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Running title: Intranasal immunization with lipoteichoic acid

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Abstracts

Objective: *Streptococcus* (*S.*) *pyogenes* is common cause of acute tonsillitis. Lipoteichoic acid (LTA), which is a common constitute of the cell surface of most gram positive bacteria, is known to act as a substance of bacterial site for adherence to epithelium and antiserum to LTA is reported to inhibit bacterial attachment to epithelial cells *in vitro*. Cholera toxin subunit B (CT-B) is known to be a mucosal adjuvant. The purpose of this study is to investigate whether intranasal immunization with LTA and CT-B may be a possible candidate for vaccine formulation. **Methods**: Six-week-old male BALB/c mice were assigned to 3 experimental groups, mice immunized with LTA and CT-B, with LTA alone and with phosphate buffered saline (PBS) as a control. Immunizations were performed intranasally every 2 days for 2 weeks in every group. At the 21 days after immunization, sera, pharyngeal washings and pharyngeal epithelial cells were taken. The levels of serum IgG and pharyngeal IgA antibodies to LTA were measured by enzyme-linked immunosorbent assay (ELISA). The adherence rates of *S. pyogenes* pretreated by pharyngeal washings to pharyngeal epithelial cells from the mice were determined by *in vitro* adherence assay. **Results**: The serum anti-LTA IgG antibody levels of either mice immunized with LTA and CT-B or mice immunized with LTA alone were significantly higher than those of mice administered with PBS alone. The pharyngeal anti-LTA IgA antibody levels of the mice immunized with LTA and CT-B were significantly higher than those of either mice with LTA alone or mice with PBS alone. The streptococcal adherence rates to pharyngeal epithelial cells were significantly decreased by pretreatment with pharyngeal washings from the mice immunized with LTA and CT-B as compared to pretreatment with those from either mice with PBS or mice with LTA alone. **Conclusions**: These data shows that

intranasal immunization with LTA and CT-B evokes a good pharyngeal IgA response as well as systemic IgG response to LTA and inhibits streptococcal adherence to pharyngeal epithelial cells, suggesting that intranasal immunization with LTA and CT-B may be an effective approach to prevent streptococcal tonsillitis.

Key Words: intranasal immunization, lipoteichoic acid, cholera toxin, *Streptococcus pyogenes*

Introduction

Recurrent episode of acute tonsillitis is a common problem in infectious disorders during childhood. Bacterial adherence to tonsillar or pharyngeal epithelium may be a potential cause of recurrent tonsillitis (RT) [1, 2]. Humoral immunity of the host may be another cause of recurrence of this phlogistic condition. Pharyngeal secretory IgA has been reported to be lower in children with RT [1].

Streptococcus (*S.*) *pyogenes* is a common pathogen of acute tonsillitis [3]. Lipoteichoic acid (LTA), a constituent of the cell surface of most streptococci including *S. pyogenes*, is characterized as glycolipid-linked polymers of glycerol phosphate units [4, 5]. LTA is homogenous among strains and species in structure and antigenicity, and to act as a substance in the bacterial site for adherence to mucosa [6-9]. It has been reported that *in vitro* streptococcal adherence to human buccal cells or hydroxyapatite was significantly reduced by treatment with anti-LTA antibody obtained from rabbit immunized systemically with LTA, demonstrating that the antibody had an inhibiting activity against bacterial adherence to epithelium [6, 7]. Therefore, LTA has been proposed as a possible candidate for vaccine formulation for *S. pyogenes* infections.

Recently, we demonstrated decreased serum IgG and pharyngeal IgA antibody levels specific to streptococcal LTA in young children with RT [10]. This immunologic failure may be potential cause of RT in childhood. It has been demonstrated that oral administration of conjugate with LTA from *S. pyogenes* and lipopolysaccharide from *Klebsilella pneumonia* enhanced the IgA and IgG antibody synthesis in the lung and in mice [11]. However, there is no attempt to enhance pharyngeal IgA response specific to streptococcal LTA and to investigate its protective effect against streptococcal infection.

Cholera toxin B subunit (CT-B) containing trace amount of the holotoxin is a potent mucosal immunogen, partly because of its high-binding to the receptor GM1 ganglioside, facilitating uptake at mucosal surfaces of both CT-B and molecules linked to it [12]. Several studies with animals have shown that CT-B used as a carrier for various protein or carbohydrate antigens can enhance the mucosal immunogenicity for the linked antigens [13, 14]. Recent studies have shown that intranasal immunization of antigen mixed with CT-B induces effectively an antigen-specific serum IgG response as well as a nasal or nasopharyngeal IgA response [15, 16].

In this study, on the basis of the backgrounds, we performed intranasal immunization of streptococcal LTA with an adjuvant CT-B in mice. In addition, we measured antibody levels to LTA in serum IgG and in pharyngeal IgA, and assessed streptococcal adherence to pharyngeal cells from the mice.

Materials and Methods

Animals and Immunization

Six-week-old male BALB/c mice were obtained from CLEA Japan Inc. (Tokyo, Japan). They were maintained under the specific pathogen free conditions and provided food and water *ad libidum*.

Immunization was performed as described previously [16]. BALB/c mice were randomly assigned to 3 experimental groups (A to C). Group A mice were intranasally immunized with 10 µl of phosphate buffered saline (PBS) containing 100 µg of LTA

purified from *S. pyogenes* (Sigma Immuno Chemical Co., St. Louis, Mo., USA) and mucosal adjuvant cholera toxin (CT) (Cholera toxin B subunit supplement with 0.17% of CT; Sigma). Group B mice were intranasally immunized with 10 µl of PBS containing 100 µg of LTA alone. Group C mice were intranasally administered with 10 µl of PBS alone as a control. Immunizations were performed on days 0, 2, 4, 6, 8, 10, 12 and 14 in every group. On days 21 days, sera, pharyngeal washings and pharyngeal epithelial cells were taken and subject to assay.

Sampling

Blood was obtained transcardially. Serum samples were separated and stored at –70°C. Pharyngeal washings were collected by rinsing with 100 µl of PBS in the pharynx and centrifuged at 1000 *g*. The supernatant fluids were collected and filtered through a 0.45 µm filter. Pharyngeal epithelial cells were obtained by scraping the pharynx with cotton swabs and suspended in sterile PBS. The cells were washed thrice with the buffer and the concentrations adjusted to 10^5 cells/ml. The cell suspension (50 µl) was seeded into tissue culture glassware slide (Flow Laboratories Limited, Irvine, Scotland) for the *in vitro* adherence assay.

Measurement of antibodies specific to LTA

The antibody levels to LTA in sera and pharyngeal washings were measured by enzyme-linked immunosorbent assay (ELISA) as described previously [17] with some modification. Wells of flat-bottomed microplates (Nunc-Immuno Plate MaxiSorp, Roskilde, Denmark) were coated with 50 µl of 10 µg/ml LTA in carbonate buffer (pH 9.6) and were incubated at 4°C overnight. The material was then aspirated and 100 µl of 3% gelatin in

PBS was added to each well to cover the unreacted sites. After standing for 60 minutes at 37°C, the wells were washed three times with PBS containing 0.05% polysorbate 20 (Tween 20; PBS-T). These wells were then added to 50 µl of serum samples diluted 1:5 in PBS-T containing 0.5% gelatin (PBS-T-G) or pharyngeal washing samples. Each sample was assayed in duplicate. After incubation for 2 hours at 37°C, the wells were washed three times with PBS-T. The plates were then incubated sequentially with 50 µl of peroxidase-labeled goat anti-mouse IgG and IgA (Sigma) diluted 1:500 each in PBS-T-G at 37°C for 2 hours. They were then reacted with 50 µl of 10 mg/ml o-phenylenediamine (OPD: Sigma) in phosphate-citrate buffer. After a 15-minute incubation at room temperature, the reaction was stopped by 50 µl of 5N sulfuric acid. The optical density of each well was measured with an automated spectrophotometer (Easy Reader EAR 400FW; SLT-Labinstruments, Grödig, Austria) at 492 nm. A serial dilution curve was run on each plate with standard mouse serum (Sigma) and used as a standard. The value of anti-LTA antibody was read from the serial dilution curve with standard mouse serum and expressed as an absolute concentration (µg/ml). To standardize test values for potential dilutional differences between pharyngeal washing samples, the levels of anti-LTA antibodies wee adjusted to a uniform IgA level by dividing them by the total IgA concentration and expressed as units. One unit was 1/100 of concentration in the total IgA. The concentration of total IgA was measured by sandwich ELISA. The lower limit of detection in this assay was 0.5 µg/ml in IgG antibody and 0.05 units per ng/ml total IgA in IgA antibody. In ELISA for both serum and pharyngeal antibodies, negative control wells contained either diluent without sample or PBS-T. The specificity of each conjugate was confirmed in a series of cross-reaction tests. To ensure the specificity of this assay, we measured the residual antibody activity of standard mouse serum with bacterial antigens.

In vitro adherence assay

To assess the effect of intranasal immunization on the bacterial adherence, inhibition of adherence of *S. pyogenes* to pharyngeal epithelial cells from mice with or without immunization were investigated. Approximately 1 x 10^7 c.f.u. (20 µl) of *S. pyogenes* (WHOSF42) were incubated with 20 µl of the pharyngeal washing samples at 37°C for 20 minutes. Then the mixture was inoculated to tissue culture glassware slides seeded with pharyngeal epithelial cells, and the slides were incubated for 1 hour at 37°C. Following the incubation period, the wells were washed free of unattached bacteria 5 times with PBS and fixed with 4% paraformaldehyde. The slides were stained with Giemza and observed under microscope. The numbers of epithelial cells and of attached bacteria to the cells on the slides were counted. The adherence rate was calculated as the number of attached bacteria per cell.

Statistics

Analysis of data was performed using appropriate nonparametric test procedures. Comparisons between over 2 groups were tested by the Friedman's test or the Kruskal-Wallis analysis with the Bonferroni method for calculation of p-value. Statistical tests were based on a level of significance of $p<0.05$.

Results

Antibody responses in sera and pharyngeal washings

The serum anti-LTA IgG antibody was detectable in only one (17%) of 6 control mice administered with PBS alone, in 6 (86%) of 7 mice with LTA alone ($p=0.029$), and in all of 6 mice immunized with LTA and CT-B ($p=0.015$). The antibody levels of mice immunized with LTA and CT-B (median:interquartile range=1.39: 0.77-1.66 μg/ml) and of mice immunized with LTA alone (1.67: 0.53-2.58 μg/ml) were significantly higher than those of mice administered with PBS alone ($p<0.01$, $p<0.05$, respectively; Fig. 1a).

The pharyngeal anti-LTA IgA antibody was detected in any groups. The pharyngeal IgA antibody levels of the mice immunized with LTA and CT-B (5.39: 4.15-9.39 units) were significantly higher than those of the mice LTA alone (0.54: 0.53-0.56 units; $p<0.01$) and of the mice with PBS alone (0.27: 0.05-0.5 units; Fig. 1b).

***In vitro* adherence assay**

Pharyngeal washings from each immunization group were mixed in equal volume and subjected to *in vitro* adherence assay. The anti-LTA antibody level was 6.35 units in pharyngeal washings from mice with LTA and CT-B, 0.53 units in those with LTA alone, and 0.4 units in those with PBS.

Table 1 shows the streptococcal adherence rates to pharyngeal epithelial cells from each immunization group according to pretreatment by pharyngeal washings. The streptococcal adherence rate to pharyngeal epithelial cells from the control mice (administered with PBS) was significantly lower when pretreated with pharyngeal washings from the mice immunized with LTA and CT-B (0.44: 0.3-0.79) as compared to either when pretreated with those from the mice with PBS (3.54: 1.66-5.01, $p<0.05$) or when treated with those from the mice with LTA alone (3.67: 2.21-5.39, $p<0.05$). As well, either

adherence rates to the cells from the LTA immunized mice or from the LTA and CT-B immunized mice were significantly lower by pretreatment with pharyngeal washings from the mice immunized with LTA and CT (0.77: 0.39-1.59, 0.68: 0.21-1.41, respectively) as compared to as compared to pretreatment with those from the mice with PBS (1.97: 1.07-2.68, $p<0.05$; 2.28: 0.56-4.39, $p<0.05$; respectively) or when treated with those from the mice with LTA alone (1.61: 0.6-2.5, $p<0.05$; 2.55: 1.27-6.13, $p<0.05$; respectively).

On the other hand, there was no different adherence rate among the cells from 3 different immunizations (PBS alone: 3.54, LTA alone: 1.97, LTA and CT: 2.28; Table 1), when pretreated with pharyngeal washings from control mice. No different adherence rate among the cells from 3 different immunizations was also shown either when treated with pharyngeal washings from the LTA immunized mice (3.67, 1.61, 2.55, respectively) or when treated with those from the LTA and CT-B immunized mice (0.44, 0.77, 0.68, respectively).

Discussion

S. pyogenes is common is a common pathogen of acute tonsillitis [3]. It has been reported that *in vitro* streptococcal adherence to human buccal cells or hydroxyapatite was significantly reduced by treatment with anti-LTA antibody obtained from rabbit immunized systemically with LTA, demonstrating that the antibody had an inhibiting activity against bacterial adherence to epithelium [6, 7]. Furthermore, LTA is homogenous among strains and species in structure [6-9], indicating that the antibody to LTA may response to a number of species and strains of streptococci. These are reason why we chose LTA as a candidate

antigen for vaccine formulation against streptococcal tonsillitis.

Kofler and Wolf [11] demonstrated that oral administration of conjugate with LTA from *S. pyogenes* and lipopolysaccharide from *Klebsilella pneumonia* enhanced the IgA and IgG antibody synthesis in the lung of mice. Several reports have demonstrated increased attachment of *S. pyogenes* to tonsillar or pharyngeal epithelium during acute tonsillitis [1, 2], suggesting bacterial adherence to epithelium may be one of the determinants for streptococcal tonsillitis. Most pathogens enter the host through the mucosal membranes and induce a local mucosal immune response, mainly represented by secretory IgA [18]. In pharyngeal secretions, secretory IgA is the predominant immunoglobulin and plays a role in the inhibition of bacterial adherence to pharyngeal mucosa [19]. Systemic immunity by IgG-specific antibody does not influence bacterial adherence to respiratory epithelium [20]. Therefore, induction of pharyngeal IgA response is important to inhibit streptococcal adherence to epithelium, resulting in prevention for streptococcal tonsillitis.

Cholera toxin B subunit (CT-B) is reported to be as a mucosal adjuvant facilitating uptake at mucosal surfaces of both CT-B and molecules linked to it [12]. Recently a number of investigators demonstrated that intranasal immunization of antigens mixed with CT-B evoked effectively an antigen-specific serum IgG response as well as a pharyngeal IgA response [15, 16, 19]. In our study, we succeeded to induce a good pharyngeal IgA response as well as systemic IgG response to LTA by the intranasal immunization with LTA and CT-B. The detectable levels of pharyngeal anti-LTA IgA antibody observed in control mice may represent preexisting antibody due to prior exposure to LTA of oral streptococci in the normal flora. This preexisting antibody has been observed also in the other experiment previously reported elsewhere [11].

In the adherence study, pretreatment with pharyngeal washings from the mice

immunized with LTA and CT-B, which contained high levels of anti-LTA IgA antibody, decreased streptococcal adherence to pharyngeal cells from the mice with any immunization. This may be mainly due to effect of anti-LTA secretory IgA antibody in the pharyngeal washings. The affects of preexisting cell bound antibody induced as a result of immunization is likely to be minor, because the number of organisms pretreated with pharyngeal washings from control mice was not different between pharyngeal cells from LTA and CT-B immunized mice and those from control mice. However, we cannot exclude influence of factors other than secretory IgA [21]. Recent study has suggested that locally synthesized IgG, different from its serum counterpart, could complement secretory IgA in the defense against pathogens [22].

Our present data suggest that intranasal immunization of LTA with CT-B may be an effective approach to protect against streptococcal adherence to pharyngeal epithelial cells, resulting in preventing streptococcal tonsillitis. On the other hand, intracutaneous immunization with LTA failed to evoke specific antibody response to LTA and to protect against dental caries in rhesus monkeys [23]. Our present study is a first attempt to develop vaccine formation for streptococcal tonsillitis. However, this is still preliminary and there is a number of problems to be dissolved. For example, we previously demonstrated decreased pharyngeal IgA antibody levels to streptococcal LTA in children with recurrent tonsillitis [10], suggesting that pharyngeal antibody response to LTA vaccine may be reduced in those children. The CT-B used as an adjuvant in this study is toxic in humans. Recently, mutant cholera toxin that is not toxic in humans has been reported to be effective to evoke mucosal immune response [24]. Additional studies for antigen delivery system using non-toxic mucosal adjuvant will be needed to develop vaccine formation for humans.

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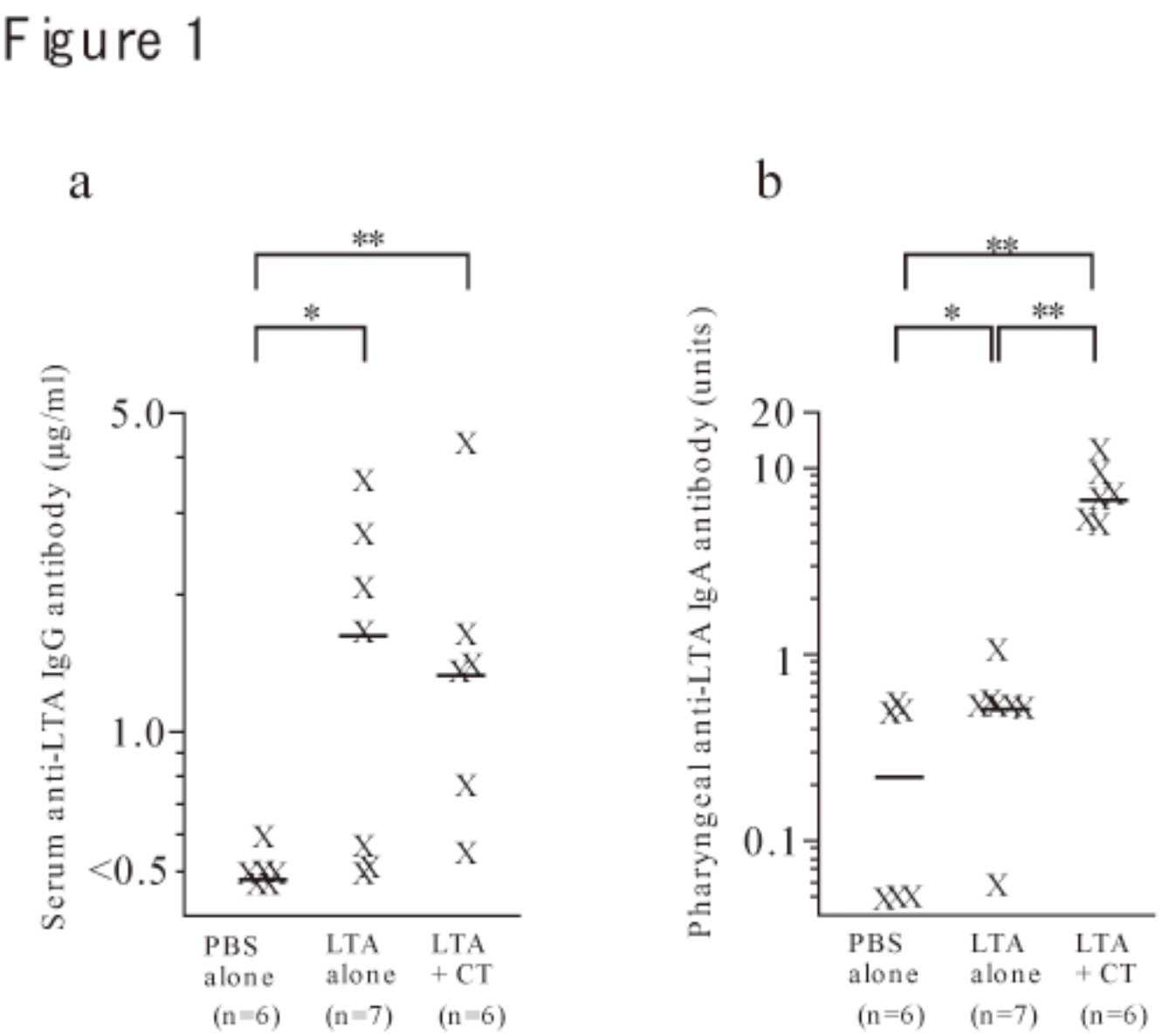
Figure legends

Figure 1. The levels of serum anti-lipoteichoic acid (LTA) IgG antibody (a) and of pharyngeal anti-LTA IgA antibody (b) in mice administered with PBS alone, mice immunized with LTA alone, and mice immunized with LTA and cholera toxin B subunit (CT). The median values are expressed as short bar (-). Kruskal-Wallis analysis with the Bonferroni method was used for calculation of p-value. *$p<0.05$, **$p<0.01$

Figure 1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Serum anti-LTA IgG antibody (μg/ml)
5.0
1.0
<0.5
PBS alone (n=6)
LTA alone (n=7)
LTA + CT (n=6)
b
**
*
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Pharyngeal anti-LTA IgA antibody (units)
20
10
1
0.1
PBS alone (n=6)
LTA alone (n=7)
LTA + CT (n=6)