Trehalose Ingestion Modifies Mucosal Immune Responses of the Small Intestine in Mice

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Trehalose has been utilized as a supplement to various products such as food and cosmetics, and has recently been reported to have multiple biological functions in vitro and in vivo. We have previously found that trehalose administered orally inhibits mouse osteoclastogenesis induced by estrogen-deficiency or by injection of lipopolysaccharide (LPS). In this study, we examined whether oral administration of trehalose to mice produced changes in the Peyer’s patches (PP) of the small intestine, that are essential for mucosal immune responses, to clarify the inhibitory mechanism of trehalose on osteoclastogenesis. Male C3H/HeN mice were orally administered trehalose 1 g/kg for 5 consecutive days. Interestingly, trehalose administration caused a significant decrease in the total number of PP lymphocytes (PPL) and in the spontaneous release of interleukin (IL)-6 by PPL compared with those of controls, without inducing apparent pathological damage. Moreover, proliferation and interferon (IFN)-γ production by PPL in response to LPS tended to increase compared with those of the control group. Considering that IL-6 and IFN-γ are factors associated with bone metabolism, our results suggest the possibility that trehalose ingestion may modify the intestinal immune environment, resulting in altered systemic immunity and bone metabolism.

Key words — trehalose, Peyer’s patch, interleukin-6, interferon-γ, lipopolysaccharide, osteoclastogenesis

INTRODUCTION

Trehalose (1-α-glucopyranosyl-α-D-glucopyranoside) is a non-reducing disaccharide composed of two D-glucose molecules, and is a well-known source of energy in many living organisms. Several years ago, a large-scale biological method for the production of trehalose was developed, providing a continuous source of the pure compound. This gave us the opportunity, to investigate the multiple functions of trehalose and to apply it in various areas such as 1) stabilization of protein and nucleic acids under dried conditions or at low temperatures, 2) protection from stress induced by freezing, heating, and drying, 3) prevention of osteoporosis.

As regards the third function, we have found that orally administered trehalose inhibited osteoclast differentiation in ovariectomized (OVX)-mice, and showed that this effect was the result of inhibition of excess interleukin (IL)-6 production by bone marrow cells under conditions of estrogen-deficiency. In an endotoxin-induced bone resorption model in mice, trehalose significantly suppressed the elevation of serum tumor necrosis factor (TNF)-α induced by lipopolysaccharide (LPS)-injection, resulting in the inhibition of increased numbers of osteoclasts in the femurs. However, we have not yet thoroughly established why trehalose ingestion leads to a down-regulation of the cytokines associated with bone resorption, such as IL-6 and TNF-α overexpressed in our osteoporosis models, nor have we identified the cells responsible for the changes in cytokine production.

To obtain some insight on the regulatory mechanism of trehalose, we first focused on the intestinal mucosa that would be directly exposed to trehalose after oral administration. In particular, the Peyer’s patches (PP) are known to be essential for mucosal immunity and subsequently, activated PP lymphocytes (PPL) have the potential to regulate systemic immune responses.

In this study, we have examined the effects of oral administration of trehalose on the total number of PPL and on the production of cytokines associated with bone metabolism by PPL to address the inhibitory mechanism of trehalose on osteoclastogenesis, according to the mouse LPS-induced bone resorption model.

MATERIALS AND METHODS

The experimental procedures used in this study met the guidelines of the Experimental Animal Usage Committee of Hayashibara Biochemical Labo-
that described previously.10) This administration schedule was in accordance with 60 µ and DW were filtered through 0.22 m membrane filters (Millex GV13, Millipore corporation, Bedford, MA, U.S.A.).

**Samples** —— Trehalose (Hayashibara, Okayama, Japan) solution (0.1 g/ml) in distilled water (DW) and tap water. Samples were forced through a 100 µm mesh (Becton Dickinson) after digestion using 0.2% (w/v) collagenase (Amano Enzyme Inc., Nagoya, Japan) in RPMI 1640 medium at 37°C for 30 min. The resultant mixtures were forced through a 40 µm mesh (Becton Dickinson) after washing three times with RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan), and then centrifuged at 1100 × g for 10 min. The lymphocytes collected from the interface between the two gradients were passed over 75% solution, and then centrifuged at 1100 g for 10 min. The lymphocytes collected from the interface between the two gradients were passed through a 40 µm mesh (Becton Dickinson) after washing three times with RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan).

**Preparation of PPL** —— Mice were sacrificed 24 hr after the last administration of trehalose and PP were individually isolated. The preparation of PPL was performed according to the method described by Wilson et al.11) with some modification. Briefly, PP were teased apart from the small intestine and digested using 0.2% (w/v) collagenase (Amano Enzyme Inc., Nagoya, Japan) in RPMI 1640 medium supplemented with 100 µM HEPES (pH 7.2), 50 µg/ml streptomycin and 60 µg/ml penicillin. Preparations of PPL were performed according to the method described by Wilson et al.11) with some modification. PPL was performed according to the method described by Wilson et al.11) with some modification.

**Administration of Trehalose** —— Trehalose was orally administered at 1 g/kg of body weight by gastric intubation for 5 consecutive days to 5-week-old mice (n = 10). In the vehicle control group, the same volume of DW was similarly administered (n = 10). This administration schedule was in accordance with that described previously.10)

**Preparation of PPL** —— Mice were sacrificed 24 hr after the last administration of trehalose and PP were individually isolated. The preparation of PPL was performed according to the method described by Wilson et al.11) with some modification. Briefly, PP were teased apart from the small intestine and digested using 0.2% (w/v) collagenase (Amano Enzyme Inc., Nagoya, Japan) in RPMI 1640 medium at 37°C for 30 min. The resultant mixtures were forced through a 100 µm mesh (Becton Dickinson, Franklin Lakes, NJ, U.S.A.) to remove undigested material, separated on a discontinuous Percoll gradient (Amersham Pharmacia Biotech, Uppsala, Sweden) made up of 45% solution layered over 75% solution, and then centrifuged at 1100 g for 10 min. The lymphocytes collected from the interface between the two gradients were passed through a 40 µm mesh (Becton Dickinson) after washing three times with RPMI 1640 medium supplemented with 5% fetal bovine serum (FBS; Dainippon Pharmaceutical Co., Ltd., Osaka, Japan).

**Responses of PPL in vitro** —— PPL from each mouse were prepared at an optimal density for each assay in RPMI 1640 medium supplemented with 10% FBS and 5 × 10⁻⁵ M 2-mercaptoethanol. For induction of cytokine production, PPL were cultured at 2 × 10⁶ cells/ml with or without LPS (E. coli O55 : B5, Difco Labs., Detroit, U.S.A.) in a 96-well microplate at 37°C for 48 hr in a humidified 5% CO₂ atmosphere. After centrifugation of the culture supernatants, cell-free samples were stored at −80°C before being assayed for cytokine levels. For the proliferation assay, PPL were stimulated at 1 × 10⁵ cells/ml with or without LPS for 48 hr and pulsed with 1 µCi of [³H]thymidine for 18 hr. The level of incorporated radioisotope was measured by a standard method.

**Measurement of Cytokines** —— Cytokines were analyzed using specific ELISA systems. IL-6 and TNF-α were estimated according to the manufacturer’s (BD PharMingen, San Diego, CA, U.S.A.) instructions.9) Purified anti-MuIL-6 monoclonal antibody (mAb) (clone No. MP5-20F3), purified anti-MuTNF-α mAb (MP6-XT22), biotinylated anti-MuIL-6 mAb (MP5-32C11), biotinylated anti-MuTNF-α polyclonal antibody (pAb) and murine recombinant IL-6 were purchased from BD PharMingen. As a TNF-α standard, murine recombinant TNF-α obtained from Genzyme (Cambridge, MA, U.S.A.) was used. IFN-γ and IL-18 were measured according to the published procedures developed at our laboratories.12,13) Purified anti-MuIFN-γ pAb, purified anti-MuIL-18 mAb, biotinylated anti-MuIL-18 mAb and the respective standards were prepared at our laboratories, and a biotinylated anti-MuIFN-γ mAb (XMG1.2) was purchased from BD PharMingen. In these ELISA systems, we used a horseradish peroxidase-conjugated avidin purchased from Zymed Laboratories, Inc. (South San Francisco, CA, U.S.A.) for detection. The lower detection limits were 50 pg/ml, 50 pg/ml, 25 pg/ml and 0.094 IU/ml for IL-6, TNF-α, IL-18 and IFN-γ, respectively.

**Histological Analysis** —— Jejunal and ileal PP were fixed with 10% buffered formalin and embedded in paraffin according to standard procedures. Sections were stained with hematoxylin and eosin (H-E) on microscope slides and mounted for histological analysis.

**Statistical Analysis** —— All experiments were performed at least 3 times. The results were expressed as means ± standard errors of means (SEM) and subjected to a Student’s t-test to compare the differences between the two groups.

**RESULTS**

**Effect of Trehalose on the Total Number of PPL**

PPL from each mouse were prepared after oral...
administration of trehalose for 5 days. Figure 1 shows the total number of PPL per mouse in the two groups. The total number of PPL from trehalose-treated mice was 4.2 ± 0.4 × 10^6 cells and was significantly less than that in the control group (6.0 ± 0.4 × 10^6 cells), (p < 0.01). Similarly, we observed that both the number and size of PP were reduced in the trehalose-treated group (data not shown). Figure 2 shows the pathological changes in the PP and the small intestine after oral administration of trehalose. In the mice administered trehalose, the follicle size and the germinal centers of PP tended to be smaller than those in the control group because the PP were thin and small. However, the general structures of the PP and the small intestine were normal and there were no evidences of necrosis or apoptosis.

**In Vitro Responses of PPL from Mice Administered Trehalose**

To compare differences between the biological functions of lymphocytes from trehalose-administered mice and control mice, we examined the spontaneous or LPS-induced immune responses of PPL. Figure 3-A shows the result of cell proliferation assays. The spontaneous proliferation of PPL isolated from the trehalose-treated mice was similar to that of PPL in the control group. However, in the presence of LPS, the proliferation of PPL from trehalose-treated mice was slightly increased. We also investigated *in vitro* cytokine production by PPL. Spontaneous IL-6 secretion by PPL from the trehalose-treated mice was significantly less than that of PPL from untreated control mice (p < 0.05) (Fig. 3-B). Similarly, spontaneous IFN-γ release was slightly lower than that in the untreated group (Fig. 3-C). However, when stimulated with LPS, IFN-γ production by PPL had a tendency to increase after trehalose administration while IL-6 production tended to decrease further. TNF-α and IL-18 levels produced in the supernatants during the 6–48 hr incubation period were under the detection limits of their respective ELISA systems (data not shown).

**DISCUSSION**

Gut-associated lymphoid tissue is a unique immune site for defense from infection and autoimmunity because it is always exposed to various en-
teric microbe antigens and dietary factors. In particular, the PP in the intestine are well-known as sites that initiate mucosal immune responses. PPL activated with antigens within the PP migrate, home to the lamina propria, interintestinal lymph nodes and other immunologically-active sites, and contribute to systemic immunity. Therefore, in an experimental model employing oral administration of a biologically active agent, it is reasonable to study the influence of the sample on PPL and the possible effects on systemic immunity.

We have previously shown that oral administration of trehalose suppressed LPS-induced osteoclastogenesis in mice as a result of inhibiting the excessive induction of TNF-α in the serum. Although the elevation of glucose and insulin in the serum has been found to diminish TNF-α production induced after LPS injection, the levels of these parameters were within the normal range in the sera of mice administered trehalose just before LPS injection. We also reported that oral administration of trehalose decreased excess IL-6 secretion by bone marrow cells, leading to prevention of bone loss in OVX-mice. However, the inhibitory mechanism of trehalose on osteoclastogenesis-associated cytokine production has not been thoroughly clarified. To explain the inhibitory effects of trehalose on osteoclastogenesis, we examined changes in PP induced by trehalose administration, focusing mainly on the production of bone metabolism-associated factors (TNF-α, IL-6, IFN-γ and IL-18). In our previous mouse LPS-induced bone resorption model, oral administration of trehalose at 1 g/kg of body weight significantly inhibited osteoclastogenesis. Therefore, we applied the same dose of trehalose in the present experiments. Surprisingly, when PPL were prepared from individual mice after administration of trehalose 5 times, the total numbers of PPL were significantly decreased and the responses of PPL in vitro were different from those of controls. As regards cytokine production in vitro, ingestion of trehalose resulted in a significant decrease in the spontaneous production of IL-6, and a slight decline in the spontaneous IFN-γ production by PPL, although TNF-α and IL-18 were undetectable in the culture supernatants. In the presence of LPS, IFN-γ levels were slightly greater than those of controls, while those of IL-6 were lower. These results indicate that trehalose affected PPL, directly or indirectly. Recently, IFN-γ has been reported to inhibit osteoclastogenesis by interfering with the receptor activator of NF-κB (RANK) - RANK ligand (RANKL) / osteoprotegerin ligand (OPGL) signaling pathway. Moreover, in mice lacking the IFN-γ receptor, resorption of calvarial bone induced by LPS-injection was notably exacerbated. In contrast, IL-6 is a major factor in osteoporosis induced by estrogen-deficiency. Taken together with these reports, our study suggests that oral administration of trehalose may subtly alter the ratio of IFN-γ/IL-6
in PP, resulting in the systemic suppression of osteoclastogenesis. As regards the decrease IL-6 production by PPL after trehalose administration, we could not define whether this was due to the lower number of cells producing the cytokine or due to functional changes in the producing cells.

Most interestingly, oral administration of trehalose induced a significant decrease in the total number of PPL accompanied with a reduction in the number and in the size of the PP. Histological analysis revealed that this phenomenon was not due to toxicity induced by trehalose. We also examined the distribution of T cells, B cells and monocytes in the PP by immunohistochemical analysis (CD4 and CD8 for T cells, CD19 and CD45R for B cells, and CD11b for monocytes). In the PP taken from the trehalose-administered mice, these cells were normally distributed and exhibited no apparent differences from those of the control mice (data not shown). Results showed that the general structure of the PP was preserved in trehalose-administered mice. In a number of reports, PP have been shown to develop and to be activated upon changes in the intestinal environment or with aging, possibly due to increased antigenic stimulus.

In germ-free mice, the germinal centers in the PP, which are a B cell-rich area, enlarged after oral infection with virus. Concerning the biological function of PPL, there are some reports showing that IL-6 production by PPL and splenic cells increased with aging possibly resulting from increased antigenic stimulus. Taking into account the decrease in vitro IL-6 release from PPL induced by trehalose-administration in our study, we consider that the responses of PP lymphoid tissues are altered because oral administration of trehalose may improve the intestinal environment and decrease antigenic stimulation in the form of microbes and food antigens.

When mice administered trehalose were injected with LPS, the increase in the number of osteoclasts in the bone marrow and the elevation of serum TNF-α levels induced by LPS-injection were inhibited. Recently, several investigators have singled out TNF family-proteins as potential factors related to both osteoclastogenesis and the development/or- ganization of lymphoid tissues. In TNF-α knockout mice, PP size was slightly reduced regardless of retainment of normal structure. Similarly, a deficiency in OPG, which is also a member of the TNF family and is essential for the formation of mature osteoclasts, leads to a reduction in PP size. These reports together with the findings that reduction in the number and size of the PP was observed in the trehalose-administered mice, raise the possibilities that trehalose may regulate the development/organization of PP, and LPS-induced osteoclast development through down-regulation of TNF family-proteins. Although TNF-α was not detectable in the culture supernatants of PPL in this experiment, it is worthwhile examining the involvement of other proteins of the TNF family.

Most recently, in in vitro experiments using a human intestinal epithelial cell line, exposure to trehalose reportedly up-regulated the production of OPG, which inhibits osteoclastogenesis by inhibiting the interaction between OPGL/RANKL and RANK. This finding also suggests that trehalose may affect the intestinal epithelium directly.

In conclusion, we propose that trehalose may regulate the production of cytokines such as IL-6 and IFN-γ that are associated with bone metabolism via effects on the PP. At present, we are performing further studies to clarify the influence of trehalose on other intestinal tissues to address the possibility of other mechanisms of action of trehalose.

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