Lipid A of *Salmenella typhimurium* Suppressed T-cell Mitogen-Induced Proliferation of Murine spleen Cells in the Presence of Macrophage

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Received December 7, 2006 / Accepted January 18, 2007

Infection with virulent or attenuated *Salmonella typhimuriumhas* known to induce reduction in proliferative responses of spleen cells. We investigated a role of lipid A from *S. typhimurium*, a B cell mitogen, on proliferation of spleen cells by T cell mitogens such as concanavaline A and phytohemagglutinin under *in vitro* and *ex vivo* conditions. Lipid A alone induced proliferation of spleen cells *in vitro*in a dose-dependent manner. However, subsequent treatment of concanavaline A or phytohemagglutin in after lipid A treatment induced proliferation suppression of murine spleen cells *in vitro* and *ex vivo*. Removal of macrophages from spleen cells, which were obtained from a lipid A-injected mouse, restored proliferation by concanavaline A and phytohemagglutinin, indicating that macrophages appeared to play a role in lipid A-induced suppression. Secreted molecules from macrophages did not accounted for the suppression because suppressive effect was not achieved when the supernatant from macrophage-containing spleen cell culture was conditioned to macrophage-depleted spleen cell culture. Co-culture of spleen cells from lipid A-treated and - untreated mice showed proliferation suppression as increasing cell numbers of lipid A-treated mouse. These data suggested that the cell-to-cell contact of macrophage with splenic lymphocyte cells is responsible for immune responses against lipid A, which is applicable to the case of human *S. typhi* infection.

Key words – *Salmonella typhimurium*, lipid A, immunosuppression, lymphocyte proliferation

Introduction

Murine infection of *Salmonella typhimurium*, mouse typhoid, is a model for human typhus induced by *Salmonella typhi*. *Salmonella typhimurium* is a facultative intracellular pathogen of mice that causes a systemic infection[8]. Severe infection results in bacteremia, and sublethal infection results in the persistence of *Salmonella*in liver and spleen for several weeks with concomitant immunity, otherwise the pathogen is eliminated by cellular immune responses. Therefore, the interaction of the invading bacteria and their products with macrophages and lymphocytes is very important for the resistance to infection of this pathogen[9].

Many studies have demonstrated that *Salmonella* infection with avirulent strain or attenuated bacteria induced reduction in proliferative responses of spleen cells from infected mice to B and T cell mitogens[2,3,5,7,13]. Such antiproliferative activity of *Salmonella* infection was also demonstrated by Brunner and Kroll[8] in *Salmonella* resistant CBA/J-mice with a virulent strain. They found that antiproliferative activity on spleen cells reached maximum at one week post-infection and that *Salmonella*-infected mice showed a raised resistance against a superinfection with other bacteria, for example, *Staphylococcus aureus*. Matsui et al.,[19-21] reported that treatment with cell-free *S. typhimurium* extract suppressed proliferation of mitogen-induced spleen T cells. Their data showed that the immunosuppression of T cells in *Salmonella*-infected mice was caused dose-dependently by ST1, a 87 KDa protein, from the bacterial cells.

Several cell-wall components of Gram negative bacteria have been widely used as polyclonal B-cell activators. Among these are lipopolysaccharide (LPS), lipid A, endotoxin protein and lipoprotein. Each of these has been shown to be a potent inducer of lymphocyte proliferation and secretion of nonspecific antibody by B cells[11,14]. LPS is an amphiphilic molecule composed of O-specific polysaccharide, core polysaccharide and lipid A. The physiological and immunological effects of LPS have been extensively studied[7,11]. A current model of the innate immune recognition of bacterial LPS is supported with LPS transferring proteins based on discovery of LPS-binding

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protein[28], CD14[35], and Toll-like receptor 4 (TLR4) [26,27]. Furthermore, CR3 (CD11b/CD18) is also known to coordinate with CD14 and TLR4 for LPS-mediated signal in macrophage[25].

Lipid A from many bacteria shares a common backbone consisting of a -1,6-linked D-glucosamine disaccharide carrying ester- and amide-linked fatty acids, and phosphate groups at positions C-1 and C-4. Lipid A has been thought to be the active center of endotoxin[17] and 2-keto-3-deoxy-octulosonic acid (KDO) residues seem to play a role[5]. However, little study has been investigated the effects of *in vivo* administered lipid A without other LPS components on the proliferation of spleen cells.

In this report, we describe the effects of mitogens on the proliferation of spleen cells *in vitro* and *ex vivo*. Proliferation of lipid A-treated spleen cells was suppressed when mitogens such as Concanavalin A (Con A) and Phytohemagglutinin (PHA) were subsequently treated. Also, the suppressive role of macrophages in anti-proliferative response of spleen cells by mitogens was discussed.

Materials and Methods

Experimental animals and spleen cell primary culture

Six-to-ten week old, specific pathogen-free, randomly selected, female mice (CBA/J) were purchased from Bomholtgard, Denmark. CBA/J mice are genetically resistant to *S. typhimurium* infection through the dominant allele Ity^r on chromosome 1 and carry the Lps^n(normal) allele[16]. Single spleen cell suspensions were prepared as previously described[3]. Briefly, spleens were removed aseptically and homogenized in 5 ml RPMI 1640 medium. Homogenized sample was then centrifuged at 1,000 rpm for 5 min at room temperature, and red blood cells were lysed for two min by adding 5 ml of 0.16 M NH_4Cl. After red blood cell lysis, spleen cells were washed 3 times with RPMI1640 and resuspended to 2 $x10^6$cells/ml in a complete medium (RPMI 1640 supplemented with 2 mM glutamine, 100 U/ml of penicillin G, 100 μg/ml of streptomycin, and 10% of fetal bovine serum). Unless otherwise indicated, 200 μl of the spleen cell suspension were added to flat-bottomed, 96 well microtiter plates. To remove macrophages from spleen cells, a gel filtration chromatography with Sephadex G-10 was used[18]. Cells from the column were collected in a sterile siliconized tube. Cells were then counted and incubated in 96 well plates for assay. After Sephadex G-10 chromatography macrophages were less than 5.5% of the total cells. Percentage of macrophages was determined by monocyte esterase staining using an esterase staining kit (Technicon, Neuwied).

In vitro assay of mitogen-induced proliferation

To assay the effect of lipid A on mitogen-induced cell proliferation, whole spleen cells and macrophage-depleted spleen cells were treated with various concentrations of lipid A. After 1 hr pretreatment with lipid A, the cells were cultured further with the indicated mitogens in the presence of lipid A for 68 hr. Con A (Amersham Bioscience) and PHA (Welcome Diagnostics, Dartford) were used as T cell mitogens whereas lipopolysaccharide (LPS) was used as a B cell mitogen. LPS and lipid A of *S. typhimurium*LT12 were obtained from Sigma. Con A and LPS were used at the same final concentration of 2 μg/ml, and the concentration of PHA was 100 μg/ml unless otherwise indicated. Phosphate-buffered saline (PBS) was used for a negative control. Spleen cells were labeled by the addition of 0.5 μCi/well ^{3}[H]-thymidine (2.0 Ci/mmol, Amersham Bioscience) for 6 hr prior to harvest. Spleen cells were harvested onto glass fiber filters, fixed by washing with 5% methanol and dried at 110°C. The filters were then measured for ^{3}[H]-thymidine incorporation by Rackbeta 1216 liquid scintillation counter (Amersham Bioscience). Each experiment was carried out independently three times with triplicated samples.

In vivo effect of lipid A on spleen cell proliferation

Lipid A was dissolved by bath sonication for 2 min to a concentration of 5 mg/ml in saline. To determine a time point for *in vivo* effect of lipid A, suspension culture of spleen cells was prepared at 8 hr, 24 hr, 48 hr, 72 hr after 200 μl of lipid A (200 μg/mouse) injection. Whole spleen cells and macrophage-depleted spleen cells obtained from lipid A-injected mice were treated with 2 μg/ml of Con A for 68 hr *in vitro*. For assay of the *in vivo* effect of lipid A on antiproliferation, 200 μl of lipid A solutions in various concentrations was intraperitoneally administered into mice At 48 hr post-administration the spleen cells obtained from mice, that were administered with various concentrations of lipid A, were treated with Con A, PHA and

LPS as done the *in vitro* assay. Proliferation of spleen cells was measured with ^{3}[H]-thymidine incorporation as described above.

Effect of cell-to-cell interaction

Suspension culture of spleen cells was prepared at 48 hr after lipid A injection (200 μg/mouse). Whole spleen cells obtained from lipid A-treated and -untreated mice were mixed to total $4x10^{5}$ cells in the ratio of 0.05:1, 0.5:1, and 1:1, respectively and incubated 72 hr in the presence of Con A (2 μg/ml) and PHA (100 μg/ml). The same number of untreated cells only to each co-culture was used as a control to calculate percent of suppression. Proliferation of spleen cells was measured with ^{3}[H]-thymidine incorporation as described above.

Results

Mitogenic effect of lipid A on spleen cells *in vitro*

We first tested mitogenic effect of lipid A with various concentrations on primary-cultured spleen cells *in vitro*. After treated with different concentration of lipid A, spleen cells were examined for proliferation by measuring incorporation of ^{3}[H]-thymidine. As expected, proliferation of spleen cells was induced in a lipid A dose-dependent manner, reaching the maximum proliferation at the concentration of 200 μg/ml of lipid A (Fig.1). Therefore, 200 μg/ml of lipid A concentration was determined for *in vitro* and *in vivo* experiments.

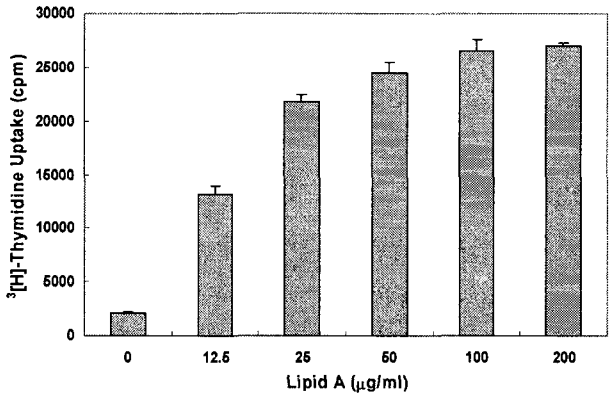

Fig. 1. Mitogenic effect of lipid A on spleen cell proliferation *in vitro*. Murine spleen cells ($4x10^{6}$ cells) were treated with the indicated concentration of lipid A for 68 hr. Spleen cell proliferation was measured as ^{3}[H]- thymidine uptake.

Lipid A suppresses mitogen-induced proliferation of spleen cells *in vitro*

Spleen cells with or without macrophages were pretreated with various concentrations of lipid A for 1 hr and cultured continuously in the presence of lipid A together with other mitogens. Proliferation responses of spleen cells to T cell mitogenic lectins, PHA and Con A, were suppressed by lipid A in the presence of macrophages but not suppressed when macrophages were depleted (Fig. 2). Lipid A suppressed ~27% and ~50% mitogenic activities of

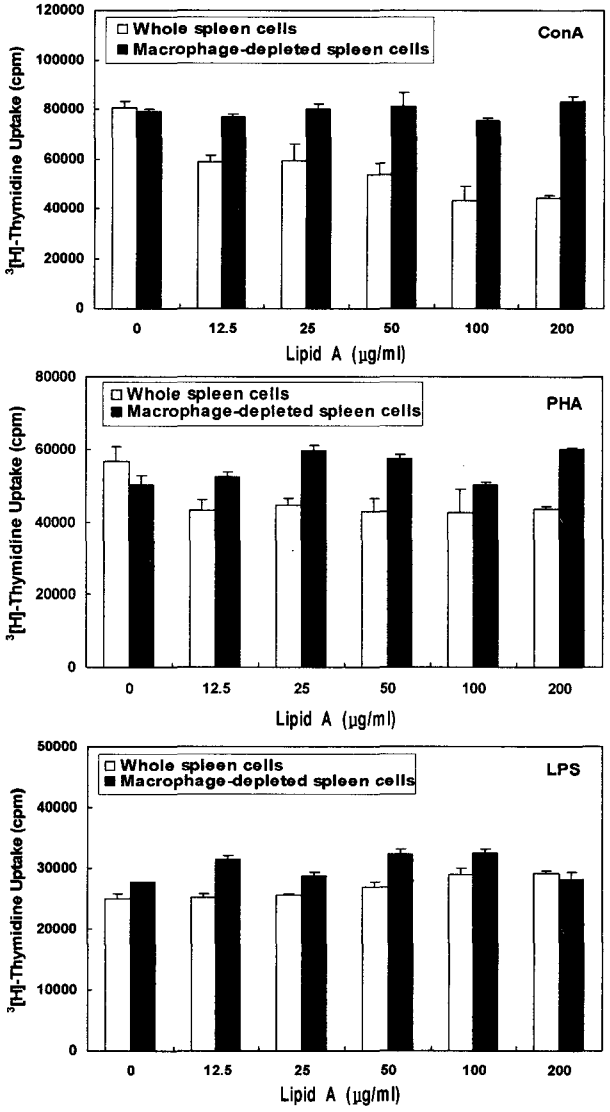

Fig. 2. Effect of lipid A on mitogen-induced proliferation of spleen cells *in vitro*. Murine spleen cells were incubated with the indicated concentration of lipid A. Each of LPS, PHA, Con A was additionally added to the culture, and spleen cells were further incubated for 68 hr. Spleen cell proliferation was measured as described in Materials and Methods.

PHA and Con A, respectively. Suppression in the presence of Con A was greater than that of PHA and was increased in a lipid A dose-dependent manner. In contrast, a B cell mitogen, LPS, in the presence of lipid A did not show any suppressive effect regardless the presence or absence of macrophages.

In vivo treatment of lipid A inhibits mitogen-induced proliferation of spleen cells *ex vivo*

In vivo treatment of lipid A in proliferation suppression was investigated. A time course for *in vivo* effect of lipid A was first determined. Lipid A (200 μg/mouse) was administered into mice intraperitoneally. *Ex vivo* proliferation suppression was assayed with spleen cells from 8 hr, 24 hr, 48 hr and 72 hr of post-lipid A injected mice. The incorporation of ^{3}[H]-thymidine was performed with those cell in the presence of Con A for 68 hr incubation. The incorporation of ^{3}[H]-thymidine was markedly reduced in the spleen cells from 48hr and 72 hr post-lipid A injected mice (Fig. 3). To determine administration amount of lipid A to induce suppression the indicated amounts of lipid A were injected and spleen cells were obtained from 48 hr post-injected mice. The obtained spleen cells, which were exposed to the indicated amounts of lipid A *in vivo*, were then incubated further in the presence of Con A, PHA, and LPS for 68 hr. Similarly to the *in vitro* data, T cell mitogen-induced proliferation of lipid A-injected mouse was suppressed significantly in comparison with that of cells from untreated mouse. In both cases of PHA and Con A, proliferation suppression by lipid A was ~80% and 90% with 200 μg/mouse of lipid A injection, respectively and

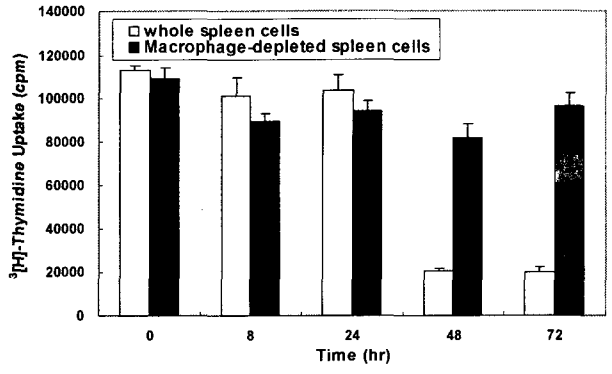

Fig. 3. A time course of lipid A response *in vivo*. Lipid A (200 μg/mouse) was injected intraperitoneally to mice, and spleen cells were obtained at 8, 24, 48, 72 hr after lipid A injection. Proliferation of spleen cells was assayed in the presence of Con A (2 μg/ml) for 68 hr.

was reduced as the amounts of injected-lipid A decreased (Fig. 4). 50 μg/mouse of lipid A was the minimum amount to produce suppressive effect on the T cell mitogen-induced proliferation. No effect of the lipid A suppression on the B cell mitogen-induced cell proliferation was observed, which is consistent with *in vitro* data. Macrophages appeared again to play a role in suppressing the T cell mitogen-induced proliferation by lipid A because only macrophage-depleted spleen cells showed proliferation suppression.

Therefore, suppressive effect of lipid A *in vivo* was time-dependent and dose-dependent. It was also noted that

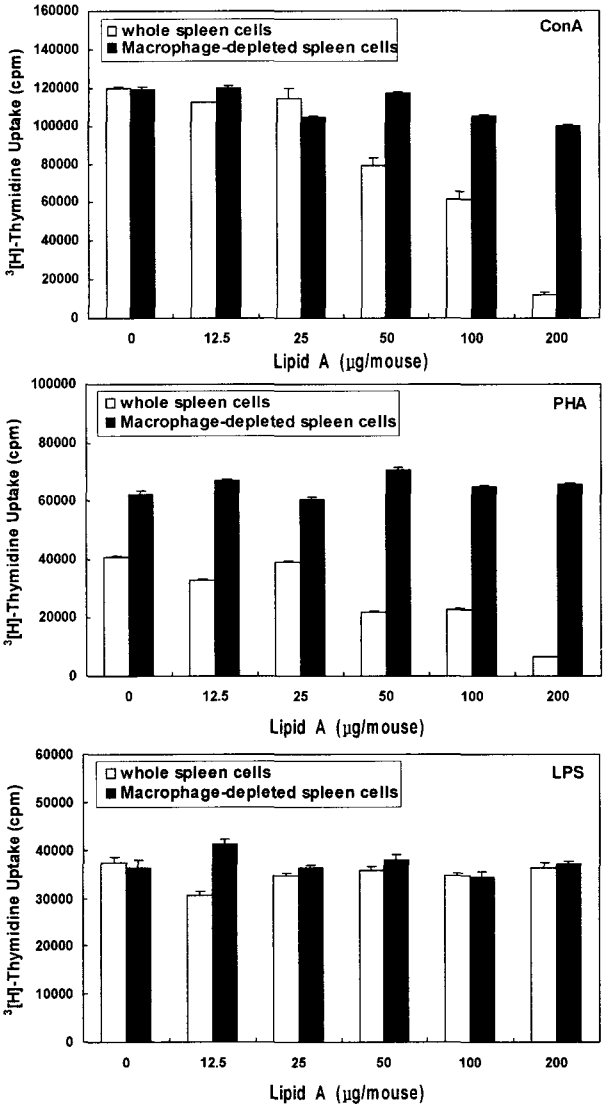

Fig. 4. Effect of lipid A *in vivo* injection on *ex vivo* mitogen-induced proliferation of spleen cells. The indicated concentrations of lipid A were injected intraperitoneally to mice, and spleen cells were obtained from each mouse at 48 hr after lipid A injection. Spleen cell proliferation was measured as described in Materials and Methods.

lipid A-injected mice showed marked splenomegaly, which was detectable at 24 hr and kept increasing up to 72 hr after lipid A injection (Table 1).

Role of Macrophage contact to lymphocytes

The observation that splenomegaly accompanied with mitogenic suppression suggested that macrophages might be involved in mediating the suppression phenomenon. Accordingly, a potential role of macrophages on mitogenicity was tested by comparing whole and macrophage-depleted spleen cells. Removal of macrophages from whole spleen cell suspension resulted in restoration of mitogenic responses of spleen cells after lipid A treatment both *in vitro* and *in vivo* (Fig. 2 and Fig. 4). These data indicated that macrophages mediate mitogen-induced antiproliferation activity possibly by secreted regulators from macrophages or cell surface molecules on macrophages.

We investigated whether the suppressive effect of macrophage on spleen cells is transferable by culture supernatant. Macrophages isolated from lipid A-injected mice were cultured for 72 hr and the supernatant of macrophage culture was collected. The supernatant was then transferred to the macrophage-depleted spleen cell cultures and the conditioned culture was incubated for 72 hr with mitogens. The transferred supernatant did not show any suppressive effect against proliferation response to the mitogen (data not shown).

We, therefore, tested inhibition effect of cell-to-cell interaction by co-culture of spleen cells containing macrophage from a lipid A-treated mouse to spleen cells of a lipid A-untreated mouse. There was no significant suppression of mitogen-induced proliferation when $1x10^4$ spleen cells containing macrophages from lipid A-treated mouse were added to $2x10^5$ of macrophage-depleted spleen cells from a lipid A-untreated mouse. Increasing the number of lipid A-treated cells in co-culture resulted up to 80% suppression of the proliferation response to T cell mitogens as shown in Table 2. To rule out the possibility that the observed reduction is due to cell crowding, lipid A-untreated spleen cells ($4x10^5$ cells) were cultured alone with mitogens. ^{3}[H]-thymidine uptake by high-populated lipid A-untreated spleen cells was not reduced (data not shown). Thus, direct cell-cell contact appeared to be important for the suppression in the presence of T cell mitogens.

Table 1. Splenomegaly of Lipid A-injected Mice

Mouse	Spleen weights (gm)*			
	8 hr	24 hr	48 hr	72 hr
Control	0.17±0.02	0.15±0.03	0.18±0.04	0.17±0.05
Lipid A-injected	0.20±0.02	0.32±0.04	0.54±0.04	0.91±0.06

*Means of three mice ± SD at the indicated post-injection times.

Table 2. Mitogen-induced proliferation suppression observed in co-culture of whole spleen cells from lipid A-untreated and -treated mice

Cells numbers in co-culture[a]			Suppression (%)[b]	
Untreated	Treated	(Ratio)	Con A	PHA
$2x10^5$	$1x10^4$	(1:0.05)	4.0±1.5	9.7± 3.7
$2x10^5$	$1x10^5$	(1:0.5)	43.7±8.4	54.1±11.0
$2x10^5$	$2x10^5$	(1:1)	78.4±2.7	69.3± 9.5

[a]The indicated number of spleen cells were co-cultured in the presence of mitogens and ^{3}[H]-thymidine uptake was measured at 96 hr of incubation. [b]Calculated with respect to ^{3}[H]-Thymidin uptake of the control ($2x10^5$ untreated cells only). The results are the means±SD of triplicate cultures.

Disscusion

We investigated the effect of an endotoxin, lipid A, of *S. typhimurium* on suppression of mitogen-induced proliferation of spleen cells. *In vitro*, LPS and lipid A, which have been well characterized as a B cell mitogen, showed mitogenic activity on spleen cells[24]. When spleen cells were treated with lipid A in the presence of T cell mitogens *in vitro*, spleen cells showed decreased proliferation. Furthermore, our data showed that the intraperitoneal injection of lipid A to mice induced significant suppression on mitogen-induced proliferation to T cell mitogens such as Con A and PHA. Maximum *ex vivo* suppression was observed at 48 hr after lipid A injection into mouse. The suppressive activity by lipid A appeared to be mediated by macrophages since their removal restored mitogen responsiveness of spleen cells to T cell mitogens. The data indicated that *in vivo* administration of endotoxin (lipid A) of *S. typhimurium* can cause *ex vivo* inhibition of mitogen-induced lymphocyte proliferation in the presence of macrophages. The results of this study, thus, supported the hypothesis that endotoxin of *S. typhimurium* causes the suppressed spleen cell proliferation in the infection of *S. typhimurium*.

The mechanism by which lipid A induces suppression on the immune responses is not clear yet. The results pre-

sented here suggest that lipid A-induced suppression of lymphoproliferative response to mitogens appeared to be associated with macrophages. Previous studies also demonstrated that chronic disease pathogens such as Leishmania, Trypanosoma and Toxoplasma induce macrophage-mediated suppression of mitogen-induced lymphocyte proliferation[6,15,22-34]. Failure in mitogen response has been frequently interpreted as a poor immune status of the host induced by the microorganisms and suppressor macrophages have been described in trypanosomiasis[34] and Leishmania infection[15].

It has been reported that macrophages suppress lymphocyte responses and are associated with the production of arachidonic acid (prostaglandins) [10,22,30,32], peroxide [4] and nitric oxid (NO) [1,13,32]. In addition, secretory proteins of macrophages, for instance interleukin-10, have been reported to be involved in inhibiting a lymphocytic response[12]. Arachidonic acid derivatives have many biological effects, among which is their anti-inflammatory effect by inhibiting cytokine production in macrophages[29]. Peroxide and NO have cytotoxic capacity such as parasitocidal and bactericidal effects to the cells. Furthermore, NO appears to contribute to the maintenance of immunologic homeostasis in T cell suppression by activated macrophages[1,11]. Our results with addition of supernatant from lipid A-treated spleen cell culture to lipid A-untreated spleen cells indicated that soluble factors of macrophages alone do not account for the suppression of T-cell response. However, we do not exclude NO-mediated suppression in our case because the half-life of NO is too short for it to be transferred by culture supernatant as reported previously[23,29]. Our results also showed that co-culturing of spleen cells from lipid A-untreated and treated mice induced suppression of proliferative response to T cell mitogenes. These data together with conditioned culture results suggest that cell-to-cell interaction is at least necessary for the suppressive effects. It is supported by the finding of B7-1-like molecule as a suppressive signal transmitting molecule from macrophages to T cells[31].

In conclusion, *in vivo* administration of endotoxin induces a reduced proliferative response of spleen cells to T cell mitogens and macrophages play a critical role in this suppression. Lipid A alone is enough to induce the macrophage-mediated suppression of spleen cells. The mechanism of immunosuppression appears in a cooperation of direct interactions between macrophages and spleen lymphocytes. The further study may focus on what surface molecule(s) on macrophage is responsible for suppression effect.

Acknowledgements

This research was supported by the Dong-Eui University Research Grants in 2004AA098.

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초록 : *Salmonella typhimurium* lipid A를 처리한 식세포 존재 조건에서 mitogen에 유도되는 이자세포의 증식억제

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사람의 장티푸스 연구는 생쥐에 감염되는 *Salmonella typhimurium*를 모델로 연구되고 있으며, 생쥐에 있어서 *S. typhimurium*의 감염은 이자세포의 증식반응을 감소시키는 것으로 알려져 있다. *S. typhimurium* lipid A의 처리가 T 세포 mitogen에 의한 이자 세포의 증식에 어떤 영향을 주는 가를 *in vitro*와 *ex vivo* 조건에서 알아 보았다. Lipid A 단독 처리는 이자 세포의 증식을 보였으나, lipid A 처리 후 T 세포 mitogen인 concanavalin A (Con A)와 phytohemagglutinin (PHA)에 의한 *in vitro*와 *ex vivo* 조건에서의 이차 처리는 오히려 세포증식이 억제되었다. Lipid A를 주사한 생쥐로부터 분리한 이자 세포에서 대식세포를 제거하였을 조건에서는 T 세포 mitogen에 의한 증식 효과가 유지되었으나 대식세포를 제거하지 않았을 경우에는 T 세포 mitogen에 의한 증식 효과가 억제되었다. Lipid A를 주사한 생쥐에서 얻은 대식세포를 포함한 이자세포의 숫자를 증가하면서 Lipid A를 주사하지 않은 생쥐에서 얻은 이자세포와 혼합 배양하였을 때 Lipid A를 주사한 생쥐에서 얻은 대식세포를 포함한 이자세포의 숫자가 높을수록 Con A와 PHA에 의한 증식억제가 높게 측정되었다. 이러한 결과는 Con A와 PHA의 이자세포 증식 기능이 lipid A의 전처리에 의해 활성화된 대식세포의 직접적인 접촉 작용으로 억제된 것으로 생각된다. 본 연구의 결과를 바탕으로 억제에 관여하는 대식세포 표면분자를 밝히는 것이 사람의 장티푸스 연구에 도움이 되리라 생각된다.