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**Galectin-3 inhibits cytokine-inducing activity of
periodontopathic bacterial endotoxin in murine splenocytes**

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Abstract

Galectins are a family of animal lectins involved in not only development and differentiation, but also immunoregulation and host–pathogen interactions. Galectin-3 interacts with lipopolysaccharides (LPS) from gram-negative bacteria such as *Salmonella minnesota* and *Pseudomonas aeruginosa*. The present study investigated whether galectin-3 inhibited the cytokine-inducing activity of LPS from periodontopathic bacteria using splenocytes derived from mice of different ages. Splenocytes were prepared from 1- or 7-month-old C57BL/6 mice. LPS was extracted from *Aggregatibacter (Actinobacillus) actinomycetemcomitans* Y4 and then purified. To culture solutions, either *A. actinomycetemcomitans* Y4 LPS alone, or LPS and murine galectin-3 were added, and after 17 h of incubation, the release of IL-6 and IFN- γ from splenocytes was measured using ELISA. In all mice tested, LPS stimulation significantly increased production of IL-6 and IFN- γ ($p < 0.02$). In the splenocytes of 1-month-old mice, murine galectin-3 suppressed LPS-induced cytokine production, but this suppressive effect was not seen with splenocytes from 7-month-old mice. The pre-stimulation level of cytokines was also significantly higher for 7-month-old mice than for 1-month-old mice ($p < 0.05$).

Introduction

Periodontal disease is an infectious disease caused by dental plaque: oral biofilm formed by multiple organisms (Listgarten, 1965; Loesche *et al.*, 1980). *Aggregatibacter actinomycetemcomitans* is widely studied human periodontopathogen and have been considered to have a role in periodontal disease. *A. actinomycetemcomitans* is found in several sites of destructive periodontitis, have a strong association with aggressive periodontitis. Furthermore, *A. actinomycetemcomitans* possesses several interesting virulence factors (Wilson *et al.*, 1995; Fives-Taylor *et al.*, 1996, Kato *et al.*, 1996; Kimizuka *et al.*, 1996; Kurita-Ochiai & Ochiai, 1996; Sugai *et al.*, 1998). Lipopolysaccharide (LPS) of this bacterial is involved in the progression of periodontitis by inducing production of inflammatory cytokines such as interleukin-1 β (IL-1 β), interleukin-6 (IL-6), and tumor necrosis factor- α (TNF- α) as endotoxin (Takahashi *et al.*, 1991; Kato *et al.*, 2000). Of particular significance is the ability of IL-6 to induce bone resorption, both by itself and in conjunction with other bone-resorbing agents (Ishimi *et al.*, 1990). It has been reported that interferon- γ (IFN- γ) plays an important role in modulating alveolar bone loss under inflammatory conditions induced by microbial challenge in human and mouse, *in vivo* (Teng *et al.*, 2005). Yonezawa *et al.* (2005) suggested that the restraint of IFN- γ production elicited by gingipain DNA vaccine played a significant role in protection against periodontopathic *Porphyromonas gingivalis* infection in mice.

Galectins are a family of animal lectins involved in not only development and differentiation, but also immunoregulation and host–pathogen interactions (Rabinovich & Gruppi, 2005). Galectins are β -galactoside-binding lectin and can be divided into 3 families: proto type; chimera type; and tandem-repeat type (Cooper, 2002; Leffler *et al.*,

2004). Galectin-3, chimera type, interacts with lipopolysaccharides (LPS) from gram-negative bacteria such as *Salmonella minnesota* and *Pseudomonas aeruginosa* (Mey *et al.*, 1996; Gupta *et al.*, 1997). Recently, Li *et al.* (2008) demonstrated that galectin-3 directly interacts with LPS of *Escherichia coli* and blocks the LPS-induced inflammatory response. The present study investigated whether galectin-3 inhibited the cytokine-inducing activity of LPS from periodontopathic bacteria using splenocytes derived from mice.

Materials and Methods

Bacterial strain

A. actinomycetemcomitans Y4 was maintained anaerobically on blood agar plates containing Trypticase soy agar (Becton Dickinson Microbiology System, Cockeysville, MD) supplemented with 10% defibrinated horse blood, hemin ($5 \mu\text{g mL}^{-1}$; Sigma Chemical Co., St. Louis, MO) and menadione ($0.5 \mu\text{g mL}^{-1}$; Wako Pure Chemical Industries, Osaka, Japan).

LPS purification

Bacterial cells of *A. actinomycetemcomitans* Y4 were grown in Trypticase soy broth (Becton Dickinson Microbiology System) with 0.4% yeast extract anaerobically at 37 °C for 3 days. Harvested cells were washed with phosphate-buffered saline (PBS; pH 7.2) and suspended in same buffer. LPS preparation obtained by hot phenol-water method (Westphal & Jann, 1968) and purified as described previously (Kato *et al.*, 1989; Imatani *et al.*, 2001). The hot phenol-water extracts were repeatedly ultracentrifuged ($105,000 \times g$, for 2 h) and the precipitation was suspended in pyrogen-free saline. The suspension was applied to polymyxin B Sepharose 4B column (Amersham Pharmacia Biotech., Uppsala, Sweden) equilibrated with pyrogen-free saline, and bound LPS was eluted in 0.1 M Tris buffer (pH 8.0) containing 1% sodium deoxycholate. LPS fractions were assessed for the purity by SDS-polyacrylamide gel electrophoresis using silver staining. The LPS fractions included slow-migrating and repeating ladder bands forming a typical LPS pattern.

Effect of galectin-3 on cytokine-inducing activity of *A. actinomycetemcomitans* LPS

Splenocytes were prepared from 1- or 7-month-old C57BL/6 mice. Their spleens were removed, gently homogenized, and suspended in RPMI 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) supplemented with 10% fetal calf serum, penicillin G (100 U mL⁻¹) and streptomycin (100 µg mL⁻¹). Tissue fragments were left to settle for a few minutes in plastic tubes, and the cell suspension was then washed with RPMI 1640. The splenocytes adjusted to 8 X 10⁵ cells mL⁻¹ were cultured in a 24-well culture plate in a humidified chamber with 5 % CO₂ in air at 37°C. To culture solutions, either *A. actinomycetemcomitans* Y4 LPS alone (200 ng mL⁻¹), or LPS and recombinant murine galectin-3 (10 µg mL⁻¹). were added. After 17 h of incubation, the release of IL-6 and IFN-γ from splenocytes was measured using ENDOGEN ELISA kit (Pierce Biotechnology, Inc., Rockford, IL). Splenocytes without LPS and galectin-3 served as negative control. Mice were treated in accordance with “The Guidelines for the Treatment of Experimental Animals at Tokyo Dental College”.

Statistical analyses

The Mann-Whitney *U*-test was used for all experiments in this study to identify statistically significant differences.

Results

The pre-stimulation level of IL-6 and IFN- γ was significantly higher for 7-month-old mice than for 1-month-old mice ($p < 0.05$) (Fig. 1). In all mice tested, *A. actinomycetemcomitans* LPS stimulation significantly increased production of IL-6 and IFN- γ in dose dependent manner (Fig. 2). We selected 200 ng mL⁻¹ of *A. actinomycetemcomitans* LPS for galectin-3 inhibition assay. In the splenocytes of 1-month-old mice, murine galectin-3 suppressed LPS-induced cytokine production, but this suppressive effect was not seen with splenocytes from 7-month-old mice (Fig. 3).

Discussion

Galectin family is defined by a conserved carbohydrate-recognition domain with a canonical amino acid sequence and affinity for β -galactosides (Cooper, 2002; Leffler *et al.*, 2004). Galectin-3, a one- carbohydrate-recognition domain galectin, is unique in that it contains unusual tandem repeats of short amino-acid stretches fused onto the carbohydrate-recognition domain (Leffler *et al.*, 2004; Liu & Rabinovich, 2005). Galectin-3 has the ability to bind to gram-negative bacterial LPSs (Mey *et al.*, 1996). The data presented above paper demonstrate that galectin-3 is able to interact with LPS by two distinct binding sites, allowing association with rough- as well as smooth-type LPS through independent recognition of the core region and/or O-polysaccharide region of LPS. The structure and biological abilities of *A. actinomycetemcomitans* LPS and lipid A are similar to those of *Enterobacteriaceae* (Nishihara *et al.*, 1986; Masoud *et al.*, 1991). Galectin-3 also interacts with *A. actinomycetemcomitans* LPS certainly.

Galectin-3 deficient macrophages had markedly elevated LPS-induced signaling and inflammatory cytokine production compared with wild-type cells (Li *et al.*, 2008). And this enhancement could be normalized by recombinant galectin-3. These indicate that the galectin-3 is a negative regulator for LPS function. In the splenocytes of 1-month-old mice, murine galectin-3 suppressed the cytokine-inducing activity of *A. actinomycetemcomitans* LPS. The present study suggests that galectin-3 interacts to *A. actinomycetemcomitans* LPS with the result that LPS activity to the murine splenocytes was prevented. *A. actinomycetemcomitans* LPS stimulation induced IL-6 and IFN- γ productions from murine splenocytes. Usually LPS stimulates B cells and

macrophages, but not T cells. *A. actinomycetemcomitans* LPS possesses B cell mitogenic activity (Eastcott *et al.*, 1990). It is well known that IFN- γ is produced by T helper type 1 (Th1). The polysaccharide moiety of LPS possibly mediated T cell stimulation *via* macrophage (Williamson *et al.*, 1984). Excessive production of IL-6 in response to exposure to LPS has an inflammatory effect, resulting in injury. Although IFN- γ plays a pivotal role in host defense mechanisms, its excessive release has been associated with the pathogenesis of chronic inflammatory and autoimmune diseases (Farrar *et al.*, 1993; Feldmann *et al.*, 1998; Tilg *et al.*, 1999). It is important that galectin-3 has an ability to obstruct IL-6 and IFN- γ inducing activity of LPS.

The level of IL-6 and IFN- γ was higher for 7-month-old mice than for 1-month-old mice both before and after LPS stimulation. Abiko *et al.* (1998) suggest that aging in both human gingival fibroblast and periodontal ligament fibroblast is an important factor in higher production of inflammatory mediators in response to both LPS and mechanical stress. Our data did not contradict their results. The suppressive effect of galectin-3 against *A. actinomycetemcomitans* LPS was not seen with splenocytes from 7-month-old mice. A poor responsiveness of cells from 7-month-old mice is looked for by dose-dependent analysis (Fig. 2). Dose-dependent response of cells from old mice may be dulled. When higher dose of galectin-3 will be used for the cytokine-inducing assay, the reactivity of *A. actinomycetemcomitans* LPS may be inhibited by galectin-3 in the cells of 7-month-old mice.

In conclusion, murine galectin-3 suppressed the cytokine-inducing activity of *A. actinomycetemcomitans* LPS for the splenocytes of 1-month-old mice. However, this suppressive effect was not seen with splenocytes from 7-month-old mice, suggesting that responses change with age.

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Figure legends

Fig. 1

Levels of IL-6 (a) and IFN- γ (b) in the culture supernatants of murine splenocytes from 1- or 7-month-old C57BL/6 mice. * $p < 0.01$.

Fig. 2

Dose response analysis of IL-6 (a) and IFN- γ (b)-inducing activity of *A. actinomycetemcomitans* LPS in murine splenocytes.

Fig. 3

Effect of recombinant murine galectin-3 on IL-6 (a) and IFN- γ (b) induction by *A. actinomycetemcomitans* LPS. Data are the means of three duplicate experiments with standard deviation. *vs. control $p < 0.01$, ** $p < 0.02$, *** $p < 0.05$.

Fig. 1

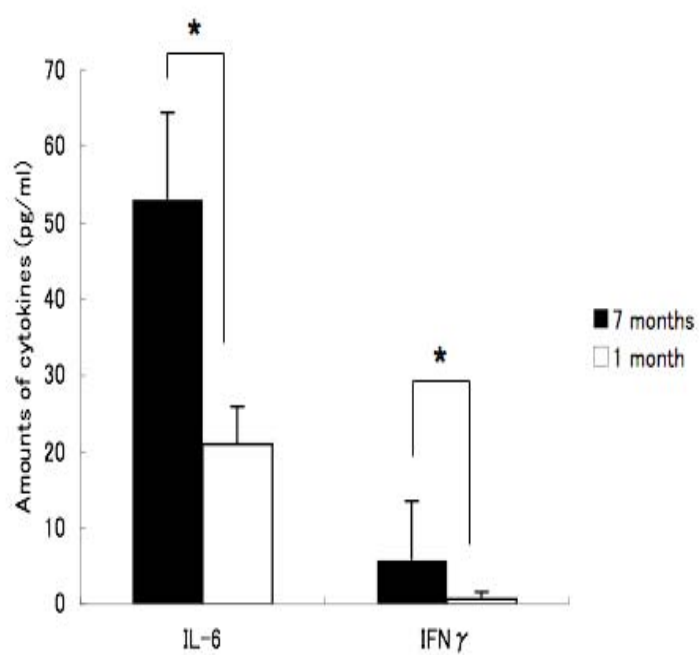


Fig. 2 (a)

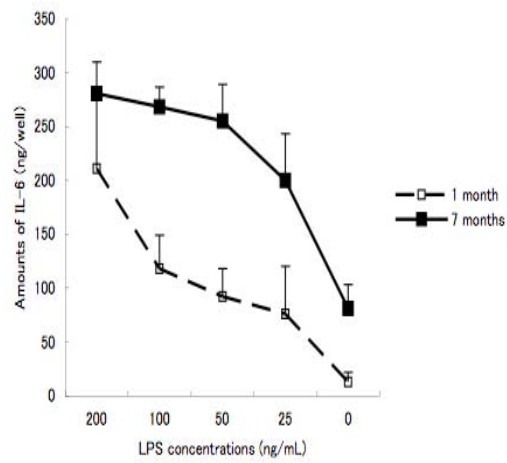


Fig. 2 (b)

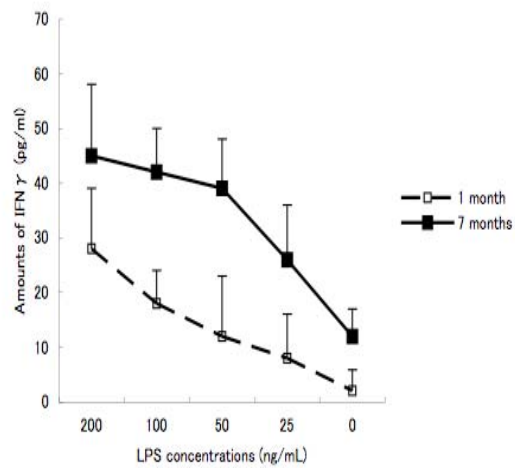


Fig. 3 (a)

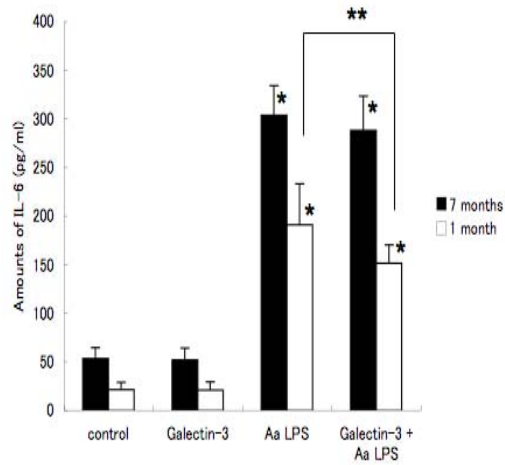


Fig. 3 (b)

