Pediatrics & Therapeutics
ISSN: 2161-0665

Bu, et al., Pediat Therapeut 2015, 5:2
DOI: 10.4172/2161-0665.1000237

Research Article Open Access

Diminished ROS Production in Neonatal Polymorphonuclear Leukocytes Stimulated with *Escherichia coli*

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Rec date: Feb 15, 2015, **Acc date:** Mar 06, 2015, **Pub date:** Mar 10, 2015

Abstract

Bacterial infection is an important cause of death and long-term morbidity in neonates, especially in immature preterm neonates. The present study clarified the defense mechanism in neonates by measuring superoxide anion (one of reactive oxygen species: ROS) production and phagocytic activity of polymorphonuclear leukocytes (PMNs) caused by live bacterial stimulation in whole blood. In neonatal PMNs, superoxide anion production was significantly diminished when stimulated with an appropriate amount of *Escherichia coli* (*E. coli*). There was no significant difference in ROS production with stimulation of *Staphylococcus aureus* (*S. aureus*) between adult and neonatal PMNs. Both adult and neonatal PMNs showed equal activity in *E. coli* phagocytosis. The expression of Toll-Like Receptor 2 (TLR2), Toll-Like Receptor 4 (TLR4) and CD14 on the PMNs showed no difference between neonate and adult blood. The p38 mitogen-activated protein kinase (p38 MAPK) was also activated equally with the stimulation of live bacteria in both PMNs. Since p38 MAPK is a key molecule in lipopolysaccharide binding protein (LBP)-TLR4 mediating intracellular pathway, the receptor mediated signal transduction pathways have no difference between adult and neonatal PMNs. Diminished superoxide production of neonatal PMNs with *E. coli* stimulation was reconstituted, when neonatal PMNs were stimulated in adult plasma. This phenomenon was abolished by the heat inactivation of adult plasma but not by the addition of anti-interferon γ or anti-tumor necrosis factor α blocking antibody. These data suggest that some heat-labile factors in whole blood might have a role in augmentation of the ROS production of neonatal PMNs, responding to *E. coli*.

Keywords: Neonate; Polymorphonuclear leukocytes; ROS production; *Escherichia coli*

Abbreviations:

ROS: Reactive Oxygen Species; PMNs: Polymorphonuclear leukocytes; TLR: Toll-Like Receptor; ERK: Extracellular signal-Regulated Kinase; JNKs: C-Jun NH2-terminal Kinase; LBP: Lipopolysaccharide Binding Protein; LPS: Lipopolysaccharide; PRRs: Pattern Recognition Receptors; IFNγ: Interferon γ; TNFα: Tumor Necrosis Factor α; MFI: Mean Fluorescence Intensity

Introduction

Bacterial infection in neonates, especially in immature preterm neonates, causes death and has a key role for long-term morbidity of those infants. The reason of this phenomenon is mainly due to the immaturity of bactericidal mechanisms in innate immunity. Neutrophils (PMNs: Polymorphonuclear leukocytes) are primary responders maintaining nonspecific innate immunity against bacterial invasion [1]. The principal functions exhibited by circulating PMNs in response to bacterial invasion and inflammation include adherence to the vascular endothelium, deformability, chemotaxis, phagocytosis and intracellular microbial killing. In neonates, the ability of PMNs adherence, deformability and chemotaxis are known to be low because of the immaturity of neutrophil structure [2]. Because the toxic reactive oxygen species (ROS) appear to be essential for the microbicidal activity of neutrophils [3], the possibility of a deficit in oxygen metabolism in neonatal neutrophils has been explored in several studies, but the results were controversial [4-9].

The intracellular signal transduction of PMNs in response to a wide variety of stimuli appears to utilize the MAP kinase cascade. Mammalian PMNs contains at least three distinct MAPKs: p42/44 extracellular signal-regulated kinase (ERK) MAPs, c-Jun NH2-terminal kinases (JNKs), and p38 MAPKs [10,11]. The response of neutrophils to cytokines and other proinflammatory mediators is also regulated by p38 MAPK. When stimulated with TNF-α, p38 MAPK of neutrophil regulates distinctly different functions, including adhesion, activation of NF-κB, synthesis of TNF-α and IL-8, superoxide anion release, chemotaxis and apoptosis [12-14]. Systemic inhibition of p38 MAPK can inhibit neutrophil responses in dependent of other host responses [15,16]. Toll-like receptors are the first family of pattern recognition receptors (PRRs) and their signal transduction pathways are well understood. Although, all TLRs except TLR3 are expressed in neutrophils [17,18], TLR2 and TLR4 are well characterized for bacterial response. Neutrophils express lower level of TLR2, TLR4 and CD14 comparing to monocytes. TLR2 is activated with Staphylococcus peptidoglycan and TLR4 is activated with LPS, activation of both receptors revealed changes in adhesion molecules, expression of respiratory burst and IL-8 generation. But TLR4 is a major regulator of neutrophil survival and TLR2 has a less effects for preventing neutrophil apoptosis. While TLRs are important regulator of

neutrophil activation and survival, TLR2 and TLR4 have distinct and separate roles in neutrophil response [19,20].

The present study was designed to clarify the defense mechanism in neonates by measuring superoxide anion, one of the ROS, produced by PMNs following live bacterial challenge in whole blood. And the characteristic features of neonatal signal transduction of PMNs and factors influencing ROS production were evaluated. To confirm the ability of neonatal PMNs against bacterial infection is important for clinical decision of treatment in sepsis. If neonatal PMNs have lower ability of bacterial killing, blood exchange or white blood cells infusion is more effective than opsonization enhancing therapy for neonatal sepsis. We found that no significant difference in ability of superoxide production, when adult and cord blood PMNs were stimulated with *S. aureus*. On the other hand, the ability of ROS production with *E. coli* stimulation was significantly diminished in cord blood. This bacteria dependent ROS production was reconstituted with heat labile humoral factors in adult plasma. These results suggest that opsonization of the bacteria is important for the treatment of neonatal sepsis, but the efficiency of the treatment depends on bacterial species.

Materials and Methods

Reagents

Calcium and magnesium free Dulbecco's phosphate-buffered saline (PBS) was purchased from GIBCO (Grand Island, USA), anhydrous glucose was purchased from Wako (Osaka, Japan) and PBS containing 0.9% anhydrous glucose was signified as PBSg. Hydroethidine (HE) was purchased from Sigma (St Louis, USA), and dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mM and stored at -80°C. Before using, the stock solutions were thawed and diluted with PBS to the final concentration of 2 μM. LPS was purchased from Phycoerthrin (PE) conjugated anti-human Toll-like receptor 4 (TLR4) and Fluorescein isothiocyanate (FITC) conjugated anti-human Toll-like receptor 2 (TLR2) were purchased from eBioscience (San Diego, USA). Phospho-p38 MAPK mouse monoclonal antibody and p38 MAP kinase antibody were purchased from Cell Signaling technology (Beverly, USA). Affinity purified mouse monoclonal anti-human IFN-γ and affinity purified goat polyclonal anti-human TNF-α antibodies were purchased from DAKO JAPAN (Kyoto, Japan), R&D systems (Minneapolis, USA) respectively.

Staphylococcus aureus (*S. aureus*, strain 209P JC-1) and *Escherichia coli* (*E. coli*, strain NIHJ JC-2) were cultured in trypticase soy agar plate (Eiken Chemical Tokyo, Japan) at 37°C overnight. Then the concentration of bacteria suspension was adjusted to 2×10^9/ml by spectrophotometer, UV-2500PC (Shimadzu, Kyoto, Japan). FITC conjugated *Escherichia coli* (strain K-12) was purchased from Molecular Probes (Oregon, USA) and prepared according to manufacturer's protocol. Before using, FITC conjugated *E. coli* was adjusted to the same concentration as live bacteria suspension with PBS.

Whole blood sample preparation

Mie Chuo Medical Center Institutional Review Board approved the study and informed consent was obtained from the parents of all newborn infants, heparinized (10 IU/ml) umbilical cord blood of 25 neonates (mean ± SD, gestational age, 39.6 ± 0.8 weeks; birth weight, 3050 ± 320 g, uncomplicated pregnancy, labor, and normal vaginal delivery) were collected at birth. For control study, heparinized venous blood samples were collected from 11 healthy adults volunteer.

Superoxide production and phagocytosis of PMNs

Measurement of superoxide anion (one of ROS) production and phagocytosis of PMNs in whole blood was modified from Perticarari's method [21]. All assays were performed within 12 hours after collection. One hundred μl of heparinized cord or adult whole blood sample was made in aliquots in 12 × 75 mm round bottom tubes, mixed well with 1μl-diluted HE (final concentration of 2 μM). Prepared *S. aureus*, *E. coli* bacteria suspension and FITC conjugated *E. coli* were added to samples respectively for bacterial challenge. To determine the suitable volume of bacteria suspension which added to whole blood, preceding experiments with a series of neutrophil-bacteria ratio were performed. The most suitable ratio of PMNs to bacteria, 1:80 was used in the following bacterial challenge experiments. The number of PMNs was determined by microscopic count of Giemsa staining of blood smear and trypan blue dye exclusion method. After the incubation of 37°C in water bath shaker at 150/min for 60 minutes, each sample was lysed at room temperature with 2 ml of diluted lysing solution which purchased from Becton Dickinson (San Jose, USA) for 15 minutes. After washing, the cell pellets were resuspended in PBSg and a total of 1×10^4 cells were immediately analyzed on FACScan using CellQuest software (San Jose, USA). Quantitation of superoxide production and phagocytic activity were estimated by red and green mean fluorescence intensity (MFI) per cell respectively.

Analysis of surface molecules on PMNs

The expression of CD14, TLR2 and TLR4 were analyzed using FACScan after staining with fluorescent-conjugated monoclonal antibodies (mAbs). Adult or cord blood was incubated with the corresponding mAbs for 30 min at 4°C in the dark. Then, cells were lyzed with lyzing solution and washed with PBS. After washing, the cell pellets were resuspended in PBS and a total of 1×10^4 cells were immediately analyzed on FACScan.

Preparation of PMNs

In some experiments, enriched PMNs are prepared for intracellular signaling study. For enrichment of PMNs, Rosette Sep Myeloid enrichment kit (Stem Cell Technology Inc. Vancouver, Canada) was used as manufacture's protocol. Preparations were microscopically checked with Giemsa stain and these >95% purity cells were used for further preparation. Enriched PMNs were lysed with RIPA buffer (PBS with 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS) containing protease inhibitors (0.15 μM aprotinin, 1 μM leupeptin and 1 mM PMSF).

SDS-PAGE and western blotting

Samples were normalized for total protein content using DC protein assay kit (BIO-RAD Hercules, USA) according to the manufacturer's protocol. Forty micrograms of each sample were heated at 95°C for 5 min, and the proteins were separated on a SDS-12% polyacrylamide gel with reduced condition as Laemmli's method. Then the gels were electroblotted to nitrocellulose filters for immunoblotting assays. Antibodies used for immunoblotting assays included the affinity-purified Phospho-p38 MAPK mouse monoclonal antibody (used at 1:2000 dilution) and p38 MAP kinase antibody (used at 1:1000 dilution). Antibodies were detected on filters using

horseradish peroxidase conjugated goat anti-rabbit IgG serum followed by the enhanced chemiluminescence (ECL) reagents (Amersham Biosciences Piscataway, USA). In some experiments fluorographic intensity of interest bands were measured and calculated with Macworks (Scanalytics, Inc. Fairfax, VA).

Adult plasma treatment for inhibition assays

In order to study the factors in adult plasma, superoxide production and bacterial phagocytosis were examined with various conditions adult plasma. In some experiments, adult plasma was heat inactivated (56°C, 30 min) or pre adsorbed with enough amount of *E. coli*. For antibody blocking experiments, neutralizing anti-cytokines (mouse monoclonal anti-human IFN-γ, goat polyclonal anti-human TNF-β were used as manufacture's protocol.

Statistical analysis

All the data were presented as mean ± SD. Statistical analysis was performed by the student's T test method, significance was accepted at $P<0.05$.

Results

ROS production of PMNs with bacterial stimulation

PMNs were discriminated in terms of forward and side light scatter on FACScan (Figure 1A). Red fluorescence from ethidium bromide, the oxidized product by PMNs was displayed as a single histogram. The superoxide producing PMNs stimulated with bacteria showed high fluorescent population in the histogram (Figure 1B and 1C). When PMNs were stimulated with *E. coli* or *S. aureus*, adult PMNs seemed to produce higher level of superoxide anion both in *E. coli* and *S. aureus* stimulation. But a significant difference of superoxide anion production was observed between cord and adult PMNs with *E. coli* stimulation (Figure 1D).

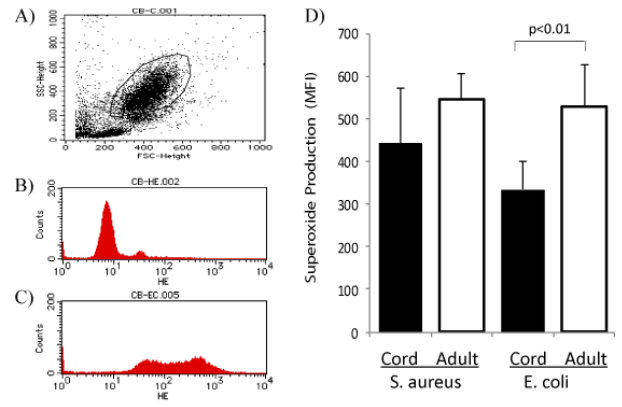

Figure 1: ROS production of PMNs with bacterial stimulation.

Superoxide production of cord and adult PMNs. PMNs were stimulated with Escherichia coli or *Staphylococcus aureus*. Flowcytometric analyses were shown in A, B and C. ROS production was measured in gated population (A). The ability of superoxide production of PMNs is shown in histogram of negative control (B) and of *E. coli* stimulation (C). Superoxide production of cord (closed column) and adult (open column) PMNs with *E. coli* or *S. aureus* stimulation was presented by mean fluorescence intensity (MFI) acquired by FACScan (D). (Cord/S. aureus: MFI 443 ± 129; Adult/*S. aureus*: MFI 546 ± 61; Cord/E. coli: MFI 334 ± 66; Adult/*E. coli*: MFI 530 ± 98) Data are presented as the mean ± SD; statistical analysis by student's T test method, significance was accepted at $P<0.05$.

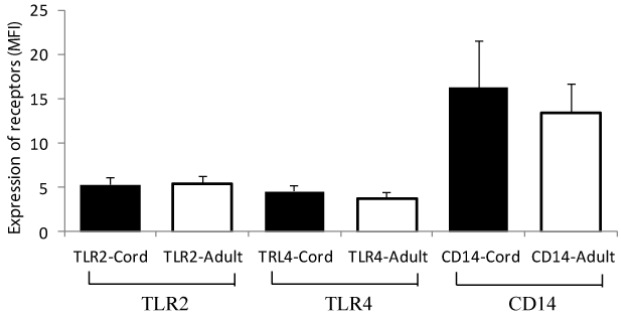

Figure 2: TLR2, TLR4 and CD14 expression on adult and cord PMNs.

The expression of TLR2, TLR4 and CD14 surface molecules of PMNs are presented by mean fluorescence intensity (MFI) acquired from green or red fluorescence on granulocyte gate by FACScan. (Cord/TLR2: MFI 5.28 ± 0.79; Adult/ TLR2: MFI 5.3 ± 0.94; Cord/ TLR4: MFI 4.55 ± 0.61; Adult/TLR4: MFI 3.73 ± 0.65; Cord/CD14: MFI 16.23 ± 5.32; Adult/CD14: MFI 13.35 ± 3.37) MFI measured in granulocyte gated all PMNs of adults (open column) and neonates (closed column) is presented as the mean ± SD.

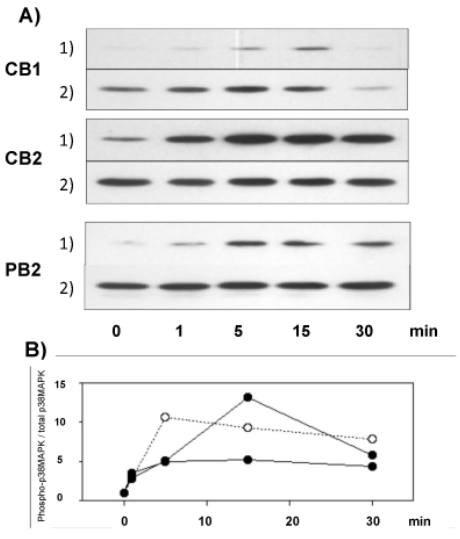

Figure 3: The time course of p38 MAPK activation.

Activation of p38 MAPK is measured by Western blot and plotted in time course. Western blot revealed total amount of p38 MAPK (2) and phosphorylated active form p38 MPAK (1). Each lane shows 0,1,5,15,30 minutes after *E. coli* incubation (A). Active p38 MPAK/

total p38 MPAK ratio was calculated with densitmetric analysis and plotted in time course (B).

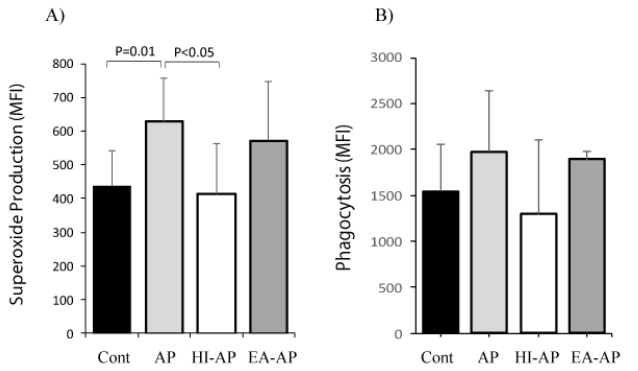

Figure 4: The effects of adult plasma and heat inactivated or *E. coli* adsorbed adult plasma on cord PMNs activities.

(A) Superoxide production and (B) phagocytotic activity of PMNs with the condition substituting adult plasma for neonatal plasma were shown. Neonatal blood cells were incubated with *E. coli* or FITC-conjugated *E. coli* in autologous plasma or in various conditioned adult plasma. Then mean fluorescence intensity (MFI) was measured by FACScan. (ROS/Cont: MFI 439 ± 103; ROS/AP: MFI 630 ± 129; ROS/HI-AP: MFI 413 ± 150; ROS/EA-AP: MFI 572 ± 178; Phagocytosis/Cont: MFI 1545 ± 509; Phagocytosis/AP: MFI 1976 ± 665; Phagocytosis/HI-AP: MFI 1296 ± 805; Phagocytosis/EA-AP: MFI 1894 ± 81) Data are presented as the mean ± SD of plasma exchanging condition (gray and open column), and autologous control (closed column). In some experiments, heat inactivated adult plasma (light gray column) or bacterial adsorbed adult plasma (dark gray column) was used for substitution. MFI measured in adults autologous condition is also revealed as open column. Statistical analysis by student's T test method was performed and significance was accepted at $P<0.05$.

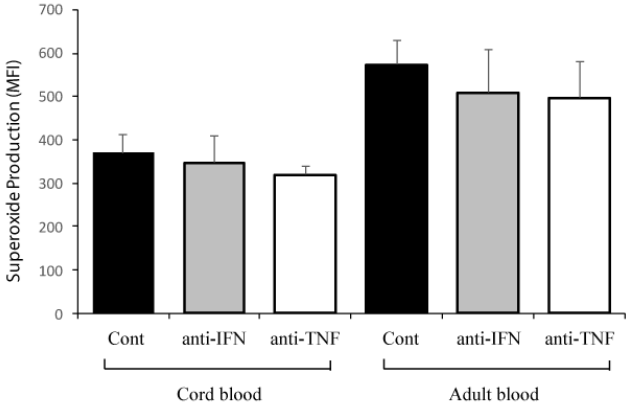

Figure 5: The effects of anti-IFNγ and anti-TNFα on ROS production of PMNs.

Effects of blocking antibodies (interferon-γ and TNF-α) on superoxide production of PMNs are shown. Enough amounts of each antibody were added to whole blood and bacterial mixture according to manufacturer's protocol. (Cord/Cont: MFI 371 ± 43; Cord/IFN: MFI 347 ± 63; Cord/TNF: MFI 320 ± 21; Adult/Cont: MFI 574 ± 58; Adult/IFN: MFI 510 ± 97; Adult/TNF: MFI 498 ± 84) MFI measured in the presence of anti-interferon-γ (dark column) or anti-TNF-α (open column) is presented as the mean ± SD. Closed columns are controls with no antibody.

TLR and CD14 expression on cord and adult PMNs has no significant difference

To investigate the mechanism of the different superoxide production between cord and adult PMNs, we analyzed the expression of TLR2, TLR4 and CD14, which are important receptors for the response to bacterial stimulation, on the cell surface of PMNs. As shown in Figure 2, the expression of TLR2, TLR4 on PMNs was very low in resting phase and flowcytometric analysis revealed no difference in the expression of TLR2, TLR4 and CD14 in cord and adult PMNs. CD14 expression on PMNs of cord blood was slightly higher than that on adult controls, although there was no significant difference in statistical analysis.

Intracellular activation is similar in cord and adult PMNs

Then we investigated the activation of an intracellular signaling molecule p38 MAPK to elucidate the different response of cord and adult PMNs to *E. coli* challenge. The activation of p38 MAPK has a major role in LPS stimulated PMNs. We examined the *E. coli* mediated activation of p38 MAPK in adults and cord PMNs using Western blotting which clarifies the levels of p38 MAPK phosphorylation (Figure 3A). *E. coli* induced dramatic activation of p38 MAPK in a few minutes and the activation reached maximum at 5 to 15 minutes in all samples that we examined (Figure 3A). Time course analysis of the p38 MAPK activation was performed using active p38 MAPK to total p38 MAPK ratio (Figure 3B). *E. coli* induced p38 MAPK activity reached maximum level within 15 minutes and gradually decreased. There was no difference between cells from cord and adult blood.

Plasma exchange reconstitutes superoxide production of cord blood PMNs

Since cell signaling molecules and phagocytic activity showed no difference in adults and neonates, we evaluated the humoral factors in plasma. We separated adult plasma from AB type blood and replaced cord blood plasma to this adult plasma. As shown in Figure 4A, *E. coli* induced superoxide production of cord PMNs were significantly increased when plasma replacement was done. For further evaluation of humoral factors in plasma, heat sensitivity was checked. We found a clear effect of plasma replacement to increase superoxide production but heat inactivated plasma replacement failed to reconstitute superoxide production (Figure 4A). When adult plasma was mixed with cord blood cells, superoxide production of the cells was increased to almost the same level of adult blood, but heat inactivated plasma lost the effect completely. On the other hand, *E. coli* adsorbed plasma exchange revealed the same effect in ROS production as adult plasma exchange (Figure 4A). Phagocytosis of PMNs causes some stimulation for the superoxide production. We investigated the phagocytic activity of cord and adult PMNs with the stimulation of *E. coli* using flowcytometric analysis. MFI acquired from PMNs gate on flowcytometry was used as an indicator for the ability of PMNs

phagocytosis. We found that flowcytometric analysis showed no difference in the ability of phagocytosis between cord and adult PMNs and we confirmed it by the observation with a Laser confocal microscope (data not shown). We performed plasma exchange experiments to evaluate changes in phagocytic activity of PMNs (Figure 4B). The effects of intact adult plasma, heat inactivated plasma and *E. coli* adsorbed plasma seemed similar pattern to ROS production experiments with various plasma exchange, but we could not find any statistical difference.

The effects of anti-IFNγ and anti-TNFα on ROS production of PMNs

We performed neutralizing experiments for cytokines by blocking antibodies to evaluate the effects of cytokines such as TNFα and INFγ in plasma exchange effects (Figure 5). Anti-TNFα and anti-INFγ was added to samples as enough amounts to abolish effects of these cytokines. We found slightly decreased super oxide production in samples adding these antibodies, but there was no statistical difference between cord and adult samples with or without antibodies.

Discussion

ROS generation is the initial phase of the respiratory burst and the importance of this respiratory burst in bacterial killing is well characterized. We found that the ability of superoxide production by *E. coli* stimulation was lower in neonatal neutrophils than in adult. To clear the mechanism of impaired superoxide production in neonatal neutrophils, we analyzed receptors and intracellular signaling pathways of neutrophils. The signal transduction pathway in stimulated neutrophils with bacterial components, peptidoglycans and lipopolysaccharide (LPS), includes TLR2 and 4 receptors on the cell surface. TLR2 and 4 receptors signaling pathway are mediated by MyD88, IARKs and TAK1 [18]. Then the microbial stimulation induced by Gram negative rods such as *E. coli* transmitted to NF-κB and MAP kinases following transcriptional regulation. CD14 is expressed on the surface of monocytes, macrophages and neutrophils and acts as a co-receptor along with TLR4 and MD-2 for the detection of LPS [22,23].

First, we examined the distributions of TLR2, TLR4 and CD14 on the cell surface of neonatal and adults neutrophils. Although low expression of these molecules was observed on neutrophils, there was no significant difference between adult and neonatal neutrophils. From these results, neonatal neutrophils are thought to have almost the same ability responding to *E. coli* at receptor level.

Second, we explored the activation and kinetics of p38 MAPK which is a key enzyme in LPS-stimulated signal transduction pathway of neutrophils. Nick et al. reported that exposure of neutrophils to LPS results in the phosphorylation and activation of p38 MAPK [24]. The signal transduction pathway necessary for induction of interferon-stimulated genes in neutrophils involves p38 MAPK, is activated by a TLR 4 ligand but not activated by TLR 2 ligand [25]. The expression of active p38 MAPK and its time course of adult and neonatal neutrophils revealed no difference. Our findings suggest the intracellular signaling pathway of neonatal neutrophils works well as adult neutrophils and some extracellular factors make impaired response of superoxide production against *E. coli*.

It has been shown that neutrophils from newborn infants are not primed in response to LPS, in contrast to neutrophils from adults under similar in vitro condition [26,27]. In addition, Fujiwara et al reported the significance of plasma effects on phagocytic activity and hydrogen peroxide production by neutrophils in neonates [28]. There are some more reports showing significance of plasma for bacterial killing. Bonner et al reported the necessity of plasma for LPS-mediated ERK activation. In human neutrophils, LPS-mediated activation of ERK 1 and 2 requires plasma [29]. Furthermore there is a report that some cytokines such as TNF can act on neutrophils to increase respiratory burst capacity [30].

We explored the factors in adult plasma with heat inactivation and *E. coli* adsorption for plasma exchange experiments. *E. coli* adsorbed plasma; anti-IFN-γ or anti-TNF-α added plasma were all revealed no significant effect to the ROS production of in neonatal neutrophils. On the other hand, heat inactivated plasma failed to reconstitute the ability of superoxide production in neonatal neutrophils [31]. Therefore the rapid uptake which leads to the intracellular killing of *E. coli* by neutrophils depends on the presence of heat-labile plasma factors. But in terms of phagocytosis, opsonization of neonatal neutrophils is thought to be normal in our experiments and insufficiency of compliment system is unlikely.

In our experiments, ROS production of neutrophils revealed no significant decrease in *S. aureus* stimulation experiments. And the phagocytosis of *S. aureus* [32] and *E. coli* [33] was reported to be equal in both adult and neonatal neutrophils. This means that neutrophils of term neonate opsonize and recognize *E. coli* and *S. aureus* as well as adult neutrophils do. There are some reports that *S. aureus* has developed several strategies to escape killing by neutrophils including sophisticated oxidative stress response [18,34,35]. Therefore *S. aureus* might have some restricted mechanism to avoid ROS production in neutrophils. The process of phagocytosis and killing by neutrophils is mediated through receptors for both compliment and the Fc domain of immunoglobulin. Therefore opsonization of pathogenic organism is the most important for superoxide production in phagocytic activation. Generally, microorganisms are engulfed with phagocytosis after they have been properly opsonized, that is, loaded with IgG and activated C3 [36].

From these data, we have concluded that cellular components are not impaired but some plasma factors except for concerning opsonization might cause diminished superoxide production of neutrophils in term neonate. Although the antibiotics are a powerful therapeutic tool for bacterial infections, antibiotics resistant bacteria production and side effects of long term treatment are problems of antibiotics. In addition to antibiotics treatment, some supportive and innate immunity enhancing treatments are preferable for neonatal bacterial infection in future. Immunoglobulins and complements are important for bacterial opsonization but cytokines, bacteria specific monoclonal antibodies and some humoral factors which observed in our experiments are candidates for innate immunity supporting therapy.

Acknowledgments

This was supported in part by a Grant-in-Aid 1999 from the Mie Medical Research Foundation. The authors declare that no functional conflict of interest exists.

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