Influence of Bacterial Endotoxin on Mucosal Immune Response to Phosphorylcholine

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Abstract

Bacterial lipopolysaccharide (LPS), a component of the outer membrane of gram-negative bacteria that initiates inflammation by activation innate immune responses through Toll-like receptor 4 (TLR4). However, the influence of LPS on the mucosal immune reactions remains to be addressed. This study was examined the effect of LPS in nasal vaccination model. BALB/c and C57BL/6 mice were nasally immunized with keyhole limpet hemocyanin (KLH) conjugated with hapten phosphorylcholine (PC) or trinitrophenol (TNP) with LPS as a mucosal adjuvant, in the presence or absence of cholera toxin (CT). The antibody titers were measured in serum, saliva, and nasal wash fluids by an enzyme-linked immunosorbent assay (ELISA) in IgM, IgG, and IgA isotype-specific manner. The epitope-specific antibody production induced in blood and mucosal fluid was further enhanced by LPS for all isotypes examined. Besides, LPS, which has rarely been regarded as a mucosal adjuvant, was tested for its adjuvanticity by comparing the nasal immunization with PC-KLH plus LPS or with PC-KLH plus CT. LPS showed high adjuvanticity almost equal to CT. Possible differences of LPS from CT as a mucosal adjuvant remains to be elucidated.

Keywords: -

1. Introduction

Bacterial lipopolysaccharide (LPS) is a component of the outer membrane of gram-negative bacteria and is composed of a proximal hydrophobic lipid A moiety, a distal hydrophilic O-antigen polysaccharide region, and a core oligosaccharide that joins the lipid A and O-antigen structures [1]. LPS extracted from cell walls of gram-negative bacteria has a number of effects on cells of the immune system. LPS is a mitogen for B lymphocytes [2]. It is also a powerful adjuvant for antibody production [3]. The innate immune system recognizes LPS by the pattern recognition receptors (PRRs) [4]. Among the PRRs, Toll-like receptor 4 (TLR4) is the critical surface component on the mammalian cells for the LPS signaling pathway. It mediates the action of LPS [5, 6], followed by involvement of MyD88, IL-1 receptor-associated kinase (IRAK), IRAK-2, and TNF receptor-associated factor-6 (TRAF-6) [7]. TLR4 is expressed on the surface of antigen-presenting dendritic cells, LPS thus enhances the early stages of the adaptive immune system by activating the antigen presentation process [8, 9]. Thus, LPS acts as a promising, systemic and mucosal adjuvant that stimulates both innate and adaptive immune responses.

Adjuvants are substances that accelerate an antigen-specific immune response while antigen alone is not well recognized by the immune system. Thus the purpose of an adjuvant is to make an antigen visible to the eyes (macrophages/dendritic cells) of the immune system. Recognition of antigens by antigen presenting cells (APCs) is essential to initiate the critical cascade of events leading to localized inflammation, which recruits APCs and ultimately leads to the initiation of antibody-mediated immune responses [10]. An appropriate mucosal adjuvant is needed to elicit maximal levels of antigen-specific immune responses in both mucosal and systemic lymphoid tissues. Nasal vaccines, which fall into the category of mucosal vaccines, also require the use of an effective adjuvant to produce specific immunity [11, 12]. Cholera toxin (CT), the enterotoxin generated by Vibrio cholerae, which is most commonly used as a mucosal adjuvant in animal models for the induction of systemic and mucosal immunity, induces secretory-IgA (S-IgA) and systemic IgG of subclasses IgG1 and IgG2b, and systemic IgE antibody responses [13, 14]. Among the Th2 cytokines, interleukin-4 (IL-4) plays a key role in mediating the adjuvanticity of CT [15].

Phosphorylcholine (PC) is a structural component of cells from prokaryotes to mammalians and also possesses immunomodulatory properties [16]. Choline is one of the nutritional requirements of some bacteria and is taken up from the growth medium and incorporated as PC into teichoic acid and lipoteichoic acid of the cell wall [17]. PC is also known as an immunodominant epitope found on Streptococcus pneumoniae and is a candidate antigen for a potential protective vaccine against S. pneumoniae infection [16]. Actually, in the early phase of pneumococcal infection, serum PC-specific IgM and IgG3 antibodies, which are produced by marginal zone B cells of the

spleen, play an important role in immunological defense against this pathogen [18]. PC-specific immune responses induced by nasal administration of plasmid encoding Flt3 ligand (pFL) with PC-KLH resulted in enhanced clearance of S. pneumoniae [19]. The TEPC15 myeloma protein (T15) is known to be a representative mouse anti-PC monoclonal antibody (mAb) [20], and response to PC is dominated by the T15 idiotype, encoded by germline genes VS107 and Vκ22 [21]. It has been reported that the T15 idiotype in serum is the most protective antibody against pneumococcal infection22, and is mainly produced by peritoneal CD5+ B (B-1 B) cells [23]. In addition to their reactivity to PC, B-1 B cells are also shown to be an important source of IgA against cell wall protein antigens of commensal bacteria occurring in intestinal mucosal tissue [24]. It is also previously reported that B-1 B cells in submandibular glands and nasal passages play critical roles in the induction of antigen-specific mucosal responses by interacting with mucosal dendritic cells when mice were nasally treated with native CT and antigen [25].

The development of nasal vaccine may also contribute to the better preventive medicine when compared with systemic vaccination. However, most mucosal antigens are poorly immunogenic and require the use of potent mucosal vaccine adjuvants. CT adjuvants elicit strong humoral immunity following mucosal administration. Intranasal vaccination with PC conjugated keyhole limpet hemocyanin (KLH) together with CT induced PC-specific mucosal immune response mediated by Th2- as well as Th1-type cells and eliminated Streptococcus pneumoniae and Haemophilus influenzae from the nasal cavity [26]. However, CT possesses the risk of systemic toxicity and especially neurotoxicity, which renders the current CT adjuvants unsuitable for use in the human vaccine. Therefore, there remains a need for potentially safer and more potent mucosal adjuvants. LPS may act as adjuvant because of its ability to induce mitogenesis in B cells. It was wondered whether LPS could be used effectively as mucosal vaccine adjuvants. This study investigated the mucosal adjuvant properties of LPS and also examined whether a combination of LPS and CT as a mucosal adjuvant would be augmented both mucosal and systemic antibody responses.

2. Material and Method

2.1. Mice

BALB/c and C57BL/6 mice (6 to 8 weeks-old) were obtained from the Japan SLC, Inc. (Hamamatsu, Japan). Mice were transferred to sterile microisolators and maintained in an experimental animal facility at the University of Tokushima under specific pathogen-free conditions. All experimental procedures were approved and carried out following the guidelines established by the Ethics Committee of the University of Tokushima.

2.2. Immunization and sample collection

Mice were lightly anaesthetized before being immunized nasally with 50 µg of phosphorylcholine (PC)-keyhole limpet hemocyanin (KLH) or trinitrophenol (TNP)-KLH (Biosearch Technologies, Novato, CA, USA) in the presence or absence of 10 µg lipopolysaccharide (LPS) (Sigma-Aldrich, St. Luis, MO, USA). Other groups of mice were given intranasally 50 µg of PC-KLH or TNP-KLH and 1 µg of cholera toxin (CT) (List Biological Laboratories, Campbell, CA, USA) with or without 10 µg of LPS. Mice were immunized three times at weekly intervals in all experiments. On day 0, 7, 14, and 21, blood and saliva were harvested. Saliva samples were obtained following intraperitoneal injection of pilocarpine (100 µl of 1 mg/ml) diluted in sterile phosphate buffered saline (PBS). When the mice were sacrificed on day 21 (7 days after the last immunization), nasal washes (NWs) were obtained by the installation of 1 ml PBS on three occasions into the posterior opening of the nasal cavity with a hypodermic needle.

2.3. Analysis of antigen-specific antibodies

Anti-PC antibody titers in the serum, saliva, and NWs samples were determined by an enzyme-linked immunosorbent assay (ELISA). Polystyrene microtiter plates (Sumitomo Bakelite, Tokyo, Japan) were coated with 5 µg/ml of PC-bovine serum albumin (BSA) or TNP-BSA (Biosearch Technologies, Novato, CA, USA) dissolved in PBS. After blocking with 1% BSA in PBS, 2-fold serial dilution samples were added and incubated 2 hours at room temperature. Horseradish peroxidase (HRP)-conjugated anti-mouse IgM, IgG, and IgA (Southern Biotechnology Associates, Birmingham, AL, USA) were added to individual wells. The color reaction was developed for 50 minutes at room temperature with 100 µl of 1.1 mM 2,2'-azino bis(3-ethylbenzothiazoline-6-sulfonic acid) in 0.1 M citrate-phosphate buffer (pH 4.2) containing 0.01% H2O2. Endpoint titers were expressed as the reciprocal log2 of the last dilution that gave an OD at 405 nm of 0.05 greater than the background.

2.4. Detection of PC-specific Ab-forming cellsm(AFCs) by ELISPOT

Mononuclear cells from the spleen, cervical lymph nodes (CLNs), and nasal passages (NPs) were isolated using mechanical dissociation method by gentle teasing trough stainless steel screens. Mononuclear cells from submandibular glands (SMGs) were isolated by an enzymatic dissociation procedure with collagenase type IV (0.5 mg/ml; (Sigma-Aldrich, St. Luis, MO, USA), followed by a discontinuous Percoll gradient centrifugation (GE Healthcare, Little Chalfont, UK). Mononuclear cells in the interface between the 40 and 75% layer were removed, washed, and resuspended in RPMI 1640 containing 10% FCS. Mononuclear cells from systemic lymphoid and local mucosal tissues were subjected to an enzyme-linked immunospot assay (ELISPOT) to detect the numbers of anti-PC AFCs. Briefly, 96-well filtration plates with a nitrocellulose base (Millititer HA; Millipore, Billerica, MA, USA) were coated

with 5 μg/ml of PC-BSA. Single cell suspensions of mononuclear cells obtained from different tissues were added at various concentrations and then incubated at 37°C for 4 hours in the air with 5% CO2 and 95% humidity. After incubation and washing, 1:5000 diluted HRP-conjugated anti-mouse IgM, IgG, and IgA was added to the plate. The spots were developed using 3-amino-9-methylcarbazole (Sigma-Aldrich, St. Luis, MO, USA) and the numbers of anti-PC AFCs were quantified with the aid of a stereomicroscope.

2.5. Statistical analysis

The results are expressed as the mean ± standard deviation (S.D.), and treated-mouse groups were compared with the control group using the one-way ANOVA and post-hoc multiple comparisons. A p-value of < 0.05 or 0.01 were considered statistically significant.

3. Results

3.1. Nasal administration of LPS enhances systemic and mucosal antibody responses

First, it is examined whether nasal administration of LPS with PC-KLH as an antigen would strengthen both systemic and mucosal antibody responses. Higher levels of anti-PC IgM, IgG, and IgA antibody titers were seen in serum and NWs of mice nasally immunized with PC-KLH plus LPS than in those of mice given PC-KLH alone (Fig. 1, A and C). Increased level of anti-PC salivary IgA antibody responses was found, but IgM and IgG in saliva samples were not detected (Fig. 1B). The antibody responses reached the peak on day 14, and an additional immunization did not increase the level until day 21 (Fig. 2). The antibody responses induced by PC-KLH plus LPS in serum and mucosal secretions were comparable to those induced by PC-KLH plus CT (Fig. 2). These results indicate that the nasal administration of LPS, as well as CT, induced systemic and mucosal antibody responses. To further support these findings, numbers of living cells producing-specific antibody were investigated in organs which were possibly related mucosa immune responses following nasal immunization. Mice immunized with PC-KLH plus LPS exhibited higher numbers of anti-PC IgM, IgG, and IgA AFCs in spleen and CLN than those administrated PC-KLH alone (Fig. 3A). Moreover, anti-PC IgA AFCs were elevated in SMG and NPs of mice immunized with PC-KLH plus LPS, when compared with mice given nasal PC-KLH alone (Fig. 3B). Induction of IgM- and IgG-producing AFCs in SMG and NPs were not seen (data not shown).

To confirm the mucosal adjuvanticity of LPS, a different model of hapten-antigen, TNP-KLH, was used for mouse immunization. Mice immunized with TNP-KLH plus LPS exhibited higher levels of anti-TNP IgG and IgA antibody responses in serum and NWs when compared with those of mice given TNP-KLH alone (Fig. 4, A and C). Anti-TNP IgM antibody response was not increased after immunization; the level was already high prior to immunization (Fig. 4A). Nasal administration of LPS with

TNP-KLH induced anti-TNP IgA antibody responses in saliva (Fig. 4B). In contrast, mice immunized with TNP-KLH alone did not produce anti-TNP IgA antibody responses. Anti-TNP IgM and IgG were not detected in saliva samples (Fig. 4B). The antibody responses to TNP-KLH plus LPS and TNP-KLH plus CT on immunized mice showed the similar levels (Fig. 4, A, B, and C). These results were appropriate with the antibody responses when PC-KLH was used as a model antigen. These findings indicate that LPS, as a mucosal adjuvant, effectively elicits systemic and mucosal antibody responses.

3.2. The antibody response induced by nasal administration of LPS was antigen-specific

LPS is well-known to be a strong polyclonal B-cell activating substance. Therefore, it is necessary to check whether nasal administration of LPS would induce non-specific antibody responses. Assays relying on anti-PC and anti-TNP antibody responses in both PC- and TNP-immunized mice were performed to investigate the antigen-specificity of the antibody responses induced by nasal administration of LPS. The anti- PC antibody responses in serum, saliva, and NWs were only detected in PC-immunized mice but not in TNP-immunized mice in the presence of LPS, and the increased of anti-TNP antibody responses were only detected in TNP-immunized mice (Fig. 5, A, B, and C). Anti-TNP antibody responses especially IgM which found in PC-immunized mice were somewhat non-specific antibody response. Those antibodies already existed prior to the immunization (Fig. 5, A, B, and C). Therefore, the antibody responses induced by LPS were results of antigen-specific antibody responses.

3.3. A combination of LPS and CT enhanced co-administrated antigen e.g., anti-PC antibody responses similar to those induceed by a single nasal adjuvant regimen (either LPS or CT)

To investigate whether the combination of LPS and CT as a nasal adjuvant would enhance antibody responses, the combination of LPS and cholera toxin were used for the nasal immunization with PC-KLH. Mice nasally immunized with PC-KLH and a combination of LPS and CT exhibited anti-PC IgM, IgG and IgA antibody responses in serum and NWs (Table 1.). The levels of antibody responses induced by the combination of LPS and CT were similar to those of mice give PC-KLH and either LPS or CT alone. Elevated levels of anti-PC IgA antibody responses were observed in saliva of mice nasally immunized with PC-KLH and a combination of LPS and CT as well as mice given PC-KLH nasally with either LPS or CT as an adjuvant (Table 1.). These results indicated that a combination of LPS and CT as nasal adjuvant did not express any advantages to enhance co-administrated antigen systemic and mucosal antibody responses in isotype levels. There was no particular difference between the antibody responses regarding the different doses of LPS.

4. Discussion

The adjuvant activity of bacterial LPS has been first revealed in mice to enhance the antibody production to ovalbumin [27]. The adjuvant activity occurs under conditions in which LPS can also function as a B cell mitogen [2]. Like many natural adjuvants, LPS stimulates a TLR4, which activates signaling pathways mediated by the adaptor proteins myeloid differentiation primary response gene 88 (MyD88) and TIR domain-containing adaptor inducing interferon-beta (TIRF) [6]. TIRF induces type I interferons (IFNs) through interferon regulatory factor 3, stimulating dendritic cells to express the co-stimulatory molecules CD40, CD80, and CD86 [28]. LPS is a potent adjuvant because injecting it systemically within a day after antigen exposure increases the level of T cell clonal expansion, long-term survival, IFN-[illegible] production, and migration to nonlymphoid tissues [7]. LPS also promotes the accumulation of memory CD4 T cells into bone marrow [29]. However, the capacity of LPS as a mucosal adjuvant to enhance immune response in nasal vaccination model has not yet been studied.

In the present study, nasal administration of LPS enhanced co-administrated antigen both systemic and mucosal antibody responses. Nasal immunization with PC-KLH and LPS induced anti-PC IgM, IgG, and IgA antibody responses in serum and NWs, and salivary IgA. At the same time, increased numbers of anti-PC IgM, IgG, and IgA AFCs were found in both systemic and local mucosal compartments. This report is, to my knowledge, the first to directly show the effectiveness of LPS as a mucosal adjuvant for the induction of systemic and mucosal antibody responses.

Anti-PC serum antibodies were found in unimmunized mice and increased by nasal immunization with PC-KLH. This finding was corresponding with the result that has been reported by Tanaka et al. [26] Also, Briles et al. [32, 30] indicated that serum antibody responses to PC developed in normal mice that were protective against intravenous infection with S. pneumoniae. These findings suggest that serum antibodies specific to PC are normally present and are increased by nasal immunization with PC-KLH.

Mucosal immune responses to PC in the upper airway have been investigated by Tanaka et al. [26] and Tselmeg et al. [19]. The former study has demonstrated that mice immunized with intranasal PC-KLH plus CT or plasmid encoding Flt3 ligand (pFL) as a mucosal adjuvant showed increased PC-specific IgM, IgG, IgA in serum, IgA in saliva and NWs, and numbers of PC-specific nasal and splenic AFCs. Further, it has been reported that PC-specific immune responses induced by nasal immunization with PC-KLH plus CT or pFL resulted in the clearance of live S. pneumoniae from upper and lower airways of mice. Based on these findings, it is most likely possible that anti-PC antibody response induced by nasal vaccination with PC-KLH plus a mucosal adjuvant might be an effective way to protect against infection

by bacteria that express PC such as S. pneumoniae.

To validate the mucosal adjuvant activity of LPS, a different model of antigen, TNP-KLH, was used for mouse immunization. The high levels of anti-TNP serum IgM were found in both control and immunized mice. It was not increased by the immunization with TNP-KLH; anti-TNP IgM antibody level was already high prior to immunization. Nasal administration of LPS with TNP-KLH results in the induction of anti-TNP mucosal IgA in addition to serum IgG antibody responses. Together with the results of anti-PC antibody responses, this study shows that LPS used as a mucosal adjuvant effectively enhanced systemic and mucosal antibody responses.

LPS has been known for a long time to be a potent activator of B lymphocytes [2] and activates various cell types of the innate and adaptive immune responses [4, 9]. Therefore, it is necessary to check whether nasal administration of LPS would induce non-specific antibody responses. In the present study, the levels of anti- PC antibodies were higher than anti-TNP antibodies in mice immunized with PC-KLH plus LPS. Moreover, TNP-immunized mice in the presence of LPS displayed higher levels of anti-TNP than anti-PC antibodies. Those findings indicated that LPS as a mucosal adjuvant induced antigen-specific antibody responses.

By varying the dose of LPS as a mucosal adjuvant, it was found that LPS did not regulate antibody responses in a dose-dependent manner. In small amount (2 μg) of LPS as an adjuvant gave the equal effect with high amount (50 μg). It indicated that 10 μg of LPS that used for the nasal adjuvant was appropriate.

The effect of adjuvants can be altered by different formulations. In the present study, interaction between LPS and CT was examined. CT is the most widely used adjuvant in animal vaccines currently. The results revealed that nasal delivery of a combination of LPS and CT enhances co-administrated antigen, e.g., anti-PC mucosal and systemic antibody responses similar to those induced by a single nasal adjuvant regimen (either CT or LPS). The effect of the combined LPS and CT as a mucosal adjuvant is additive rather than synergistic, the isotype levels of anti-PC antibody responses were similar to that obtained by single adjuvant. A further investigation remains to be done to examine the effect of the combination of adjuvant activity.

5. Conclusion

The present study demonstrated that LPS successfully enhances antigen-specific systemic and mucosal antibody responses, when it used as a mucosal adjuvant. The stimulatory effect of LPS appeared to be similar to CT when it was evaluated by antibody responses at the isotype level. Therefore, LPS can be used as an alternative to CT that is the most effective mucosal adjuvant in animal models. Possible differences of LPS from CT as a mucosal adjuvant remain to be investigated

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7. References

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