. Fractions identified as containing PA from Mono-Q chromatography were chromatographed on Superose 12 gel and gel-filtered by hydrophobic-interaction chromatography using a 1ml phenyl-superose HR 5/5 column. Protein was eluted in a 20ml linear gradient of decreasing $(NH4)_2SO_4$ from 1.7M to 0M with a flow rate of 0.2ml/min. The predominant peak in each elution profile was collected for accessment of activity and purity of PA.

2) Sonicated cellular antigen of *B anthracis* (S-Ag) : For the preparation of S-Ag, *B anthracis* isolate was grown in BHI broth overnight, washed with phosphated buffered saline (PBS, pH 7.5) three times by centrifugation, then sonicated for 10 min three times at 50% duty cycle (Sonics materials) and finally centrifuged at 10,000 × g for 40 min to clarify. The supernatant was collected after centrifugation and filtered with 0.45μm membrane filter (Costar). Protein concentration was measured by Lowry method[17]. Samples were stored at -20℃ until use.

3) formalin-inactivated whole cell antigen (W-Ag) : In the preparation of W-Ag, the bacterium was grown in BHI broth overnight and formalin was added into the bacterial culture to 3% final concentration. After overnight incubation of the culture at room temperature, the culture was washed twice with PBS by centrifugation at 10,000 × g for 40 min and the cell pellet was suspended in RPMI medium 1640. Number of the bacterial cell was determined by a standard curve produced with optical density at 560nm based on standard agar plate count method before inactivation.

**Analysis of *B anthracis* antigens** : Antigens prepared from *B anthracis* were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)[18] and Western blot analysis[19]. Fifty ml of each sample was analyzed on 12% SDS-PAGE gel and electrophoresis was carried out in Tris-glycine buffer (25mM Tris base, 250mM glycine, 0.1% SDS) at 10mA for 1hr and 15mA for 4hrs. After SDS-PAGE, the gels were either stained with Coomassie Brilliant Blue R-250 solution or electrophoretically transferred onto nitrocellulose membrane (Immobilin-P, Millipore) in the electrode buffer (20mM Tris base, 150mM glycine, and 20% (v/v) methanol). The membrane was probed with a 1 : 2,000 dilution of the monoclonal or polyclonal antibodies against PA. The PA specific band was detected by incubation with horseradish peroxidase-conjugated goat-anti-mouse immunoglobulin G (Cappel) followed by incubation with 0.06% (w/v) 4-chloro-1-naphthol and 0.06% (v/v) $H_2O_2$ as a substrate.

**Mouse peritoneal macrophages (PMs)** : PMs were prepared by peritoneal lavage after intraperitoneal injection of 10ml of sterile medium RPMI 1640 without phenol red. The cell was harvested and washed twice with the medium RPMI 1640 by centrifugation at 1,000 × g for 10 min at 4℃. Approximately, $1 \times 10^5$ cells were plated into each well of a 48 well tissue culture plate and cultured overnight at 37℃ in a humidified atmosphere in the presence of 5% $CO_2$ in RPMI 1640 without phenol red supplemented with 2% fetal bovine serum (FBS ; Irvine Scientific, Santa-Ana, CA, USA). Nonadherent cells were removed by washing twice with PBS, and adherent cells were incubated with fresh medium as described for individual experiments. Adherent populations were 〉95% macrophages and 〉98% viable as determined by nonspecific esterase staining using a commercial kit (Sigma Co.) and trypan blue dye exclusion, respectively. Inducers were added directly to existing medium at the following morning to avoid nonspecific stimulation of quiescent cells.

**Stimulation of peritoneal macrophages** : To stimulate the PMs, the cells were cultured in the presence of antigens prepared from *B anthracis* Sterne 34F2. Concentrations of the antigens, rmIFNγ, polymyxin B, and iNOS competitive inhibitor, L-NMMA were varied as described in individual experiments. The culture media were collected and cen-

trifuged for 10 min at 1,000×g. Supernatants of PM-conditioned media were used to determine concentration of nitrite as an indicator of production of NO, concentrations of IL-6 and TNF$\alpha$. Each treatment was performed in replicates of three and the data pooled.

**RNA extraction** : Total RNA was extracted from PMs cultured in the presence or absence of stimuli by the acid guanidinium thiocyanate method as described by Chemczynski and Sacchi[20] with some modifications. Briefly, all solutions used for RNA purification were treated with 0.05% diethylpyrocarbonate (DEPC) and then autoclaved to remove DEPC. Glassware were baked overnight at 200℃. Adherent cells exposed to various stimuli were lysed in 0.5ml of solution D (5M guanidine thiocyanate, 50mM Tris pH 7.4, 25 mM EDTA, 0.5% sarcosyl, 1% $\beta$-mercaptoethanol) and sonicated for 20 sec in a sterile eppendorf tube at 50% frequency. After adding 65μl of 2M sodium acetate (pH 4.0), the sonicated PMs were then extracted with 650μl of phenol and 260μl of chloroform : isoamylalcohol (49 : 1), and RNA was precipitated with 2 vol of absolute ethanol followed by ice incubation for 10 min. RNA was recovered by centrifugation at 14,000×g for 10 min after incubation. The RNA pellet was rinsed with 70% ethanol, air-dried and resuspended in 50ml of DEPC-treated water. RNA concentration was determined by measuring absorbance at 260 nm after incubation at 68℃ for 15 min, and the RNA samples were stored in 2.5 vol of ethanol at -70℃ until further use.

**mRNA analysis by reverse transcriptase-polymersase chain reaction (RT-PCR)** : RT-PCR was done by RT-PCR kit (Bioneer, DaeJeon, Chungnam, Korea) using a thermalcycler (Perkin Elmer Gene Amp PCR System 9600). The RT-PCR was performed as following. One μg of total RNA, 50pM of sense and antisense primers and nuclease-free water were added into the kit up to 50μl of total volume. cDNA was synthesized at 42℃ for 1hr after RNA denaturation at 57℃ for 10 min. After synthesis of the cDNA, PCR condition used were forty cycles of denaturation at 94℃ for 30 seconds, annealing at 56℃ for 1 min, polymerization at 74℃ for 1 min and after an initial denaturation at 94℃ for 5 min. The PCR products were analyzed by electrophoresis in 1.5% agarose gel in 0.5×Tris-borate buffer (44.5mM Tris-borate, 1mM EDTA, pH 8.3) at 60V for 2hr. After electrophoresis, the DNA were visualized on a UV transilluminator and photographed with a Polaroid camera. The primers used to amplify mouse iNOS and glyceraldehyde phosphate dehydrogenase (GAPDH) cDNAs were adapted from Alder *et al*[21]. Mouse iNOS primer (372bp product) : sense 5-TAGAGGAACATCTGCCCAGG-3 ; antisense 5-TGGCAGGGTCCCCTCTGATG-3. Human GAPDH primers (356bp products) : sense 5-GAGATGATGACCCTTTTGGC-3 ; and antisense 5-GTGAAGGTCGGAGTCAACG-3.

**Determination of NO release** : NO was measured indirectly by determination of one of the stable end products, $NO_2^-$[22]. Briefly, 100μl of aliquots removed from PM-conditioned media were mixed with equal volumes of Griess reagent (1% sulfanilamide in 2.5% $H_3PO_4$ and 0.1% naphthyl-ethylenediamine-dihydrochloride) and incubated for 10 min at room temperature. The absorbance at 550nm was measured in a microplate ELISA reader (Molecular Device Corp., Menlo Park, CA, USA). Concentrations of nitrite were determined by extrapolation based on a standard curve established with sodium nitrite. Each sample was tested in triplicate.

**Determination of interleukin-6 (IL-6) and tumor necrosis factor $\alpha$ (TNF$\alpha$)** : Amounts of IL-6 and TNF$\alpha$ produced from PM stimulated with various antigens of *B anthracis* were analyzed by mouse IL-6 MiniKit™ and mouse TNF MiniKit™ (Endogen, Cambridge, MA, USA), respectively. ELISA was carried out as described in a method by manufacturer. Briefly, immunoplate was incubated overnight at room temperature (RT) after addition of 100μl of coating antibody as recommended by the manufacturer (40pg/well). After incubation, empty the plate of coating solution. The plate was blocked with 200μl of assay buffer (PBS with 2% BSA, 0.01% thimerosal, pH 7.2 to 7.4) by incubation for 1hr at RT. After the blocking, the plate was washed three times with washing buffer (50mM Tris, 0.2% Tween-20, pH 7.0~7.5) and then 50μl of test and diluted standard samples (20-1950pg/ml) were added into each well containing 50μl of assay buffer. The plate was then incubated overnight at RT. After washing the plate three times with washing buffer, 100μl of biotinylated detecting antibody diluted as recommended was added into the each well of the plate and in-

cubated the plate at RT for 30 min. One hundred μl of TMB substrate solution was added to all wells and incubated at RT for 30 min in the dark. After the incubation, the reaction was stopped by addition of 100μl of stop solution (0.18M Sulfuric Acid). Optical density was measured with a microplate ELISA reader (Molecular Device Corp., Menlo Park, CA, USA). Concentrations of IL-6 or TNF$\alpha$ were expressed in nanograms/ml which were calculated by extrapolation based on a standard curve using rmIL-6 or rm TNF$\alpha$.

**Statistical analysis** : All the results were expressed as the mean±standard deviation (SD). Comparisons between groups were made by two-way analysis of variance (ANOVA). A p-value of less than 0.01 was considered as significant.

## Results

**Preparation and analysis of *B anthracis* antigens** : PA was purified from culture supernatant of *B anthracis* Sterne 34F2 using a FPLC system with anion exchange chromatography (MonoQ). S-Ag and W-Ag were also prepared by sonication and formalin-inactivation of the bacterium, respectively from overnight cultures in BHI broth. The antigens were analysed by SDS-PAGE and Western blot with monoclonal antibody against purified PA. SDS-PAGE analysis of the antigens revealed that only 83 kDa in the purified PA while 150, 97, 84, 60 and 36kDa in the S-Ag and 97, 70, 66 and 50kDa in the W-Ag were present (Panel A in Fig 1). However, 83kDa of PA fragment was detected only in the purified PA by Western blot analysis with monoclonal antibody against purified PA (Panel B in Fig 1).

**iNOS gene expression and NO production in the mouse peritoneal macrophages** : Expression of the iNOS gene in response to various stimuli was analyzed by RT-PCR. Expression of the iNOS gene was very sensitive to S-Ag and W-Ag, but the gene was not expressed by purified PA alone (Fig 2). The amount of gene expression by S-Ag

Fig 1. Electrophoretic analysis of *Bacillus anthracis* antigens used in this experiment. The antigens were analyzed by 12% SDS-polyacrylamide gel electrophoresis (pannel A) and Western blot (panel B) with monoclonal antibody against purified PA of *B anthracis*. Lane H in panel A; molecular weight marker and the number indicates the size of the proteins. Panel A and B, lane 1; purified protective antigen (PA), lane 2 ; Sonicated antigen (S-Ag) and lane 3 Whole cell antigen (W-Ag).

Fig 2. Gene expression of inducible nitric oxide synthase (iNOS) in mouse peritoneal macrophages stimulated with three different *B anthracis* antigens. Mouse peritoneal macrophages were cultured and stimulated with *B anthracis* PA, S-Ag and W-Ag or without any stimuli for 24hrs. Total RNA was purified from the cells and iNOS gene in the total RNA was amplified using RT-PCR kit with a thermocycler. The amplified DNAs were electrophoretically analyzed in 1.5% agarose gel. Panel A and B indicated the gene expressions of glyceraldehyde phosphate-dehydrogenase (GADPH) and iNOS, respectively. In the panels A and B, lane M; molecular weight marker (100bp ladder, Gibco/BRL), lane 1, control (no RNA), lane 2, total RNA from cells stimulated with LPS (5μg/ml), lane 3, total RNA from cells with purified PA(5μg/ml), lane 4 total RNA from cells with S-Ag (375μg/ml) and lane 5, total RNA with W-Ag ($6\times10^8$cells).

and W-Ag was very similar to that by *E coli* LPS. Production of NO from PMs in response to various stimuli was analyzed by measuring concentration of nitrite, a final product of NO in the culture supernatant of the PM cells. As shown in Fig 3, the production of NO was very sensitive

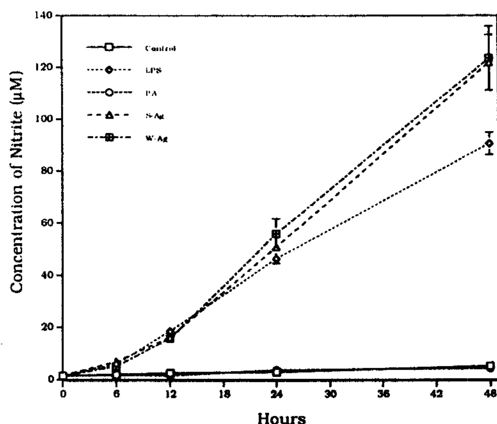


Fig 3. Time-dependent production of nitric oxide (NO) from mouse peritoneal macrophages cultured in the presence of various stimuli. Mouse peritoneal macrophages were cultured with different antigens for indicated time. The concentration of nitrite as an indicator of NO production in the conditioned media were measured by the Griess reaction as described in materials and methods. The values represent the means ± SD from three independent experiments.

and fast in the PMs by various stimuli except purified PA alone like as the gene expression of iNOS. The PA alone could not induce the NO production from the cells. The NO was time- and dose-dependently produced by S-Ag, W-Ag and *E coli* LPS even though NO production was decreased over concentrations of $1.2 \times 10^8$cells/ml of W-Ag in the PM (Fig 4). Production of NO in PMs stimulated with all stimuli was inhibited by L-NMMA, a structural analogue of L-arginine while polymyxin B could inhibit the production only in the LPS-stimulated PMs. However, IFNν enhanced the production in the PMs activated with all antigens except purified PA (Fig 5).

**Production of IL-6 and TNFα from mouse peritoneal macrophages** : IL-6 was also time-dependently produced from PMs. Results as shown in Fig 6 revealed that production of IL-6 was observed at 6hr that peaked at 12hrs

Fig 4. Dose-dependent production of NO from mouse peritoneal macrophages stimulated with various concentration of *E coli* LPS and *B anthracis* antigens with. The cells were cultured in the presence of four different antigens (LPS, PA, S-Ag, and W-Ag) at 37℃ for 24hrs under 5% $CO_2$ and amounts of nitrite in the conditioned media were measured by the Griess reaction as described in materials and methods. The values represent means ± SD from three independent experiments.

Fig 5. Enhancement or inhibition in the production of NO from mouse peritoneal macrophages stimulated with *B anthracis* antigens and *E coli* LPS. Mouse peritoneal macrophages were cultured in the presence of one of the antigens with L-NMMA (2.5mM) or polymyxin B(20μg/ml) or IFN-ν (1,000U/ml) at 37℃ for 24hrs. Amounts of NO were measured by the Griess reaction as described in the materials and methods. The values represent means ± SD from three independent experiments.

and continued at 48hrs from PMs stimulated with W-Ag and LPS. But, IL-6 production induced by S-Ag stimulated

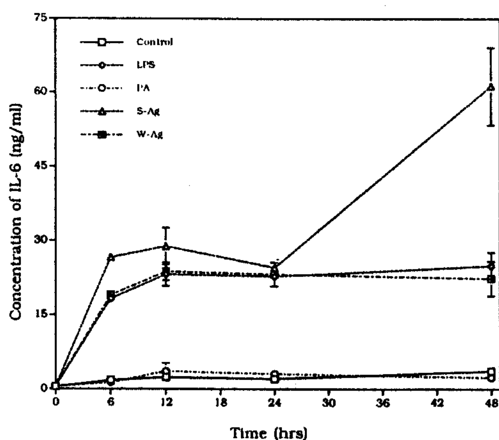


Fig 6. Time-dependent production of interleukin-6 from mouse peritoneal macrophages activated with various antigens for indicated length of time. Mouse peritoneal macrophages were cultured in the presence of *E coli* LPS (1μg/ml) or PA (10μg/ml) or S-Ag (375μg/ml) or W-Ag ($6\times10^8$cells) of *B anthracis* at 37℃ for the indicated length of time under 5% $CO_2$. Concentrations of IL-6 were measured with mouse IL-6 Minikit™ as described by the manufacture. The values represent means ±SD from three independent experiments.

Fig 7. Dose-dependent production of interleukin-6 from mouse peritoneal macrophages activated with various concentrations of the antigens at 37℃ for 24hrs. Mouse peritoneal macrophages were cultured in the presence of the antigens as the indicated concentrations under 5% $CO_2$. After the culture, concentrations of IL-6 in the conditioned media were measured with mouse IL-6 MinKit™ as described by the manufacture. The values represent means±SD from three independent experiments.

PMs also continued to increase and peaked at 24h. The production peaked at 1μg/ml of LPS, 375μg/ml of S-Ag and $1.2\times10^8$cells/ml of W-Ag. However, purified PA alone did not induce IL-6 up to 10μg/ml of the concentration (Fig 7). TNF$\alpha$ was also dose-dependently produced from PMs stimulated with S-Ag, W-Ag, and LPS but not with purified PA alone. The production of each analyte decreased over the $1.2\times10^8$cells/ml of W-Ag. PA alone did not induce the production of TNF$\alpha$ similar to the production of NO and IL-6 in the PMs (Fig 8). With polymyxin B, a specific inhibitor of LPS, the production was not inhibited in the production of IL-6 and TNF$\alpha$ except LPS-stimulated PMs (data not shown). And, identity of the reaction was confirmed by neutralization of the reaction with antibodies against mouse mrIL-6 and mrTNF$\alpha$.

Fig 8. Time-dependent production of TNF$\alpha$ from mouse peritoneal macrophages activated with various concentrations of *E coli* LPS, purified PA, S-Ag, and W-Ag of *B anthracis*. Mouse peritoneal macrophages were cultured in the presence of indicated concentration of the antigens at 37℃ for 24hrs under 5% concentration of $CO_2$. Concentration of TNF$\alpha$ in the conditioned media were measured with mouse TNF$\alpha$ MiniKit™ as described by the manufacture. The values represent means±SD from three independent experiments.

## Discussion

Anthrax caused by *Bacillus anthracis* is associated with the presence of two major virulence factors, capsular polyglutamic acid and exotoxins encoded by plasmids pXO2

and pXO1, respectively. The exotoxins consist of two binary toxins sharing a common binding components and possessing separate effector components, each expressing little or no individual toxicity. PA has been identified as a central component in the pathogenesis of anthrax with limited proteolysis for the activation of each toxin, lethal toxin consisting of PA and LF and edema toxin consisting of PA and EF. Similarities between the systemic shock caused by *B anthracis* infections in human or animals, or by LeTx test in animals and gram-negative bacterial sepsis (endotoxic or LPS-mediated shock) have been demonstrated with an exception of the characteristic sudden death observed in the terminal phase of anthrax[23]. In the microbial infections, a variety of cytokines such as TNF$\alpha$ and IL-1$\beta$ that mediate damaging inflammatory cascade, shock and death, are produced from macrophages stimulated with LPS or other cellular factors. The induction of inflammatory cytokines, IL-1 and TNF$\alpha$ was reported in the toxin sensitive cultured macrophages (RAW 264.7 cells) cell lines by sublethal concentration of lethal toxin[6]. However, roles of PA by itself and other cellular components of the bacterium in the production of inflammatory mediators have not reported yet.

Macrophages which are the first defense line produce inflammatory mediators against various external stimuli including bacterial infection[8~11]. Also, the macrophage is one of the most important target cell in the pathogenesis of anthrax. Bacterial cellular components such as LPS, lipoteichoic acid, outer membrane proteins and bacterial exotoxins have been described as inducers of the inflammatory mediators from the cells[12~14]. However, studies on the production of inflammatory mediators with individual virulence factors and other cellular components of *B anthracis* are just beginning now. Therefore, we investigated the production of inflammatory mediators from mouse peritoneal macrophages by PA and cellular antigens of *B anthracis*.

To purify the virulence factors of *B anthracis*, several modifications have been applied to medium, bacterial strains etc.[1,15]. Recently, yield of PA was evaluated using two different media, RM media and modified RM media that xanthine was substituted for adenine and ferrous sulfate was added because it has been known that, of components in anthrax holotoxin-producing medium, adenine sulfate inhibits the yield of PA, while xanthine enhances it[16]. Based on these evaluations, we used RM medium to produce PA. Presence of PA in the prepared antigens of *B anthracis* was confirmed by analysis of the antigens by SDS-PAGE and Western blot with monoclonal and polyclonal antibodies. No contamination of PA with the other cellular antigens and purity of our PA preparation were confirmed by the analysis. We chose mouse peritoneal macrophages as a target cell because of several advantages such as ease of handling, ability to purify and culture as well as economical. Also, the cells play an important role in the innate immunity against infection. In addition, immunogenic properties of the cells were well-documented[12].

Involvement of reactive oxygen intermediate (ROI) in the pathogenesis of anthrax has been reported[6,7]. High concentrations of the molecules are disastrous to the cell due to modification of vital residues in $Ca^{2+}$ homeostasis and a destructive peroxidative cascade[24]. The ROI were produced from macrophages stimulated with a sublytic concentration of lethal toxin (LeTx). This ROI may play a dual role in the overall pathogenesis of anthrax, first (at low levels) inducing macrophage cytokine expression and then (at high levels) bursting the cell. Sublytic concentration of this oxidants can modify gene expression patterns of immune cells via activation of transcriptional regulators[25]. Additionally, involvement of nitric oxide (NO) and other reactive nitrogen intermediates (RNI) were investigated in the lysis of macrophages. However, these molecules seem not to be involved in LeTx-induced macrophage cytotoxicity[7]. In this experiment, NO was produced from mouse peritoneal macrophages activated with various cellular antigens except PA. The inductions were dose-and time-dependent. Also, their productions were specifically inhibited by L-NMMA, an inhibitor of NO synthetase. Patterns in the production and inhibition of this molecule are congruent with those from a previous study[7]. Even though this molecule is not involved in the cytotoxicity of host cells, it might enhance the host-defense mechanism by bacterial killing. *B anthracis* cellular antigens have been shown to induce TNF$\alpha$ and IL-6 which could mediate cellular damage[6,26]. These results suggested

that purified PA did not induce NO, IL-6 and TNF$\alpha$ while other antigens (W-Ag and S-Ag) did similar LPS which is a known as an inducer of proinflammatory mediators. Our results of the NO and cytokine production closely parallel those from a previous report[6]. The results from the present study suggested that cellular components of *B anthracis* in addition to exotoxins might be important virulence factors in the pathogenesis of anthrax, especially in the initial stage of the disease.

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